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Single and mixture effects of pesticides and a degradation product on fluvial biofilms

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Abstract The Morcille River located in the Beaujolais vineyard area (Eastern France) is subjected to strong vine-growing pressure leading to the contamination by a range of herbicides and fungicides of the surrounding freshwater environment. Particularly high concentrations of norflurazon, desmethyl norflurazon and tebuconazole were recorded in spring 2010 at the downstream site of the river. Despite their occurrence in rivers, scarce toxicity data are available for these products, in particular in the case of desmethyl norflurazon (main norflurazon degradation product). Furthermore, the toxicity data are generally available only for single compounds and are issued from single species toxicity tests, leading to a lack of ecological relevance. Consequently, this study was undertaken to evaluate the toxic effects of norflurazon, desmethyl norflurazon and tebuconazole singly and in a ternary mixture on fluvial biofilm. Toxicity tests were performed in microplates for 48 h. Photosynthetic endpoints were measured using pulse amplitude-modulated fluorometry; diatom densities and taxonomic composition were determined. After 48 h of exposure, significant effects on optimal quantum yield (F_v/F_m) for desmethyl norflurazon and mixture were observed.

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Introduction

The European Union's Water Framework Directive has set the target to achieve good ecological status for all aquatic environments in Europe by 2015. Among environmental pollutants, pesticides are of particular concern and greatly affect water quality management. Providing knowledge and ecotoxicological data about pesticides is a challenge for prioritization of substances. Nevertheless, the question of breakdown products and mixture toxicities is rarely taken into account in water quality management despite recent studies underlining the toxicity of some metabolites (Amorós et al. 2000; Pesce et al. 2010) or highlighting possible synergic effects of pesticides acting with similar or dissimilar modes of action (Magnusson et al. 2010).

The Morcille River located in the Beaujolais vineyard area (Eastern France) is subjected to strong agricultural pressure, essentially exerted by vineyard treatments. Herbicides and fungicides were found in particularly high concentrations in the surrounding freshwater environment and particularly high levels of norflurazon (NFZ; a herbicide belonging to the pyridazinones), of its main biodegradation product, desmethyl norflurazon (DMN) and of tebuconazole (TBZ; an azole fungicide) were recorded in the spring of 2010 at the downstream site of the river.

In this context, the objective of this work was to evaluate the single and joint effects of NFZ, DMN and TBZ on river biofilms. Attached microbial communities play a fundamental role in the ecological functioning of river systems, by their key place in the trophic web and their important contribution to primary production. Moreover, river biofilms interact strongly with dissolved substances present in water such as pesticides and are likely to respond quickly to contaminant pressures and can so be regarded as early warning systems and powerful indicators for ecosystem health (Sabater et al. 2007). Moreover, testing the effects of toxic compounds on natural communities instead of using classic single species tests is an approach particularly appropriate because organisms are grouped in complex biological communities in the environment; this reasoning integrates then high ecological relevance (Clements and Rohr 2009).

Toxicity tests on natural biofilm using pulse amplitude-modulated fluorometry were performed directly on microplates in order to minimize the volumes of toxicant needed. Decreasing amount of compound in toxicological studies can be a crucial issue in particular when testing expensive substances like metabolites; moreover, the use of microvolumes decreases the quantity of waste to treat and so the environmental and monetary cost of the study.

Biofilm was exposed to a range of pesticide concentrations for 48 h. Then, photosynthetic parameters (optimal quantum yield (F_v/F_m) and effective quantum yield of photosystem II (Φ_{PSII})), diatom densities and taxonomic composition of diatom communities were determined.

Previous studies already showed toxicity of NFZ and TBZ on aquatic organisms (Guseinova et al. 2005; Ochoa-Acuña et al. 2009), so we hypothesized that these two pesticides might have toxic effects on natural biofilms and particularly NFZ with regard to its photosynthetic efficiency due to its mode of action (inhibition of carotene synthesis). Moreover, based on the scarce data available in the literature, a lower toxicity of DMN compared to its parent compound was expected, due to an increase of polarity from NFZ to DMN and thus an assumed decrease in its ability to influence carotenoid synthesis in the lipophilic chloroplast thylakoid (Wilkinson 1987).

