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Environmental effects of realistic pesticide mixtures on natural biofilm communities with different exposure histories

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HIGHLIGHTS

- We assessed the effects of pesticides exposure on biofilms using passive samplers.
- The sensitivity of biofilm to pesticides revealed the past history of communities.
- Pesticides had significant effects on growth-related and structural endpoints.
- History exposure had a crucial role in biofilm responses to pesticides exposure.
- POCIS extracts were highly relevant for assessment of chronic effects of mixture.

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ABSTRACT

This study deals with the use of Polar Organic Chemical Integrative Sampler (POCIS) extracts to assess the impact of low-dose pesticide mixtures on natural biofilm communities originating from either a chronically contaminated or a reference field site. To investigate how natural biofilm communities, pre-exposed to pesticides in situ or not might respond to environmentally realistic changes in pesticide pressure, they were exposed to either clean water or to POCIS extracts (PE) in order to represent toxic pressure with a realistic pesticide mixture directly isolated from the field. The impacts of PE were assessed on structure, physiology and growth of biofilms. Initial levels of tolerance of phototrophic communities to PE were also estimated at day 0.

PE exposure led to negative effects on diatom growth kinetics independently of in-field biofilm exposure history. In contrast, the impacts observed on dry weight, ash-free dry mass and algal fluorescence-related parameters followed different trends depending on biofilm origin. Exposure to PE induced changes in diatom assemblages for the biofilm originating from the reference field site with higher relative abundance of *Eolimna minima* and *Nitzschia palea* with PE exposure. Initial tolerance of phototrophic communities to PE was 8-fold higher for the biofilm originating from the chronically contaminated site compared to the reference field site.

The use of POCIS extracts allowed us to highlight both chronic impacts of low doses of a mixture of pesticides on natural communities with regard to biofilm exposure history as well as initial levels of tolerance of phototrophic communities.

1. Introduction

The traditional approach to environmental risk assessment consists of evaluating the toxicity of a single compound on single species. But, this reasoning lacks ecological relevance because organisms are grouped in complex biological communities exposed to chemical mixtures in the environment. The development of new tools is thus needed to reach a more environmentally realistic and integrative approach in risk assessment studies. Attached microbial communities could respond to such needs as they play a fundamental role in the ecological functioning of river systems, owing to their key position in the trophic web and their important contribution as primary producers and microbial decomposers. Moreover, such communities interact strongly with dissolved substances such as pesticides present in water and are likely to respond quickly to contaminant pressures making river biofilms useful early warning systems for the detection of the effects of toxicants (Montuelle et al., 2010). Among the variety of methods available, physiological approaches may be appropriate for the detection of acute effects whereas persistent or chronic effects should act on other biofilm indicators, for example growth or

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biomass-related factors, or community composition (in particular the diatom community) (Sabater et al., 2007). It is for these qualities that attached microbial communities have been used as biological models in the study of various toxicants in controlled laboratory conditions (Laviale et al., 2011; Corcoll et al., 2012a,b; Tlili et al., 2008). Nevertheless, most of these studies dealt with single compounds or binary mixtures. On the other hand, translocation experiments with attached microbial communities have been used to reach more realistic exposure conditions (in particular with regard to toxicant exposure) (Morin et al., 2010a; Arini et al., 2012). Reference biofilms transferred to contaminated sites generally presented, after some weeks, the characteristics of communities from polluted sites. Conversely, recovery of communities after translocation from a contaminated to a less contaminated site is observed after a few weeks to a few months. Nevertheless these approaches do not allow clear identification of the extent to which the toxicant(s) of interest is (are) responsible for the observed effects on biofilms, since abiotic (e.g. light, temperature, current velocity) and biotic (e.g. species drift) parameters are not controlled and multiple contamination often occurs.

Passive sampling devices like the Polar Organic Chemical Integrative Sampler (POCIS) are useful tools for monitoring trace levels of chemicals in aquatic environments since they concentrate many organic chemicals from large volumes of water (Mazzella et al., 2010). This high concentration of compounds makes the POCIS a powerful instrument for the assessment of extract toxicity via biological testing. The use of the POCIS in combination with bioassays has the advantage of being more relevant from an ecotoxicological perspective because of the pollutant mixtures it provides. Moreover, this approach gives an estimation of an integrative measure of the toxic potential of a group of compounds including unknown toxicants (a non a priori approach). While POCIS extracts in combination with short-term toxicity tests have been successfully used in particular in order to reveal the exposure history of biofilms (Pesce et al., 2011b), evaluation of potential extract toxicity in long-term studies is still in a challenge. The first and only study dealing with chronic low-dose effects of passive sampler extracts on biofilms was conducted by Morin et al. (2012b). The experiment was a first attempt to evaluate whether the chronic effects of pesticides in a mixture could be approached by the use of POCIS extracts. The study highlighted the methodological issues of dealing with low contaminant doses in long-term experiments, particularly the difficulty of monitoring the concentration of contaminants in large volumes of water. It nevertheless reported the promising perspectives of the approach for further ecotoxicology studies.

The objective of the present study was to evaluate the efficiency of the use of PE in order to study the impact of changes in pesticide pressure on biofilms. In order to reach this goal, biofilm communities were collected from a reference and from a chronically contaminated site of a small river located in a French vineyard area, and then communities were chronically exposed. Initial levels of tolerance of phototrophic communities to pesticide mixtures were also estimated using a short-term photosynthetic bioassay with the PE. Chronic effects were assessed by exposing biofilms either to clean water or to low doses of PE. Diatom growth kinetics, dry weight, ash-free dry mass, algal fluorescence-related parameters, effective quantum yield of Photosystem II and diatom community structure and composition were determined after 0, 3, 7 and 13 days of exposure in experimental channels. Exposure to PE was expected to provoke structural and/or functional changes in the communities originating either from the chronically contaminated or from the reference field site.

2. Material and methods

2.1. Study site and sampling procedure

The study was carried out on the Morcille River, located in the Beaujolais vineyard watershed of eastern France. The Morcille River has been extensively studied over the past decades (Dorigo et al., 2007; Montuelle et al., 2010; Rabiet et al., 2010). The area is subjected to strong agricultural pressure – essentially exerted by vineyards – and is characterized by an increasing multi-contaminant gradient. Pesticides overall and to a lesser extent metal and nutrient concentrations increase from upstream to downstream.

Glass slides fixed in perforated plastic boxes were used as artificial substrates to allow biofilm colonization for 4 weeks (from 24th May to 21st June 2011) at the 2 stations located upstream (reference site) and downstream (the more contaminated site). The minimum number of slides to run the analyses considered here was estimated at 120 slides of 14 cm^2 (60 at each station). Consequently, 100 slides were immerged at each site (in order to have sufficient biofilm taking into consideration possible breakage of glass slides in the river).

"Quantitative" POCIS were used for pesticide quantification of the majority of the compounds found in the water (Mazzella et al., 2010; Lissalde et al., 2011), while grab sampling was used to determine the concentration of compounds that were not calibrated by POCIS in the present study (average of the 5 grab samples taken from 24th May–21st June 2011).

A Performance and Reference Compound (PRC) was introduced in a "Quantitative" POCIS. The devices were immersed in the current at upstream and downstream stations for two weeks and then replaced by new ones for two extra weeks for pesticide quantification and characterization of biofilms in situ past exposure. After collection, all POCIS were kept at −4 °C until extraction and chemical analysis.

"Accumulative" POCIS used for the toxicity tests (Morin et al., 2012b) were immersed at the downstream station during the biofilm colonization period to concentrate pesticides. After 4 weeks in the river, the glass slides and POCIS were brought back to the laboratory. The biofilms were put in aquariums filled with water from their respective sites supplemented with nutrients following the composition of WC culture medium (Guillard and Lorenzen, 1972) given in Table 1 for one week before the beginning of the experiment (corresponding to the time required to carry out POCIS extraction).

