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Genetic and physiological responses of three freshwater diatoms to realistic diuron exposures

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Abstract This study examined the effects of diuron on strains of three major freshwater diatom species, *Eolimna minima*, *Nitzschia palea* and *Planothidium lanceolatum*. These species are frequently recorded in the Morcille River, where diuron runs off during phytosanitary treatments of the vineyards around. Here, there were three diatom exposure groups for each species: 0, 1 and 10 μg/L diuron during a 14-day laboratory assessment. Diuron water concentration, cell number, photosynthetic activity and gene expression were assessed at 6 h and 2, 7 and 14 days after contamination. Diuron exposure altered photosynthetic activity in that the optimal quantum yield of photosystem II (PSII) decreased between 40 and 50 % and, for *P. lanceolatum* at 10 μg/L, there was complete inhibition. Genetic responses indicated diuron effects on both photosystem II and mitochondrial metabolism in all three species at both diuron exposure levels. Thus, analysis of the expression of *psaA*, *d1*, *cox1*, *nad5* and 12s could be an early biomarker to detect pesticide pollution. Overall, this study revealed differences in diuron sensitivity among the three species: *E. minima* and *N. palea* appeared to be more tolerant than *P. lanceolatum*. These results suggest that the development of molecular tools, and more precisely of biomarkers, will aid in early assessment of contamination and water quality.

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Introduction

The EU Water Framework Directive (WFD) (2000/60/EC) requires European countries to assess the ecological status of water bodies. This policy of preservation and restoration of aquatic ecosystems aims to achieve good ecological status of water bodies by 2015. Thus, tools and methods to evaluate contamination, as well as characterization of ecological impacts associated with aquatic systems, must be improved.

Ever-increasing agricultural activities have led to the contamination of surface and ground waters, creating strong, long-lasting degradation of aquatic ecosystems. Thus, streams running through these polluted areas are very sensitive to contamination by phytosanitary compounds, like diuron, which is frequently found in rivers in France and throughout Europe (Loos et al. 2009; SOeS 2013). Furthermore, although diuron is prohibited in most European countries, including France since 2009, it is often found at levels above the maximum acceptable concentration of 1.8 μg/L fixed by the WFD.

In streams and rivers, most microorganism activity is from periphytic communities which are key players in the general functioning of aquatic ecosystems, and more precisely diatoms which, at certain times of the year, represent the majority of periphyta (McIntire et al. 1996). Because of their position at the base of trophic food webs, they play an important role in major ecological processes, such as photosynthesis and nutrient recycling. Furthermore, their capacity for adaptation and their tolerance of environmental factors (light, temperature, etc.) and aquatic pollution make them relevant bioindicators

(Potapova and Charles 2002). Indeed, facing environmental pressure, these communities can adapt irreversibly or temporarily, leading to structural, diversity and functional changes.

Over the past decades, the development of transcriptomics, genomics and proteomics has allowed, via molecular biology, development of new genetic descriptors called biomarkers. These are not only assets for determining cellular impacts associated with environmental pollutants but they can also help to better define the levels of contamination likely to have toxic effects on communities. Use of these new genetic descriptors is currently lacking for the freshwater microalgal compartment. Thus, only a few diatom nucleotide sequences are available in genomic databases and only two marine diatom species have been entirely sequenced (Armbrust et al. 2004; Bowler et al. 2008).

Pesticide penetration into cells can induce oxidative stress which (i) might be responsible for DNA breakage and/or (ii) might affect mitochondrial and/or photosynthetic metabolism and induce apoptotic mechanisms leading to cell death (Fukuyama et al. 2009; Rutherford and Krieger-Liszkay 2001; Slaninova et al. 2009). Molecular descriptors could be indicators of this type of damage by revealing early and specific cellular effects. Kim Tiam et al. (2012) successfully used these descriptors in experimental cadmium contamination and showed their sensitivity and early response compared to more integrative measures (growth rates). Furthermore, they can complete index methods based on identification of the structure of diatom communities, which are still insufficiently sensible to evidence effects of toxic compounds.