Materials and methods

Experimental design

Biofilm sampling and study site

Biofilms were collected in the Morcille River, located in the Beaujolais vineyard area (Eastern France). The Morcille River is subjected to strong agricultural pressure, essentially exerted by vineyards, and is characterized by an increasing pesticide gradient from upstream (<0.1 μg/L total pesticide concentrations) to downstream (>1.7 μg/L total pesticide concentrations) (Morin et al. 2012). Sampling took place in April 2011 at the upstream station (Fig. 1) characterized by very low pesticide concentrations (Montuelle et al. 2010). A composite biofilm sample was collected by scraping streambed rocks using a razor blade and was re-suspended in river water from the upstream site. The biofilm was maintained at 19–20 °C with a photon flux density of 25 μmol m^{-2} s^{-1} and a 12:12-h light/dark cycle before use for the short-term bioassay (within 1 week).

Chemicals

Norflurazon (CAS reg. 27314-13-2, purity=94 %), desmethyl norflurazon (CAS reg. 23576-24-1, purity> 99 %) and tebuconazole (CAS reg. 107534-96-3, purity>98 %) were purchased from Dr. Ehenstarler GmbH, Augsburg. Stock solutions were prepared in ultrapure water at a final concentration of 6,000 μg/L.

In order for it to be representative of environmental conditions, the mixture composition was chosen according to the concentration ratio of the three products found in the Morcille River at the downstream site in the spring of 2010: 5.5 % of NFZ, 54 % of DMN and 40.5 % of TBZ to obtain a stock solution with a final total pesticide concentration of 6,000 μg/L (327.6 μg NFZ/L, 3,246 μg DMN/L and 2,426.4 μg TBZ/L).

Short-term bioassay

Bioassays were performed in a 96-well microplate with a clear, flat bottom (BD Falcon, Germany). In each well, from 0 to 200 μL of pesticide stock solution was added to 200 μL of biofilm suspended in WC culture medium (10^5 diatom cells/mL) (Guillard and Lorenzen 1972);

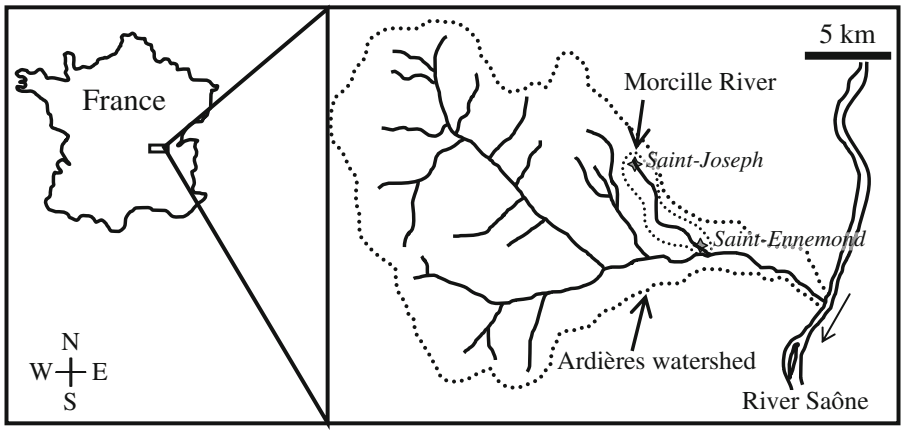

Fig. 1 Location of the study sites along the Morcille River

appropriate volumes of ultrapure water were added to reach a final volume of 400 μL.

Four conditions were run: NFZ tested alone (noted NFZ), DMN tested alone (noted DMN), TBZ tested alone (noted TBZ) and the mixture NFZ, DMN and TBZ as described above (noted Mix). Biofilm was exposed in triplicate to seven concentrations of total pesticides (C_0=0, C_1=93.75, C_2=187.5, C_3=375, C_4=750, C_5=1,500 and C_6=3,000 μg/L) and maintained at 19–20 °C throughout the 48 h of exposure with a photon flux density of 25 μmol m^{-2} s^{-1} and a 12:12-h light/dark cycle.