2.2. Laboratory experimental conditions

2.2.1. Acute toxicity testing

In order to characterize the initial tolerance of upstream and downstream biofilms to pesticides, acute toxicity tests were carried out after one week in the lab. Two conditions were tested (upstream and downstream biofilms) in triplicate (10 glass slides for each replicate) leading

Table 1
Composition of the "WC" culture medium from Guillard and Lorenzen (1972).

Freshwater "WC" medium	
Major nutrients	
$CaCl_2$, $2H_2O$	36.76 mg/L
$MgSO_4$, $7H_2O$	36.97 mg/L
$NaHCO_3$	12.6 mg/L
K_2HPO_4	8.71 mg/L
$NaNO_3$	85.01 mg/L
Na_2SiO_3, $9H_2O$	28.42 mg/L
Traces	
Na_2EDTA	4.36 mg/L
$FeCl_3$, $6H_2O$	3.15 mg/L
$CuS0_4$, $5H_2O$	0.01 mg/L
$ZnSO_4$, $7H_2O$	0.022 mg/L
$CoCl_2$, $6H_2O$	0.01 mg/L
$MnCl_2$, $4H_2O$	0.18 mg/L
Na_2MoO_4, $2H_2O$	0.006 mg/L
H_3BO_3	1.0 mg/L
Vitamins	
Thiamin, HCl	0.1 mg/L
Biotin	0.5 μg/L
B12	0.5 μg/L

to the use of a total number of 60 slides for running the acute toxicity tests. Short-term photosynthetic bioassays were performed using measurement of the optimal quantum yield (F_v / F_m) as endpoint (defined in Section 2.4.1). Biofilms were exposed to semi-logarithmic series of dilutions of the pre-concentrated extracts. For each replicate, 140 cm^2 of biofilm were scraped from 10 glass slides and resuspended in 28 mL of mineral water; then 1.4 mL of biofilm were exposed to 1.4 mL of toxicant (2.8 mL final volume) to reach the same volume in every assay. Six concentrations of POCIS extracts (from d0 to d5), a control (ultrapure water) and an extraction blank obtained with the same extraction procedure as for POCIS extraction (Blank) were tested. Only pure Blank was tested corresponding to the highest exposure concentration (d0). Biofilms were exposed for 24 h at 19–20 °C under artificial light (30 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After 30 min in the dark, fluorescence F_v / F_m emitted by the plant component of the biofilm suspensions was measured by fluorimetry (PhytoPAM, Heinz Walz GmbH, Germany).

2.2.2. Channel experiments

After one week in the lab in aquariums filled with water from their site, upstream and downstream biofilms were exposed to POCIS extracts (PE) for 13 days in indoor glass channels in WC culture medium (Guillard and Lorenzen, 1972) (Table 1). The colonized glass slides were laid directly on the bottom of the artificial channels. In total 60 slides were used for the channel experiment. Upstream (reference site) biofilms were exposed to PE at low concentrations in the range of those measured in the contaminated downstream section of the Morcille River (and in the range of PE applied for the more dilute solutions d4 and d5 in the acute toxicity testing). This modeled toxic pressure (upstream with PE) or clean water in order to represent reference conditions (upstream without PE). Downstream biofilms were exposed to PE in order to model toxic pressure maintenance (downstream with PE) or replaced in clean water in order to mimic removal of toxic pressure (downstream without PE). Each condition was performed in triplicate. Experimental channels were maintained at a temperature of 19 to 20 °C during the 13 days of exposure with a photon flux density of 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$, a 12:12 h light:dark cycle and continuous water flow.

In order to fix the contamination level and to avoid a drop in pesticide concentrations (Morin et al., 2012b), 4 POCIS-equivalent extracts were used for spiking the channel volume (9 L) one day before the beginning of the experiment. Then, the channels were spiked again on days 3 and 6 with one POCIS-equivalent to ensure constant contamination levels in channels throughout the experiment (13 days). Nutrients were added on day 6 to ensure that concentrations were sufficient to allow biofilm growth. Nutrient concentrations and physicochemical conditions were monitored throughout the experiment.

Biofilm samples were collected by scraping the glass slides with a razor blade and the film was resuspended in a known volume of mineral water for further analyses. Samples were collected in triplicate on days 0, 3, 7 and 13 for diatom densities, effective quantum yield of PSII (Φps_{II}), fluorescence of Chl *a* measured at 665 nm (Fs_{665}), fluorescence signals linked to cyanobacteria (Fs_{Bl}), diatom (Fs_{Br}), and green algae (Fs_{Gr}) group determination. Taxonomic composition was determined on days 0 and 13; dry weight (DW) and ash-free dry mass (AFDM) were determined on day 13. At each sampling time one side (14 cm^2) was used for each replicate for in vivo fluorescence measurements and then fixed for diatom analyses (diatom cell density and taxonomic analyses). At day 13, one more side was used for DW and AFDM determination. Temperature, pH, conductivity, oxygen, nitrates, orthophosphates and silica concentrations were recorded in the water channels throughout the 13 days of the experiment.

2.3. Physico-chemical parameters and pesticide analysis in water

The method developed by Lissalde et al. (2011) was used for the analysis of POCIS extracts. The solid receiving phase contained in the POCIS (Oasis HLB sorbent, Waters) was recovered in ultrapure water. Then all samples were analyzed by HPLC–ESI–MS/MS (HPLC Ultimate 3000, Dionex, equipped with a Gemini NX C18 column, 10 mm × 2 mm, 3 μm, Phenomenex, and API 2000 triple quadrupole, AB SCIEX equipped with an electrospray ionization source) and GC–MS/MS (Quantum GC, Thermo, equipped with a Rxi-5MS column 30 m × 0.25 mm × 0.25 μm, Restek). Pesticide concentrations in the water phase were determined from spot samples by solid-phase extraction on Oasis HLB cartridges followed by liquid chromatography tandem mass spectrometry (LC/MS/MS API 4000, Applied Biosystem, France). Chromatographic separation used an Atlantis T3 analytical column, 3 μm particle size, 2.1 mm × 100 mm from Waters (France), at a flow rate of 300 μL min^{-1} with a mobile phase consisting of acidified acetonitrile and water. The injection volume was 20 μL (Assoumani et al., 2013).

Analytical methods were validated in terms of calibration linearity, specificity, extraction recovery, and quantification limit according to the French standard NF T90-210. Additionally, SPE and POCIS blanks were run, and the recoveries of two levels of spiked mineral water (i.e. 0.02 and 0.2 μg/L for LC–MS/MS analysis) were evaluated for each batch. Two calibrating standards (i.e. 5 and 25 μg/L, every 10 samples) and analytical blanks were periodically checked.

pH meter (pH 3110, WTW), conductimeter (LF 340, WTW) and oxymeter (Oxi 340i, WTW) were used to determine pH, conductivity and oxygen concentration respectively. The temperature was obtained by averaging the values recorded by the 3 devices. Nitrate concentration was calculated according to French standard (NF EN ISO 13395 10/96) using Auto-analyzer Evolution II (Alliance Instruments, France). Orthophosphate and silica were determined following standard procedures (NF T90-023 09/82 and NF T90-007 02/01 respectively) with a Lambda 2 spectrophotometer (Perkin Elmer, USA).