This study was done on strains from three freshwater diatoms common to the Morcille River (Beaujolais) which are exposed to pesticides, in particular to diuron, during phytosanitary treatments of vineyards in this region (Morin et al. 2010). This herbicide, a member of the substituted urea family, targets photosynthetic processes by preventing the formation of oxygen. Diuron binds with high affinity at the Q_B-binding site of photosystem II, preventing Q_B from binding at this location and therefore blocking the electron transport chain (Zer and Ohad 1995; Trebst and Draber 1986). Although its persistence in soil is low (<1 year), it may last more than 3 years in water. This substance is detected in the aquatic compartment mainly due to soil leaching and overland flow (Brignon and Gouzy 2007).

Consequently, an objective of this work was to characterize genes involved in photosynthetic and mitochondrial metabolism. The responses of strains from three species commonly encountered in the Morcille River, *Eolimna minima* (Grunow) Lange-Bertalot, *Nitzschia palea* (Kützing) W. Smith and *Planothidium lanceolatum* (Brébisson ex Kützing) Lange-Bertalot were assessed during a 14-day laboratory exposure to 0, 1 or 10 μg/L of diuron. These moderate concentrations correspond to those frequently reported in rivers like the Morcille, and the highest is comparable to those reported during phytosanitary treatment episodes (Rabiet et al. 2010).

To determine the impact of diuron on these three major species, diuron concentrations, pulse-amplitude-modulated fluorescence measurements, cell quantification and genetic responses were followed at 6 h and 2, 7 and 14 days after exposure. Additionally, specific target genes, expected to respond earlier and more efficiently than current water quality diagnostic tools, were tested as genetic descriptors.

Materials and methods

Experimental protocol to assess diuron effects on *E. minima*, *N. palea* and *P. lanceolatum*

Algal cultures

Strains from three freshwater species were chosen for the study: *E. minima*, *N. palea* and *P. lanceolatum*. These diatoms are among the most common in the Morcille River (Beaujolais) and are easy to cultivate in the laboratory. *E. minima* was collected and isolated from periphytic biofilm sampled in the Morcille River by micropipetting under a light microscope (Roubeix et al. 2011). Species identification was confirmed by scanning electron microscopy. *N. palea* and *P. lanceolatum* strains, isolated from freshwater lotic environments, were provided by the Thonon Culture Collection (http://www6.inra.fr/carrtel-collection/, strain reference numbers, respectively, TCC583 and TCC615).

Exposure conditions

Each culture of diatoms (250 mL), in the exponential growth phase, was suspended in 500-mL Erlenmeyer flasks in a modified Dauta medium (supplemented with silica to a final concentration of 10 mg/L) (Dauta 1982), to reach an initial concentration of 12.10^4 cells/mL. The organisms were directly exposed to three concentrations of diuron (C_0=0, C_1=1 and C_2=10 μg/L) for 14 days, made from a diuron stock solution (1 mg/L, dilution in ultrapure water). All the species × concentration combinations were performed in triplicate, resulting in a total of 24 experimental units.

The cultures were maintained between 20 and 22 °C with a photon flux density of 58±8 μmol m^{-2} s^{-1} and a 12:12 h light/dark cycle. The Erlenmeyer flasks were closed with sterile cotton wool and kept on an orbital shaker (60 rpm). Flasks were set up in triplicate, resulting in a total of 27 experimental units.

Sampling and kinetic times

Diuron impact on diatoms was assessed after 6 h and 2, 7 and 14 days of exposure (hereafter called 6 h, D2, D7 and D14). At each sampling time, 6 mL of culture suspension was sampled for water diuron analyses, 2 mL for cell counting, 5 mL for pulse-amplitude-modulated fluorescence measurements and 35 mL for genetic analyses.