After exposure, the optimal quantum yield (F_v/F_m) and effective quantum yield of PSII (Φ_{PSII}) were measured directly on microplates; then, biofilms were suspended by successive pipetting and then collected and immediately fixed in Lugol for taxonomic determination and counting.

"Control pesticide wells" were used to assess the pesticide stability: 200 μL of the three stock solutions (NFZ at 6,000 μg/L, TBZ at 6,000 μg/L and DMN at 6,000 μg/L) were added at T_0 to empty wells of the microplate to assess in our experimental conditions the abiotic degradation of compounds and well wall adsorption. Pesticide concentrations were determined at T_0 and after 48 h.

Pesticide analyses

The 400-μL water samples were dried and then dissolved in 400 μL of ethyl acetate after addition of 4 μL of an internal standard solution (10 ng/μL of atrazine-*d5* and tebuconazole-*d6*), prior to analysis. Determination of pesticides and metabolites was performed with a gas chromatography system TRACE GC Ultra (Thermo Scientific) coupled with a mass spectrometer Quantum GC (Thermo Scientific) and "triple quadripole" detection. The separation was made with a TR-5MS column (5 % phenyl (equivalent) polysilphenylenesiloxane, 30 m×0.25 mm×0.25 μm, Thermo Scientific). Helium was used as mobile phase at a constant flow rate of 1.2 mL/min. One microlitre of sample was injected on splitless injector at 280 °C. The gradient consisted of an isotherm for 0.5 min at 50 °C; it then ramped to 190 °C at 17 °C/min, then to 220 °C at 4 °C/min and a last ramp to 300 °C at 20 °C/min. The temperature was kept constant at 300 °C for 8.26 min giving a total running time of 29 min. The source temperature was set at 250 °C. The ionization was operated in electron impact mode (70 eV, current of 25 μA), argon was used as collision gas at 1.2 mTorr and acquisition was performed in selected reaction monitoring (SRM) mode with a total cycle time of 200 ms. The SRM transitions were 303/145 and 305/145 for NFZ, 289/145 and 289/173 for DMN and 250/125 and 252/127 for TBZ. The SRM transitions for the internal standards, atrazine-*d5* and tebuconazole-*d6*, were 205/105 and 256/125, respectively.

Biofilm response analyses

Photosynthetic efficiency

The optimal quantum yield (F_v/F_m) and the effective quantum yield (Φ_{PSII}) of photosystem II (PSII) were measured using pulse amplitude-modulated fluorometer (version EDF, Heinz Walz GmbH, Germany). F_v/F_m

represents the potential maximal photosynthetic activity and is expected to decrease if a chemical produces alterations in the structure of the photosynthetic apparatus. On the other hand, Φ_{PSII} measures the efficiency of excitation energy capture by the open PSII reaction centres under light conditions. F_v/F_m is expected to be affected when biofilms are exposed to toxicants at high concentrations, while Φ_{PSII} is expected to respond quickly and be very sensitive to PSII inhibitor exposure (Corcoll et al. 2012).

F_v/F_m was measured after 30 min of dark adaptation, whereas Φ_{PSII} was measured under light adaptation; F_v/F_m and Φ_{PSII} are described by the following equations (Baker 2008):

$$F_v/F_m = (F_m - F_0)/F_m \quad (1)$$

$$\Phi_{PSII} = (F'_m - F_t)/F'_m \quad (2)$$

with F_0 the minimum fluorescence determined after a weak far red modulated light and F_m the maximum level of fluorescence measured during a saturating white light pulse (1) and F_t the steady state level of fluorescence under ambient light and F_m the maximum level of fluorescence measured during a saturating white light pulse (2).

Diatom cell density

Diatom cell densities were determined at T_0 and $T_{48\,h}$ for the different treatments (NFZ, TBZ, DMN and Mix) for the highest concentration (C_6) and for the control treatment. Diatom cells (200 μL of each sample) were enumerated using a Nageotte counting chamber (Marienfeld, Germany). The total number of individuals and the number of dead cells were recorded in 10 fields of the gridded area (1.25 μL each, 0.5-mm depth) under light microscopy at 400× magnification (Leitz photomicroscope). Distinction between dead and live organisms was estimated by observation of the turgescence and colour of the chloroplasts as described by Morin et al. (2010).