2.4. Biofilm response analysis

2.4.1. In vivo fluorescence measurements

In vivo fluorescence measurements used Pulse Amplified Modulated fluorometry (PhytoPAM, Heinz Walz GmbH, Germany) at four different excitation wavelengths (470, 520, 645 and 665 nm) characterizing and discriminating different groups of algae. For example green algae show a strong signal when excited at 470 nm due to Chl *b* and a weak signal at 520 nm whereas diatoms display strong signal at 470 and 520 nm when excited in relation to Chl *c*, fucoxanthin and carotenoids. For cyanobacteria a weak signal is recorded at 470 nm while excitation at 645 nm is particularly strong (Walz, 2003). By deconvolution of the signals it is then possible to estimate the fluorescence signals linked to the 3 main algal groups: cyanobacteria (Fs_{Bl}), diatoms (Fs_{Br}), and green algae (Fs_{Gr}). Measurements recorded at an excitation wavelength of 665 nm (Fs_{665}) estimate the fluorescence related to the photosynthetic component of the entire biofilm as 665 nm corresponds to the maximum excitation wavelength of the Chl *a* molecule. Fluorescence was measured over the same surface area for all samples and is expressed in relative units (ru).

Optimal quantum yield (F_v / F_m) and effective quantum yield (ΦpsII) were recorded at an excitation wavelength of 665 nm. F_v / F_m was measured after 30 min of adaptation to dark and was calculated according to (Genty et al., 1989):

$$F_v/F_m = (F_m - F_0)/F_m$$

where F_0 is the minimum fluorescence determined after a weak far red modulated light and F_m is the maximum level of fluorescence measured during a pulse of saturating light.

Samples were exposed to actinic light to determine their effective quantum yield. ΦpsII was calculated according to (Genty et al., 1989):

$$\Phi ps_{II} = \left(F'_m - F_t\right)/F'_m$$

where F_t is the minimum fluorescence determined after weak far red modulated light and F_m' is the maximum level of fluorescence measured during a saturating flash of white light while the sample was under actinic light.

2.4.2. Dry weight and ash-free dry mass

On day 13, the dry matter and the organic matter content were evaluated by calculating the dry weight (DW) and the ash-free dry weight (AFDM) following European standard NF EN 872. For each sample, 50 cm^2 of biofilm were scraped from glass slides and resuspended in 10 mL of mineral water. Biofilm suspensions were filtered through individual, previously dried, 25 mm GF/C Whatman glass fiber filters (1.2 μm pore size). Each filter was weighed, after 1 h drying at 105 °C to calculate the DW, then at 500 °C for 1 h, and weighed again to calculate the mineral matter. AFDM was calculated by subtracting the mineral matter weight from the total weight of dry matter.

2.4.3. Diatom analysis

2.4.3.1. Diatom cell density. The cells in each sample were counted using a Nageotte counting chamber (Marienfeld, Germany). 200 μL of sample were placed in the counting chamber and the total number of dead and live cells was recorded in 10 fields of the gridded area (1.25 μL each, 0.5 mm depth) under light microscopy at 400× magnification. Distinction between dead and alive organisms was estimated by the observation of the turgescence and color of the chloroplasts as described in Morin et al. (2010b).

2.4.3.2. Taxonomic analyses. Diatoms were identified from permanent slides prepared following European standard permanent slides NF EN 13946, and they were identified at 1000× magnification to the lowest taxonomic level possible using standard references (Hofmann et al., 2011).

2.5. Data analysis

The effects of pesticide exposure on photosynthetic efficiency (n = 3), fluorescence levels (Fs_{665}, Fs_{Bl}, Fs_{Gr} and Fs_{Br}, n = 3) and diatom densities (n = 3) were tested by one-way ANOVA analysis using STATISTICA 6.1 (StatSoft). The ANOVA was followed by a Tukey–HSD test. Homogeneity of variance was checked prior to data analysis. Due to the non-homogeneity of variance for DW, AFDM and Φps_{II}, statistical differences on these parameters were analyzed by Kruskal Wallis test, followed by two Mann–Whitney comparisons.

For the acute toxicity tests, EC_{25} (concentration needed to decrease F_v / F_m by 25% compared to the control) was calculated using REGTOX EV 7.0.5 (Vindimian, 2003).

Clustering analysis was performed with the stats package (version 2.12.0) of R and was based on the relative abundances of species whose abundances were above 2%. The analysis was carried out by calculating the dissimilarity matrix with Euclidean distances with dist function while hclust function was used for cluster construction with the complete link method.

3. Results and discussion

3.1. Pesticide contamination levels in the river

The main pesticides quantified in situ at the two sampling sites on the Morcille River are presented in Table 2.

The concentrations from 24th May to 21st June 2011, corresponding to the 4 weeks of biofilm colonization, were very low upstream (reference site, total pesticide concentration = 250 ng/L) in comparison to the downstream site (contaminated site, 2270 ng/L).

POCIS analysis revealed the presence of fungicides (dimetomorph, azoxystrobine, tebuconazole), herbicides (norflurazon, diuron, terbuthylazine, simazine, diflufenican) and herbicide metabolites (desmethyl norflurazon, deisopropylatrazine, DIA; dichlorophenulmethylurea, DCPMU; desethylterbuthylazine, DET; desethylatrazine, DEA) at the downstream site of the Morcille River from 24th May to 21th June 2011. Desmethyl norflurazon, norflurazon, dimetomorph, tebuconazole and diuron were the most concentrated compounds (1080, 265, 298, 259 and 138 ng/L respectively), representing 90% of the total pesticide and metabolite concentration. The contamination profile observed in spring 2011 was characteristic of a typical vineyard area contamination and similar to that reported in previous investigations at the site (Dorigo et al., 2007; Montuelle et al., 2010; Rabiet et al., 2010). We can note the persistence of diuron despite its prohibition in France since December 2008 due to its long lifetime in the soil.

3.2. Biofilm in situ exposure assessed by acute toxicity tests

Pollution-Induced Community Tolerance (PICT) was assessed on upstream and downstream biofilms on day 0 in order to evaluate the relative sensitivity of phototrophic communities to PE in relation to their in situ exposure history during the colonization period. The PICT approach illustrates the difference of tolerance to a toxicant between pre-exposed and non-exposed communities. This concept, introduced by Blanck et al. (1988), is based on the idea that when a natural community is exposed to a particular toxicant the more sensitive organisms disappear; and then when this community is exposed to this toxicant again its tolerance will be increased because of the disappearance of the sensitive species.

The final pesticide concentrations used for the short-term toxicity test with successive dilutions are presented in Table 3, the most dilute solutions (d4 and d5) were close to environmental concentrations (estimated by quantitative POCIS and spot sampling, reported in Table 2) and the strongest solution (d0) corresponded to 100- to 300-fold realistic concentrations.

Photosynthetic efficiency (F_v / F_m) expressed as percentage of the control is plotted versus PE dilutions at day 0 for upstream and downstream biofilms (Fig. 1). Statistical analysis revealed that F_v / F_m was significantly different from control for d0, d1, d2, d3 and extraction blank (Blank) for upstream communities with a particular very strong inhibition for the highest pesticide concentration (82 ± 1% for d0). On the other hand F_v / F_m was significantly different from the control only for the 2 highest pesticide concentrations (d0 and d1) for downstream biofilms and F_v / F_m was reduced by 25 ± 1% for the highest concentration (d0). The significant inhibition of F_v / F_m (10 ± 1% for Blank) for upstream biofilms exposed to extraction blank (Blank) highlights the very high sensitivity of upstream biofilms.

From these F_v / F_m values, EC_{25} were calculated and proved to be 8 times higher for downstream communities than for upstream communities reflecting the order of magnitude of the difference of in situ exposure levels with pesticide concentrations averaging 9 times higher at the downstream site than at the upstream site. The EC_{25} were equal to 0.06 ± 0.01 PE versus 0.48 ± 0.14 PE for upstream and downstream communities respectively. These values of EC_{25} correspond to 18 ± 3× and 142 ± 41× for upstream and downstream communities respectively. The results showed the difference of tolerance between upstream and downstream biofilms linked to different levels of pesticide contamination in situ as revealed by the time-weighted average pesticide concentrations measured with "Quantitative" POCIS at the two sites during the biofilm colonization period. Since diatom assemblages clearly differed between upstream and downstream sites, the increasing tolerance observed downstream

Table 2
Concentrations (μg/L) estimated at upstream and downstream stations of the Morcille River in spring 2011 (from 24th May to 21th June) and nominal concentrations applied in contaminated treatment (with PE) in the artificial channel experiment. Azoxystrobine, DCPMU, DEA, DET, DIA, dimetomorph, diuron, simazine and terbuthylazine are in situ 1 month time-weighted average (TWA) concentrations estimated with POCIS (mean of TWA concentration estimated with POCIS from 24th May to 7th June and from 7th June to 21th June). Desmethyl norflurazon, norflurazon, diflufenican and tebuconazole concentrations were estimated by average of five punctual sampling realized from 24th May to 21th June (Mean ± SD). ql: quantification limit.

Concentrations (μg/L)	In situ conditions						Experimental conditions in artificial channels
	Upstream station			Downstream station			
	(24th May–7th June)	(7th June–21th June)	Mean	(24th May–7th June)	(7th June–21th June)	Mean	
Azoxystrobine	<ql	<ql	–	0.06	0.04	**0.05**	**0.08**
DCPMU	<ql	<ql	–	0.06	0.07	**0.06**	**0.07**
DEA	0.02	0.01	**0.01**	0.01	0.01	**0.01**	**0.00**
DET	0.02	0.01	**0.02**	0.05	0.05	**0.05**	**0.02**
DIA	0.02	nd	**0.01**	0.01	0.02	**0.02**	**0.01**
Dimetomorph	0.01	0.01	**0.01**	0.15	0.45	**0.30**	**0.27**
Diuron	<ql	<ql	–	0.11	0.17	**0.14**	**0.15**
Simazine	0.01	0.01	**0.01**	0.02	0.02	**0.02**	**0.02**
Terbuthylazine	0.01	<ql	**0.00**	0.02	0.02	**0.02**	**0.01**
Diflufenican			**0.02 ± 0.025**			–	**0.01**
Tebuconazole			**0.002 ± 0.002**			**0.26 ± 0.05**	**0.22**
Norflurazon desmethyl			**0.14 ± 0.01**			**1.08 ± 0.07**	**1.18**
Norflurazon			**0.03 ± 0.01**			**0.26 ± 0.03**	**0.12**
Total			**0.25**			**2.27**	**2.16**

Bold data are means of the 2 sampling periods at each station and the nominal concentrations in artificial channels.

must be mainly due to the development of more tolerant species in this contaminated river section.

3.3. Evaluation of chronic pesticide effects on biofilms contaminated by POCIS extracts in channel experiments

3.3.1. Experimental conditions

The nominal concentrations of the main pesticides applied in the channels as well as the in situ pesticide concentrations are presented in Table 2. Appropriate dilutions of PE were used in order to reach the pesticide concentrations measured at the downstream station estimated by "Quantitative" POCIS and spot sampling in spring 2011 (from 24th May to 21th June). Total pesticide concentrations of about 2270 ng/L were found, with the presence of desmethyl norflurazon, norflurazon, dimetomorph, azoxystrobine, tebuconazole, diuron, diflufenican, terbuthylazine, simazine, DIA, DCPMU, DET and DEA.

Mean physico-chemical parameters and nutrient concentrations along the artificial channel experiment are presented in Table 4 (five samplings performed over the 13 days of the experiment). No significant differences were observed between treatments over the 13 days of the experiment; temperature, conductivity and pH presented mean values of 21 ± 0.2 °C, 367.5 ± 35 μS/cm and 7.6 ± 0.2 respectively.

3.3.2. In vivo fluorescence measurements

Exposure to PE did not significantly impact ΦpsII over the 13 days of exposure in the channels (data not shown). ΦpsII remained stable (mean for all treatments of 0.45 ± 0.01) over the whole experimental duration, nevertheless with a slight decrease on day 7 for upstream with and without PE (ΦpsII = 0.36 ± 0.01; mean for upstream with and without PE).

Regarding fluorescence of Chl *a* measured at 665 nm (Fs_{665}), fluorescence signals linked to cyanobacteria (Fs_{Bl}), diatoms (Fs_{Br}), and green algae (Fs_{Gr}), all values increased through the 13 days of the experiment in all treatments (Fig. 2). At the end of the experiment, no significant difference was shown for Fs_{Bl} and Fs_{Br} levels with or without exposure to PE for upstream and downstream biofilm. In contrast, significant differences were observed for Fs_{Gr} with regard to PE exposure or not. Thus, on day 13, for upstream biofilm, Fs_{Gr} was significantly higher in the treatment with PE compared to the treatment without PE ($p < 0.001$), while we observed a significant lower Fs_{Gr} for downstream biofilm exposed to PE compared to non-exposed biofilms ($p < 0.001$).

Fluorescence levels are often used in field or laboratory studies to assess global algal biomass (Fs_{665}) and the proportion of the different algal groups present in the biofilm (i.e. cyanobacteria, diatoms and green algae) (Barranguet et al., 2000; Serra et al., 2009). Using

Table 3
Pesticide concentrations (μg/L) used for the short term toxicity test with successive dilutions (from d0 to d5; PICT tests). Each dilution is expressed in function of environmental concentrations (mean of total pesticides concentrations) at the downstream site of Morcille River estimated with POCIS in spring 2011 (from 24th May to 21th June). 1× corresponds to environmental concentrations.

Substance group	Substance name	d0	d1	d2	d3	d4	d5
Triazine	Simazine	3.65	1.15	0.36	0.11	0.04	0.01
	DIA (Deisopropylatrazine)	1.80	0.57	0.18	0.06	0.02	0.01
	DEA (Desethyl atrazine)	0.55	0.17	0.06	0.02	0.01	0.00
	DET (Terbuthylazine desethyl)	3.90	1.23	0.39	0.12	0.04	0.01
	Terbuthylazine	2.45	0.77	0.24	0.08	0.02	0.01
Phenylurea	DCPMU	11.80	3.73	1.18	0.37	0.12	0.04
	Diuron	25.60	8.09	2.56	0.81	0.26	0.08
Strobilurin	Azoxystrobine	13.10	4.14	1.31	0.41	0.13	0.04
Morpholine	Dimetomorph	45.50	14.39	4.55	1.44	0.45	0.14
Triazole	Tebuconazole	37.75	11.94	3.78	1.19	0.38	0.12
Carboxamide	Diflufenican	1.32	0.42	0.13	0.04	0.01	0.00
Pyridazinone	Norflurazon	20.00	6.32	2;00	0.63	0.20	0.06
	Desmethyl norflurazon	201.00	63.56	20.10	6.36	2.01	0.64
Concentration factor compared to environmental contamination		148×	49×	15×	5×	1.5×	0.5×

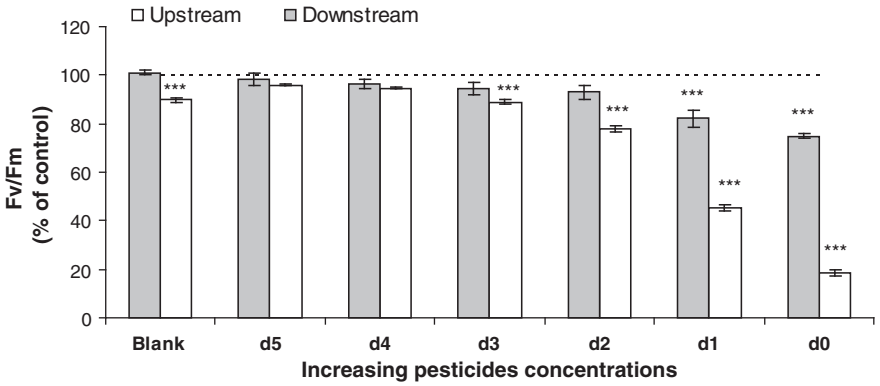

Fig. 1. F_v / F_m (expressed as % of respective controls ± standard deviation) of upstream and downstream biofilm communities exposed to serial dilutions of POCIS extracts (dx) or extraction blank (Blank) at day 0 for 24 h. White and gray bars represent biofilms originated from upstream and downstream of the river respectively. Stars indicate statistical difference from controls (n = 3, *p < 0.05, **p < 0.01, ***p < 0.001).

fluorescence levels as a proxy of biofilm growth, we can conclude that the observed increase of fluorescence levels during the 13 days of experiment was explained by the global growth of biofilm and of the different types of algae.