Taxonomic analyses

Taxonomic analyses were performed after 48 h of exposure to the different treatments (NFZ, TBZ, DMN and Mix) for the highest concentration (C_6) and for the control treatment. Diatom identifications were performed after having prepared permanent slides following the European standard NF EN 13946. Diatoms were identified at 1,000× magnification to the lowest taxonomic level possible using standard references (Hofmann et al. 2011).

Statistical analysis

The effects of pesticide exposure on photosynthetic efficiency ($n=3$) and diatom densities ($n=3$) were tested by one-way analysis of variance (ANOVA) using Statistica 6.1 (StatSoft, France). The ANOVA was followed by a Tukey–HSD test ($p<0.05$, $p<0.01$, $p<0.001$). Homogeneity of variance was checked prior to data analysis. EC_{10} and confidence intervals $\alpha_{5\%}$ were calculated using REGTOX 7.0.5 (Vindimian 2003).

Results and discussion

Pesticide stability

Pesticide stability was assessed by comparing concentrations of compounds at T_0 and $T_{48\,h}$ in the control pesticide wells for the highest pesticide exposure concentration (C_6 treatment) for each condition (DMN, NFZ, TBZ and Mix). Due to the physical characteristics of the compounds studied (low values of the octanol/water partition coefficient (K_{ow})), the latter are not likely to be adsorbed and so we considered that if no adsorption was noticed in C_6 treatment it was also null in the other treatments.

No decrease was noted for the three compounds between T_0 and $T_{48\,h}$ in their respective control pesticide well. The organisms were then considered as having been exposed to the nominal concentration of pesticides, and adsorption to the surface wells and photodegradation were considered to be negligible in our experimental conditions.

Bioaccumulation of compounds by the different biofilm components was not considered in our experiment. Measuring concentrations of pesticides in biofilm is of particular interest to evaluate the exposure history of natural biofilms; nevertheless, owing to the complexity of biofilm structure, developing analytical techniques for pesticide concentration determination in this matrix is still at an early stage and requires large amounts of contaminant (Byers, personal communication).

Photosynthetic efficiency

Φ_{PSII} was not significantly different between controls and NFZ, DMN, TBZ or Mix treatments after 48 h of exposure nor was F_v/F_m between controls and NFZ or TBZ treatments (Table 1). In contrast, a marked difference was observed when DMN and Mix treatments were compared to the control with regard to F_v/F_m (Table 1, Fig. 2).

These results show the following:

1.In our experimental conditions, F_v/F_m is a more sensitive parameter to reveal photosynthetic damage after pesticide exposure compared to Φ_{PSII}. This demonstrates the need to study complementary parameters when working with the effects of toxicants on photosynthetic efficiency. This point has already been pointed out by Laviale et al. (2011), who studied the effects of atrazine and isoproturon—two PSII inhibitors—on natural communities; they highlighted that the study of both Φ_{PSII} and F_v/F_m is necessary to avoid a partial view of periphyton photochemical response to these herbicides and limitations for interpretation of these differential effects.

2. F_v/F_m and Φ_{PSII} were not sensitive enough to reveal NFZ or TBZ toxicity on the natural community in our experiment.

As a fungicide, TBZ does not act directly on the algal compartment of the biofilm (non-target organisms). Nevertheless, the use of photosynthetic fluorescence parameters can reveal the effects of chemicals affecting metabolic processes not directly linked to photosynthetic electron transport (for example, any cellular process downstream of PSII) (Corcoll et al. 2012). For example, Ricart et al. (2010) showed a reduction of effective quantum yield (Φ_{PSII}) by up to 25 % with increasing concentrations of the antimicrobial agent triclosan on biofilms. In another study, Bonnineau et al. (2010b) underlined the toxicity of β-blockers (metoprolol, propranolol and atenolol) on fluvial biofilms with regard to photosynthetic efficiency. In our experiment, F_v/F_m and Φ_{PSII} were not relevant parameters to reveal the expected indirect effects of TBZ on the photosynthetic apparatus.