Nevertheless, fluorescence levels must be interpreted with caution because a fluorescence increase is not always linked to a biomass increase, particularly when dealing with PSII inhibitors (Corcoll et al., 2012a,b). When the photosynthetic apparatus is damaged by exposure to pesticides, the cells were proved able to induce the production of molecules of Chl *a* in order to maintain a suitable photosynthetic activity with the total number of cells remaining unchanged (greening effect) (Guasch et al., 1997; Ricart et al., 2009). Fluorescence can also increase without an increase of Chl *a* concentration when photosynthetic organisms are exposed to pesticides. When algae are exposed to PSII inhibitors, the electron transport chain is blocked, the photochemical pattern is then altered and is no longer available to evacuate the energy captured at the collective antenna, a maximum of fluorescence is then observed (Ralph, 2000). Chl *a* concentrations should not be estimated only by fluorescence measurements when dealing with exposure to pesticides targeting PSII in order to avoid global growth misinterpretation but should be confirmed by traditional Chl *a* measurements (determined spectrophotometrically), biomass determination (DW, AFDM) and/or alga counting. "Real" growth, greening effect and blockage of photosynthetic apparatus by PSII inhibitors can occur together and sometimes it is not possible to distinguish the different effects if discussion is only based on fluorescence measurements.

In our experiment the global increase of fluorescence at 665 nm (Fs_{665}) and of fluorescence signals linked to cyanobacteria (Fs_{Bl}), diatoms (Fs_{Br}), and green algae (Fs_{Gr}) between day 0 and day 13 is clearly mainly explained by biofilm growth over the 13 days of exposure in channels since the increase was observed under all treatments but could due to growth together with greening effect and blockage of photosynthetic apparatus by PSII inhibitors in the treatment exposed to PE.

3.3.3. Biofilm biomass

Dry weight and ash-free dry matter were recorded at the end of the experiment for the different exposure scenarios (Fig. 3). Statistical analyses revealed differences in DW and AFDM after 13 days of exposure to the different treatments in channels. Moreover, these two parameters followed different trends depending on biofilm origin. Exposure to PE led to a significant increase of AFDM for upstream biofilm compared to non-exposed upstream biofilm (380 ± 30 μg/cm^2 and 300 ± 10 μg/cm^2 respectively for upstream biofilm with and without PE, p = 0.049). In contrast, downstream biofilm showed an opposite trend with lower values of DW and AFDM for biofilm exposed to PE compared to non-exposed biofilm (p = 0.049). In this case, DW and AFDM were on average 1.4 and 1.6 times higher for non-exposed biofilms compared to exposed biofilms.

These results can be viewed with respect to the higher fluorescence signals recorded for the upstream biofilms with PE compared to treatment without PE and the lower fluorescence signals recorded for downstream biofilm exposed to PE compared to non-exposed biofilms. Regarding fluorescence signals and biomass at day 13, growth of biofilm was greater with PE for upstream biofilm and without PE for the downstream biofilm. In most field studies: negative effects have been observed with toxicant exposure on DW and AFDM. For example Morin et al. (2010a) recorded lower DW and AFDM in a field study on the Morcille River in spring 2008 for natural communities sampled at the downstream site (chronically pesticide contaminated) compared to the upstream site (reference).

On the other hand an increase of biomass can be observed with physical stress or toxicant exposure due to the production of more abundant extracellular polymeric substance (EPS) matrix. The increase of EPS production has already been observed as a response to physical stress, Artigas et al. (2012) observed a higher EPS content in biofilm colonized in an intermittent river compared to a biofilm growing in a permanent river. Changes in EPS production were also studied under exposure to metal and organic compounds. Fang et al. (2002) showed that marine biofilms increased the production of extracellular polymeric substances by up to 100%, when exposed to metals and chemicals, such as Cd(II), Cu(II), Pb(II), Zn(II), Al(III), Cr(III), glutaraldehyde, and phenol. This increase could be linked to the role of EPS as a protective shield for the cells against the adverse effects of the external environment.

For the upstream biofilm, the increase of DW and AFDM after PE exposure could be linked to the extracellular polymeric substance (EPS) matrix.

Table 4
Mean physico-chemical parameters (±standard deviation, n = 5) in artificial channels over the 13 days for the different treatments (upstream without PE, upstream with PE, downstream without PE and downstream with PE).

	Temp (°C)	pH	Cond. (μS/cm)	O_2 (mg/L)	N–NO_3 (mg/L)	P–PO_4 (mg/L)	Si (mg/L)
Upstream without PE	21.2 ± 0.3	7.5 ± 0.2	374.0 ± 96.9	7.7 ± 0.5	62.2 ± 11.5	3.0 ± 1.5	5.9 ± 0.3
Upstream with EP	21.4 ± 0.2	7.6 ± 0.2	365.7 ± 92.5	7.1 ± 1.0	66.0 ± 10.0	3.0 ± 1.3	6.4 ± 0.1
Downstream without PE	21.5 ± 0.2	7.7 ± 0.2	377.8 ± 96.3	7.7 ± 0.5	59.2 ± 13.0	2.7 ± 1.4	5.9 ± 0.3
Downstream with PE	21.4 ± 0.2	7.6 ± 0.3	352.5 ± 70.6	7.4 ± 0.2	54.0 ± 12.4	2.3 ± 1.3	6.3 ± 0.1

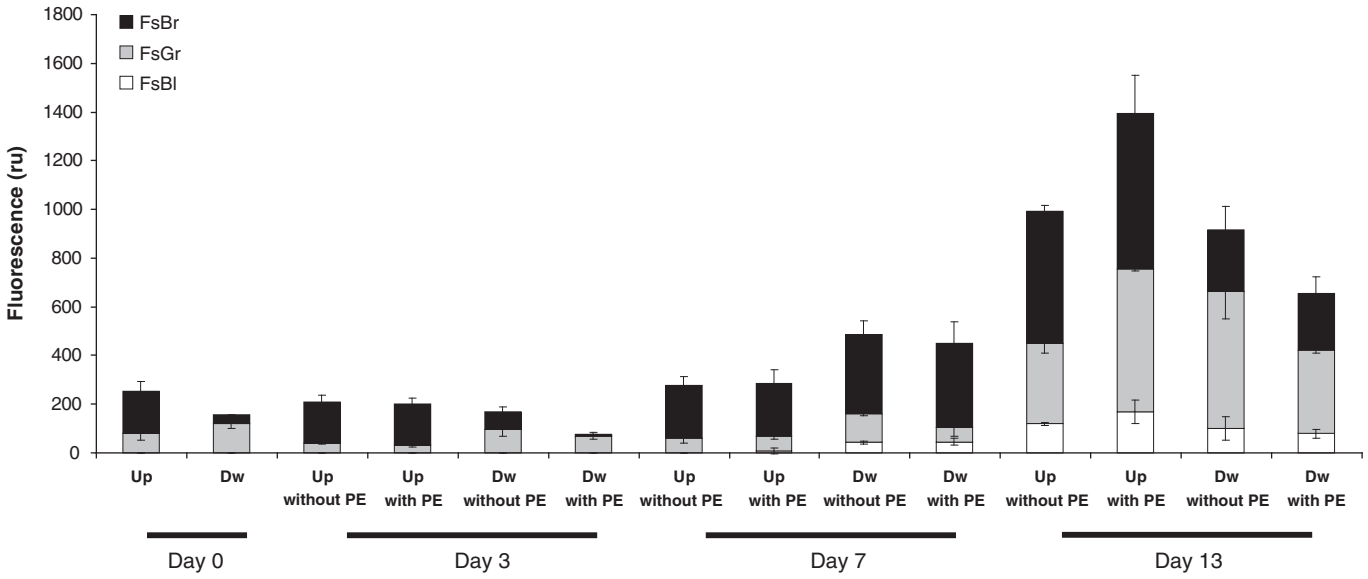

Fig. 2. Fluorescence levels (Fs) (n = 3, mean ± SD), in function of time exposure to the different treatments. Fluorescence levels related to cyanobacteria Fs_{Bl}, green algae Fs_{Gr} and diatom groups Fs_{Br} are expressed in relative units of fluorescence and are represented by the white, gray and black areas respectively. Up: Upstream biofilm; Dw: Downstream biofilm. For clarity, statistical differences are not indicated.

3.3.4. Diatom density

Diatom densities, over the 13 days of direct exposure to PE in artificial channels are plotted in Fig. 4. Statistical analysis revealed that diatom density different from day 0 to day 7 was not significant firstly between the downstream without PE and downstream with PE treatments and secondly between upstream without and upstream with PE. Significant differences in diatom cell densities were observable on day 13 between exposed and non-exposed biofilms independently of biofilm origin. For downstream biofilms the diatom cell density was 1.8 times higher without PE compared to treatment with PE (1570 ± 250 · 10^3 cells/cm^2 for downstream without PE versus 890 ± 150 · 10^3 cells/cm^2 for downstream with PE). Moreover, downstream diatom growth was null between days 7 and 13 for downstream biofilms with PE treatment (920 ± 150 · 10^3 cells/cm^2 at day 7

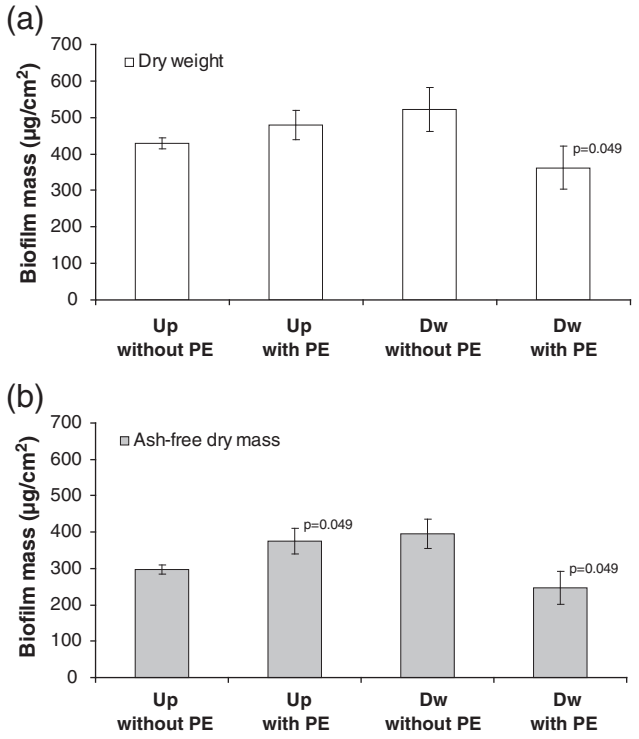

Fig. 3. Dry weight (DW) (a) and ash-free dry mass (AFDM) (b) of total biofilm recorded at day 13 only are expressed in µg/cm^2 (n = 3, mean ± SD). P-values are calculated between biofilms of the same origin with or without PE and are indicated when p < 0.05.

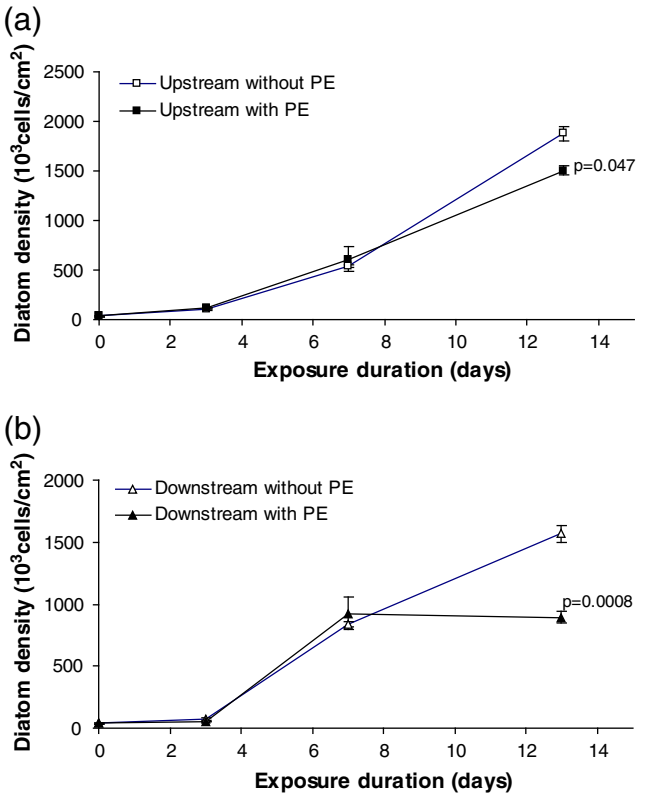

Fig. 4. Total diatom density ± standard deviation versus exposure time for the biofilms originated from upstream (a) or downstream (b) sites of Morcille River and exposed to the different treatments (upstream with PE, upstream without PE, downstream with PE and downstream without PE), p-values are calculated between biofilms of the same origin with or without PE and are indicated when p < 0.05. Solid squares and solid triangles represent contaminated conditions. Open squares and triangles represent conditions without pesticide. PE = POCIS extracts.

versus 890 ± 150 · 10^3 cells/cm^2 at day 13). For upstream biofilms, the diatom cell density was 1.2 times higher without PE compared to treatment with PE (1876 ± 72 · 10^3 cells/cm^2 for upstream control versus 1504 ± 44 · 10^3 cells/cm^2 for upstream with PE). For biofilms of both origins, note that diatom growth initiated at day 3 and densities followed the same trend with a similar order of magnitude.

The significant lower diatom densities observed on day 13 on biofilms exposed to PE compared to non-exposed biofilms highlighted the adverse effects of a mixture of pesticides at realistic environmental concentrations as produced using POCIS extracts. As pointed out in the literature, diatoms are an algal group particularly sensitive to pesticide exposure and thus show great potential for the development of new tools in biomonitoring programs focusing on toxic pollution. The responses of diatoms and more generally of aquatic organisms to chronic exposure to environmental concentrations of mixtures of toxicants are poorly documented. To our knowledge only one study has focused on low dose effects of mixtures of pesticides on river biofilms (Morin et al., 2012b). In that study, the authors exposed biofilms to PE from Morcille River but did not observe any impact of toxicants on diatom density even after 2 weeks of exposure. They attributed this result to probable difficulties in maintaining toxicants at environmentally realistic levels as very low concentrations were applied averaging 0.5 ± 0.1 μg L^{-1}(total pesticide concentrations).