In contrast, we were expecting to see toxicity effects of NFZ on biofilm photosynthetic efficiency due to its mode of action (inhibitor of the carotenoid biosynthesis), but photosynthetic damage was not revealed after acute NFZ exposure. Some authors have shown the effects of NFZ on photosynthesis efficiency of single species and natural

Table 1 Mean values and standard errors of the photosynthetic and diatom parameters as a function of treatment and time exposure. Φ_{PSII}, F_v/F_m, total diatom densities, mortality, diversity and species richness are given for C_6 for TBZ, NFZ, DMN and Mix treatments. Stars indicate significant difference compared to control at 48 h (n=3, *p<0.05, **p<0.01, ***p<0.001; when p<0.001, p value is given in round brackets)

Treatment	Time of exposure (hour)	Φ_{PSII} in C_6=3000 µg/L total pesticides (% control)	F_v/F_m in C_6=3000 µg/L total pesticides (% control)	EC_{10} (µg/L); α 5 %	Total diatom densities (10^3 cells/mL)	Mortality (%)	Diversity	Species Richness (total number of species)
Ctr	0	n.a.	n.a.	n.a.	55.6±3.5	23.6±2.9	n.a.	n.a.
Ctr	48	n.a.	n.a.	n.a.	15.3±2.9	21.5±0.1	2.3±0.1	25.7±2.9 (38)
NFZ	48	67.4±5.3	78.2±6.9	< c.l.	33.1±24.6	13.5±10.2	2.1±0.1	22.7±1.6 (32)
TBZ	48	73.5±11.5	72.6±3.9	< c.l.	12.2±1.4	21.6±4.1	2.1±0.1	18.7±1.1* (30)
DMN	48	80.8±3.8	38.2±1.6*** (0.0007)	58; [9;254]	13.8±9.9	19.7±5.0	2.2±0.1	18.7±1.5* (29)
Mix	48	64.1±22	44.2±4.2*** (0.0006)	125; [24;413][a] 68; [16;185][b]	15.8±3.0	19.8±1.9	2.0±0.2	19.7±2.2 (31)

n.a. not available; <c.l., under calculation limit

[a] Total pesticide concentration

[b] DMN concentration

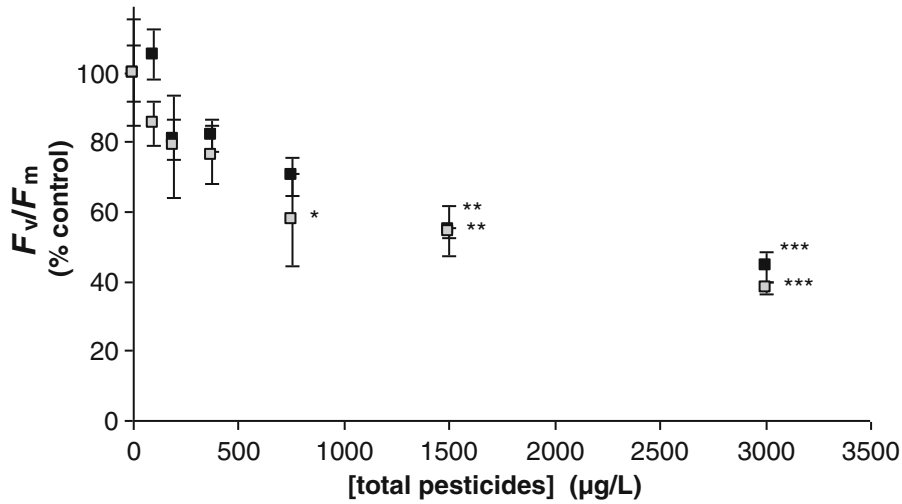

Fig. 2 Optimal quantum yield of PSII (F_v/F_m) (expressed as % control±standard error) of biofilm community exposed to DMN and Mix treatments for 48 h. PSII yield is expressed as a function of the total pesticide concentrations. *Stars* indicate statistical difference from controls ($n=3$, $*p<0.05$, $**p<0.01$, $***p<0.001$). *Grey squares* represent DMN treatments, and *black squares* represent Mix treatments

biofilms. For example, Tschiersch et al. (2002) showed a decrease of photosynthetic activity for cultures of *Euglena gracilis*; Frankart et al. (2003) showed a decrease of F_v/F_m after NFZ exposure of *Lemna minor*. Pesce showed significant effects of NFZ on natural biofilm on F_v/F_m and determined a CE_{50} close to 100 µg/L (personal communication). This difference of sensitivity could be explained by experimental conditions and variability of natural biofilms (e.g. global community structure, ratio between algal groups, taxonomic composition, life history of the biofilm, etc.).