Previous studies have shown the impact on diatom growth of exposure to a single pesticide but after longer exposure durations than in our study. For example (Pérès et al., 1996) reported the effects of low doses of herbicide with a decrease in densities between 87 and 96% compared to the controls after 34 days of direct exposure to 5 μg/L of isoproturon (a phenylurea herbicide). In our study dealing with environmentally relevant concentrations, the effect of contamination by pesticides (upstream with PE) or removal of pesticide pressure (downstream without PE) was significant only at the last sampling time (day 13). It could be interesting to investigate the evolution of diatom densities for longer exposure periods and also to focus on effects between day 7 and day 13 with a shorter time-interval in order to characterize growth kinetics in more detail.

Diatom densities estimated by the counting procedure did not follow the results found with the fluorescence measurements at day 13. No significant difference of Fs_{Br} between exposed or non-exposed biofilm was observed whereas diatom densities were significantly different with or without PE.

DW, AFDM, fluorescence levels and diatom density are often used as classical endpoints to detect long-term effects of chemicals on natural biofilms. We highlight here that biomass parameters (DW and AFDM) and fluorescence levels have to be regarded with caution. DW and AFDM reflect global biofilm growth and responses to toxicant can vary greatly with biofilm origin. Fluorescence levels cannot be directly linked to Chl *a* concentrations and users have to be aware of possible misinterpretation as highlighted by Corcoll et al. (2012a,b). Nevertheless, more global endpoints like diatom densities here showed their suitability to highlight the impacts of realistic complex pesticide mixtures for communities of different origins (upstream and downstream biofilms).

3.3.5. Diatom assemblages

A total of 69 species were identified. The 21 species occurring at more than 2% relative abundance in at least one sample are presented in Fig. 5. Sample clustering clearly differentiated diatom communities at day 0 depending on the origin of the biofilms (Fig. 5a). Species richness was on average higher for upstream biofilms than downstream biofilms (36 ± 4 and 30 ± 3 respectively). Upstream biofilms were dominated by *Nitzschia linearis* var. *linearis* (Agardh) W.M. Smith (16% relative abundance), *Planothidium lanceolatum* (Brebisson ex Kützing) Lange-Bertalot (16% relative abundance) and *Cyclotella meneghiniana* Kützing (15% relative abundance).

Downstream biofilm compositions were characterized by *P. lanceolatum* (31% relative abundance), *Nitzschia palea* (Kützing) W. Smith (12% relative abundance) and *Eolimna minima* (Grunow) Lange-Bertalot (8% relative abundance). Assemblages always presented a great abundance of *P. lanceolatum*, with higher relative abundance at the contaminated site (31% vs 16% relative abundance for downstream and upstream respectively). Moreover, *Achnanthidium minutissimum* (Kützing) Czarnecki was more abundant in upstream than in downstream biofilm (7% and 1% respectively).

After 13 days in the channels, the split already observed at day 0 between upstream and downstream biofilms was still observed (Fig. 5b). Species richness decreased with time of exposure in the laboratory for all treatments (36 ± 4 and 30 ± 3 at day 0 for upstream and downstream biofilms) but without a significant difference between conditions at the end of the experiment (19 ± 1 and 21 ± 3 for upstream without PE and upstream with PE respectively and 15 ± 1 and 14 ± 3 for downstream without PE and downstream with PE respectively). *P. lanceolatum* showed a high relative abundance in all treatments at day 13 (reaching 64% upstream without PE treatment).

The cluster dendogram for day 13 (Fig. 5b) clearly grouped biofilms from the upstream site by treatments (exposure or not to PE). Even though present in large amounts for all treatments, *P. lanceolatum* was less abundant in upstream biofilms exposed to pesticides than in upstream biofilms maintained under control conditions (18% vs 64% for upstream with PE and upstream without PE respectively).

This lower relative abundance of *P. lanceolatum* upstream with PE compared to upstream without PE coincides, in upstream biofilm with PE treatment, with an increase in the relative abundance of *E. minima* (3% and 14% for upstream without and upstream with PE respectively) and *N. palea* (6% and 24% for upstream without and upstream with PE respectively).

The massive growth of *P. lanceolatum* from day 0 to day 13 on upstream biofilms without pesticides was not observed when biofilms were exposed to PE: the relative abundance of this species remained the same as at day 0 (16% day 0 and 18% at day 13) suggesting that the inhibition of its proliferation was caused by pesticide exposure.

Likewise, this lower relative abundance of *P. lanceolatum* upstream with PE compared to upstream without PE treatment paralleled an increase in the relative abundance of *E. minima* (3% for upstream without PE and 14% for upstream with PE) and *N. palea* (6% for upstream without and 24% upstream with PE) suggesting the pesticide tolerant character of both species. Very little is known about the relative sensitivities of diatoms to toxicants. *E. minima* had been described as pesticide sensitive in numerous field studies (Hamilton et al., 1987; Morin et al., 2009; Pérès et al., 1996), but as mentioned above, pesticide gradients often parallel metal and/or nutrient gradients making it difficult to clearly identify the specific effects of pesticides on diatom assemblages in the presence of multi-contamination.

Some recent laboratory studies have investigated the relative sensitivity of single diatom species exposed to pesticides; and have reported *N. palea* and *E. minima* as being relatively tolerant to diuron (Larras et al., 2012; Moisset et al., Personal communication) and *P. lanceolatum* as relatively sensitive to diuron (Moisset et al., Personal communication).

These results suggest a change in diatom assemblages with application of pesticide pressure observable after 13 days, indicating selection by the treatment and thus different sensitivities of diatom species in our reference biofilm.

Concerning downstream biofilm exposed to POCIS extracts or not, no strong difference was observed in taxonomic composition and clustering analysis did not separate biofilm as a function of treatment. For both treatments the assemblages were dominated by *N. palea*, *P. lanceolatum*, *N. linearis*, *C. meneghiniana*, *E. minima* and *Cocconeis placentula* Ehrenberg var. *placentula* (Fig. 5b). So, the trajectory observed for downstream biofilms does not seem to be affected whether pesticide pressure is maintained or not. Several hypotheses could explain this similar evolution in diatom composition with in-channel exposure time.