Optimal quantum yield (F_v/F_m) versus toxicant concentrations for DMN and Mix 48-h treatments is presented in Fig. 2 for the whole range of contamination. Significant differences in F_v/F_m were observed for DMN and Mix treatments between the controls and the highest concentrations after 48 h of exposure. F_v/F_m was significantly different in C_4, C_5 and C_6 for DMN treatment (57.9±13.2, 53.9±1.6 and 38.2±1.6 % of control, respectively) and in C_5 and C_6 for Mix treatment (54.5±7.3 and 44.2±4.2 % of control, respectively).

In order to compare the relative toxicity of DMN and Mix treatments, optimal quantum yield (F_v/F_m) was expressed versus total pesticide concentrations and versus DMN concentrations for both treatments, and the corresponding EC_{10} (toxicant concentration needed in order to decrease F_v/F_m to 10 % compared to the control) was calculated.

EC_{10} were respectively 58 µg DMN/L, $\alpha_{5\%}$ [9;254] for DMN treatment, 125 µg/L total pesticide, $\alpha_{5\%}$ [24;413] and 68 µg DMN/L, $\alpha_{5\%}$ [16;185] for Mix treatment and were not significantly different from each other (Table 1).

To our knowledge, this is the first study demonstrating DMN toxicity on photosynthetic efficiency. Very little is known about the chemical properties and mode of action of DMN, and toxicological data are scarce. Nevertheless, this compound was reported to decrease chlorophyll content in the alga *Scenedesmus acutus* (Sandmann et al. 1981).

Moreover, we observed greater toxicity of DMN than of its parent compound on F_v/F_m (no effect of NFZ was noted at concentrations up to 3,000 µg/L). These results underline the necessity to take into account metabolite issues in pesticide toxicity assessment, as highlighted in another study dealing with the ecotoxicity of pesticides and transformation products (Sinclair and Boxall 2003). In this study, the authors identified several transformation products that were more toxic than the parent compound, for example 4-chlorophenol was found to be more toxic to algae than its parent compound the 2,4-D (a herbicide belonging to the alkylchlorophenoxy family). They pointed out four main reasons to explain the greater toxicity of the biotransformation product than the parent compound, i.e. "(1) the presence of a pesticide toxicophore; (2) the fact that the product is the active part of a propesticide; (3) the product is

accumulated to a greater extent than the parent compound; or (4) the product has a more potent mode of action than the parents" (Sinclair and Boxall 2003). Of course, explaining differences of toxicity between metabolite and its parent compound and more generally between two toxicants requires having as much toxicological data as possible for each of the compounds involved (e.g. EC_{50}, mechanisms of action of products or mechanisms of tolerance of species exposed). These data are often missing, and therefore, the scientific community has to continue its efforts to supply such information to the relevant databases.

Diatom growth

Significant differences in cell densities between controls at T_0 and the different treatments after 48 h of exposure (Ctr, NFZ, DMN, TBZ and Mix) were observed for the C_6 treatments; densities at T_0 were 3.6 times higher than densities at $T_{48\ h}$ (Table 1). This decrease of cell number could be explained by the fact that biofilm may have become deposited on the bottom of the microplate due to the absence of agitation. When sampling at $T_{48\ h}$, part of the cells may have remained adhering to the microplate leading to the decrease in overall cell density observed between T_0 and $T_{48\ h}$.

Statistical analysis revealed that densities at $T_{48\ h}$ were not significantly different in control treatment and in NFZ, DMN, TBZ and Mix treatments. Pesticide treatments did not seem to have an effect on diatom density after 48 h of exposure. This can be explained by the short time of exposure to toxicants; actually, it seems that for algae a longer exposure period is needed to observe effects on growth. Such results have been reported in the case of algae exposure to other toxicants than pesticides. For instance, Kim Tiam et al. (2012) observed the effect of high concentrations of cadmium (100 μg Cd/L) on the growth of *Eolimna minima* after 7 days of exposure. In the same way, Morin et al. (2008) reported differences in biofilm diatom densities between control and lower cadmium contaminated treatments (10 μg Cd/L) after 6 weeks of exposure.

Percentages of dead cells were not significantly different in controls (T_0 and $T_{48\ h}$) compared to contaminated conditions (Table 1). Apoptosis being the ultimate response of cells to toxicant, we can assume that during our experiment the cell damage caused by the pesticides and the metabolite did not exceed critical levels and could still be repaired by other mechanisms involving, for instance, antioxidant enzymes (Bonnineau et al. 2010a). Although no effect was observed on diatom density or mortality, sub-lethal effects can occur as shown by the decrease of F_v/F_m in DMN and Mix treatments compared to the control.

Diatom community structure

Due to the very low diatom concentrations in the samples, counting the 400 frustules as required by NF T90-354 was not feasible; around 200 frustules were counted in each replicate sample. The total number of species recorded per treatment (i.e. composite sample obtained by pooling replicates) was higher in the controls (38 taxa) than in the contaminated treatments (30.5±0.7). Average values of species richness and Shannon's diversity index were equal to 21.1±3 and 2.1±0.1, respectively, over the treatments.

Statistical analysis revealed that community composition was not significantly different in controls and with NFZ, DMN, TBZ and Mix treatments after 48 h of exposure. Communities were dominated by three main species (relative abundance >60 % in all samples): *Rhoicosphenia abbreviata*, *Achnanthidium minutissimum* and *Planothidium lanceolatum*. *Reimeria sinuata*, *E. minima* and *Nitzschia palea* were also frequently found in samples in lower proportions.

Very little is known about diatom species sensitivity to pesticides. Nevertheless, some species like *A. minutissimum* or *E. minima* have already been noted to tolerate triazines (Pérès et al. 1996; Herman et al. 1986; Kasai et al. 1993; Munoz et al. 2001; Seguin et al. 2001) or a number of pesticides (triazine and urea) in the case of *N. palea* (Kasai et al. 1993; Dorigo et al. 2004).

All samples were dominated by species belonging to the low-profile guild (79.1±1.6 %) as defined by Passy (2007) (*A. minutissimum*, *P. lanceolatum*, *R. sinuata* and *R. abbreviata*). Species from this guild were shown to cope with pesticides (Berthon et al. 2011; Roubeix et al. 2011). The fact that pesticide-tolerant species were already dominant in the inocula, although collected in a very low pesticide concentration site, may explain why no changes in community composition were observed between contaminated and control conditions and may suggest species selections occurring from very low pesticide concentrations.

Conclusions

Studies dealing with pesticides and water quality management cannot be complete and realistic without taking into account metabolite and mixture effects. In this study, the single and joint effects of NFZ, DMN and TBZ were evaluated on natural biofilm; the major implications of this work are as follows:

- Toxicity tests in microplates are of particular interest for decreasing the amounts of chemical (often expensive) needed and are already used in routine studies of single algae species with growth inhibition as endpoint. Nevertheless, physiological information is rarely investigated in this kind of study (Magnusson et al. 2010). To our knowledge, this is the first study using pulse amplitude-modulated fluorometry for toxicity tests in microplates directly on natural biofilms (more realistic than single species tests).
- However, the use of microplates showed some limitations for density calculation and taxonomic analyses (loss of part of sample on the microplate).
- DMN showed higher toxicity on photosynthetic efficiency (F_v/F_m) than its parent compound (NFZ), highlighting the importance of taking metabolites into account in toxicity assessment.
- The Mix treatment significantly affected F_v/F_m. In regard to the absence of toxicity of NFZ and TBZ when tested alone, the toxic effect observed in Mix treatment is probably due to DMN only.

This is one of the first studies dealing with metabolite and mixture toxicity of pesticides on natural biofilms in microplate; the results obtained using pulse amplitude-modulated fluorometry and microplates together as an experimental unit presage interesting perspectives in the field of toxicity evaluation of pesticides whether as single substances or mixtures.

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