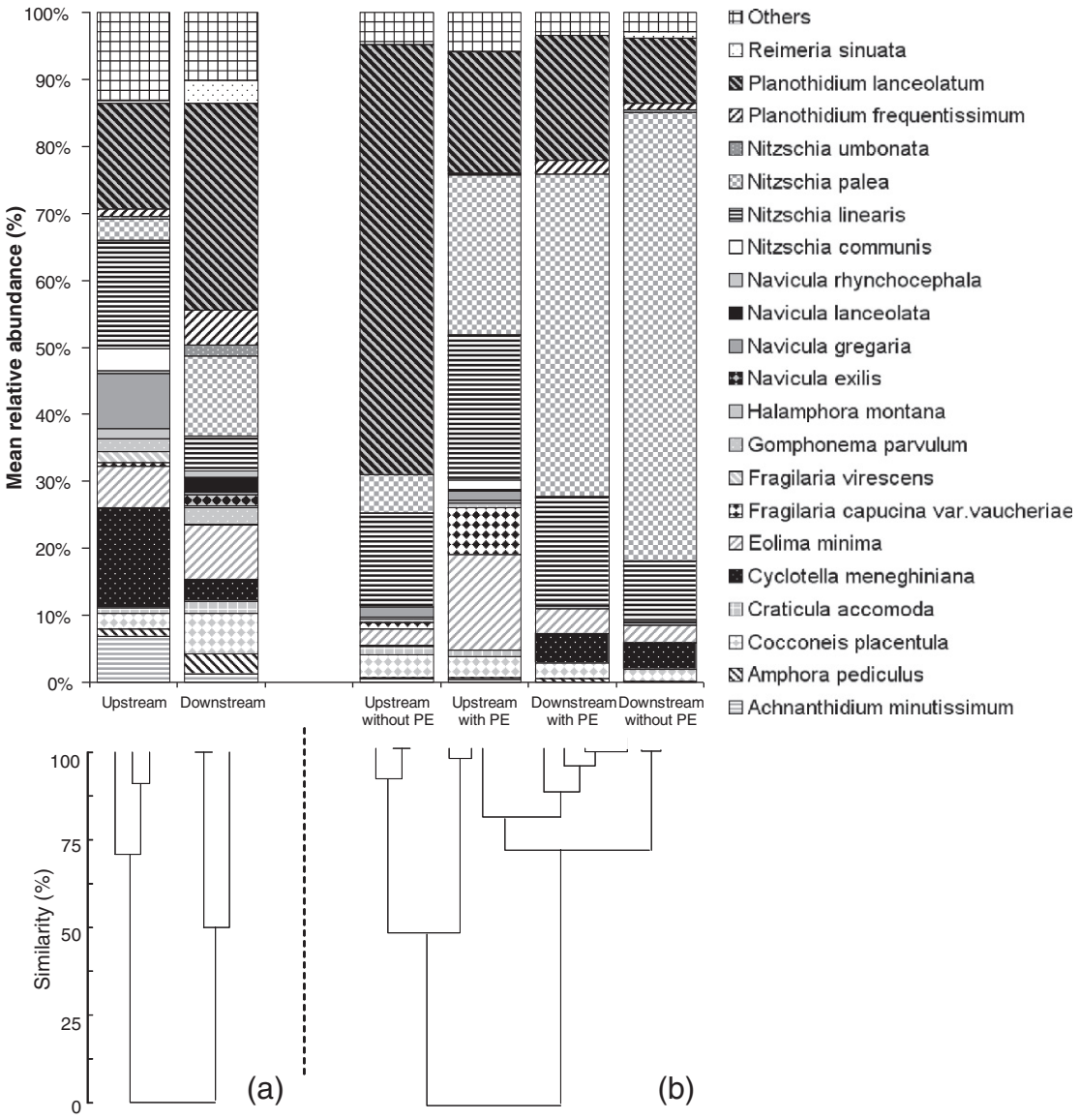

Fig. 5. Mean relative abundances of the 21 dominant diatom species (i.e. representing more than 2% relative abundance in at least one sample) at day 0 (a) and day 13 (b).

The first cause for this result could be related to POCIS selectivity. Since the POCIS preferentially samples compounds with $4 \geq \log K_{ow} \geq 0$, organic compounds outside this range of polarity and of course inorganic compounds are not taken up during sampling. In the case of the Morcille River multi-contamination occurs and the pesticide gradient parallels a metal gradient from upstream to downstream. The cocktail of compounds to which downstream biofilms are exposed in situ includes metals. Actually As and Cu have been recorded at concentrations reaching the Predicted No Effect Concentration (PNEC) at the downstream site of the river (Montuelle et al., 2010). When exposing communities from a multi-contaminated site to PE, the pressure exerted by all the compounds not sampled by POCIS but present in the field is removed. 2–4 D, chlorpyrifos (Assoumani et al., 2013) and metals are examples of such compounds. In our case, most concern lies in metal contamination since organic compounds not sampled by POCIS have been found to remain under the detection limit (data not shown) at the downstream site. Our hypothesis is that metal levels at the downstream site of the river exerted a strong selection pressure, so removal of the metal pressure is an important driver explaining the similarity in the evolution of diatom communities between days 0 and 13. So, the changes in the patterns observed for downstream biofilms do not seem so to be affected by maintenance or removal of the pesticide pressure alone in the case of communities impacted in situ by multi-contamination (metals and pesticides) highlighting the influence of the in situ exposure history on the responses to pesticide exposure.

On the other hand, the importance of immigration processes in diatom assemblage recovery has to be taken into account. Abiotic drivers are generally considered as the major structuring components for natural communities (Stevenson, 1996; Sabater, 2009), here the removal or maintenance of pesticide pressure represented by PE did not influence diatom assemblages. This importance of immigration phenomena in diatom communities has been pointed out in a recent study; Morin et al. (2012a) investigated diatom assemblage recovery in controlled laboratory conditions after chronic copper exposure and did not observe any recovery in assemblages placed under control conditions without external immigrants. In contrast, with the contribution of external immigrants, they observed divergence of assemblages with a return to a "control" assemblage within 2 weeks.

The non-divergence of diatom composition after 13 days of experimental exposure in the laboratory for the in situ pre-exposed biofilms could also be linked to the initial very high tolerance of downstream biofilms to PE (EC_{25} was equal to 142× for downstream biofilm). Some studies have demonstrated that acquired tolerance to

contaminants may remain quite a long time after removing toxic pressure (Dorigo et al., 2010). However, Pesce et al. (2010) observed that a decrease in pesticide exposure can be followed by a slight decrease in biofilm tolerance (as assessed by EC_{50}). Dorigo et al. (2010) ascribed the incomplete recovery to delayed toxicant release from the biofilm matrix. To date, very little is known about tolerance changes of biofilms in response to the reduction of pollution levels, especially in the context of multi-contamination (Pesce et al., 2011a).

The impact of PE exposure was observed with regard to diatom density, biomass, fluorescence levels (only Fs_{Gr}) and taxonomic composition for upstream biofilms. For downstream communities (pre-exposed in situ to pesticides), significant differences with or without PE were observed with regard to diatom density, biomass, and fluorescence levels (only Fs_{Gr}) but not on taxonomic composition. As discussed above for the downstream biofilm, similar diatom assemblages at day 13 could be explained by the impossibility of immigration in laboratory systems and/or by the selective extraction of the POCIS (presence of metal contamination at the downstream station). Thus, in laboratory experiments dealing with passive sampler extracts, conclusions about diatom assemblage drivers have to be regarded with precaution due to the selectivity of passive samplers and to the major role of immigration. Nevertheless, removal of pesticide pressure modeled by the replacement of downstream biofilms on clean water led to higher diatom density, DW, AFDM and Fs_{Gr} compared to biofilm exposed to PE at day 13.

The methodology applied in this study dealing with low doses of PE showed its relevance in revealing the effect of contamination by pesticides ('upstream without PE' compared to 'upstream with PE') and the effect of removal of pesticide pressure ('downstream with PE' compared to 'downstream without PE').

4. Conclusions

The use of extracts from passive sampling devices in biological tests has emerged this last decade (Harman et al., 2012). It especially allows issues related to mixtures, interactions and unknown compounds to be studied. POCIS extracts have been used in a range of acute toxicity tests for the assessment of particular toxicity (e.g. estrogens, PSII inhibitors) with very promising results but studies evaluating low-dose and long-term effects of POCIS extracts are still in their infancy (Morin et al., 2012b). This study is one of the first to deal with the use of passive sampler extracts for toxicity assessment using chronic exposure on natural biofilm communities. The sensitivity of biofilm communities to PE increases from downstream to upstream, revealing the past history of communities by in situ tolerance induction in the contaminated site of the river as expected from the PICT concept. Chronic exposure to PE in artificial channels at environmental concentrations showed significant effects on growth-related (dry weight, ash free dry mass and diatom cell densities) and structural (fluorescence levels and diatom composition) parameters. However, trends were different for some of them depending on the biofilm origin thus highlighting the crucial importance of exposure history in biofilm responses. The use of fluorescence levels to assess global biofilm growth and relative percentages of different algal groups was discussed and has to be considered with caution in studies dealing with PSII inhibitors. It was also highlighted that any conclusions about diatom assemblage drivers has to be made with prudence due to the selectivity of passive samplers and to the major role of immigration which is absent when working in controlled laboratory conditions. Our study showed the importance of taking into account chronic effects of mixtures in risk assessment studies and the relevance of the use of passive sampler (like the POCIS) extracts in this approach.

Conflict of interest

The authors do not have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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