Laboratory analysis

Diuron analysis

Culture suspension (6 mL) was filtered with regenerated cellulose membranes (0.45 μm) (Whatman, Versailles, France), and samples were stored at 4 °C. The 6-mL filtered samples from C_1 were pre-concentrated to 1 mL with a SpeedVac SPD121P (Thermo), whereas a dilution of the filtered samples from C_2 was performed. Whatever the sample, an aliquot of 1 mL was spiked with 10 μL of a diuron-d6 (Dr. Ehrenstorfer GmbH, Augsburg, Germany) solution (1 ng/μL) as an internal standard, prior to analysis by liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). Chromatographic separation was done on a Gemini-NX C18 3 μm, 110A, 100 mm×2 mm equipped with a SecurityGuard from Phenomenex (Le Pecq, France) and an HPLC Ultimate 3000 (DIONEX, Voisins-le-Bretonneux, France), and quantification was carried out with a triple quadrupole mass spectrometer API 2000 (AB SCIEX, Les Ulis, France). The two eluents were acetonitrile (A) and ultrapure water with 5 mM ammonium acetate, and a linear gradient was used: 10 % A for 1 min, then 30 % A after 4 min, 40 % A after 8 min, 80 % A after 9.5 min, followed by 90 % A until 10.5 min, then a decrease of A to 10 % after 11 min which was maintained for 4 min. The total running time was 15 min and the flow rate was kept constant at 400 μL/min. Selected reaction monitoring (SRM) mode was used for quantification of both diuron (233>72 and 233>46 SRM transitions) and Diuron-d6 (239>78). Sample injection volume was 50 μL. Further details regarding sample preparation and analysis can be found elsewhere (Lissalde et al. 2011). Instrument quantification and detection limits were respectively 1 and 0.3 μg/L.

For samples with a concentration between the quantification and detection limits, a one-half value of the quantification limit was used in the statistical analyses (Helsel 1990).

Diatom cell density

The 2-mL aliquots were immediately fixed in formalin (37 % formaldehyde, Prolabo, France) for counting. Each sample was counted using a Nageotte counting chamber (Marienfeld, Germany). After homogenization using a vortex, 200 μL of sample was placed in the counting chamber and the total number of cells were recorded in ten fields of the gridded area (1.25 μL each, 0.5 mm depth) under light microscopy at ×400 magnification (Olympus BX51 photomicroscope). Distinction between dead and live organisms before fixation was by observation of the turgescence and colour of the chloroplast as described in Morin et al. (2010).

Photosynthetic efficiency

The optimal quantum yield of photosystem II (F_v/F_m) was measured using pulse-amplitude-modulated fluorometry (PhytoPAM, Heinz Walz GmbH, Germany). F_v/F_m was measured after 30 min of dark adaptation and is described by the Genty et al. (1989) equation:

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

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.

Genetic analysis

RNA extraction In order to access the diatom genetic material, RNA extraction was performed. Falcon tubes containing the samples were defrosted in a water bath at 25 °C then centrifuged at 1,850*g* for 7 min at room temperature. The supernatant was removed and the diatom cell pellet resuspended in 1 mL of Trizol (Invitrogen) then transferred to 1.5-mL microtubes. Three hundred microlitres of glass beads (2.5 μm diameter, Sartorius AG) was added to the cell pellet, and an MPFastPrep machine (40 s at 6 m/s) crushed the diatoms. The supernatant was transferred to a clean microtube and placed in a water bath at 37 °C for 5 min. After addition of 200 μL of Chloroform RECTAPUR (VWR), the sample was vortexed for 10 s and placed in a water bath at 37 °C for 2 min. The tubes were centrifuged at 8,000*g* for 5 min at room temperature, and the aqueous phase containing the genetic material was transferred into clean tubes. The end of the extraction was performed using Absolutely RNA Miniprep Kit (Stratagene) according to the manufacturer's instructions: Briefly, 550 μL of 75 % ethanol was added to the aqueous phase; the sample was mixed, transferred to affinity columns and centrifuged for 1 min at 8,000*g* at room temperature. Filtrates were removed, and 600 μL of low-salt buffer was placed on the column which was centrifuged at 8,000*g* at room temperature for 2 min. Five microlitres of DNAse 1 [1 U] and 50 μL of activity buffer were added to the column and placed in a water bath at 37 °C for 15 min. Six hundred microlitres of high-salt buffer was added to the column which was centrifuged for 1 min at 8,000*g*. Three hundred microlitres of low-salt buffer was added and the column

was centrifuged for 2 min at 8,000*g*. The columns were transferred into clean tubes, and 30 μL of elution buffer at 60 °C was added and centrifuged for 1 min at 8,000 g at room temperature. Total RNA was analysed on a 1 % (*w*/*v*) agarose gel with ethidium bromide by UV light on an illuminator.

Reverse transcription of RNA The first strand of complementary DNA (cDNA) was synthesized from 14 μL of total RNA (3 to 5 μg) using the StrataScript first strand synthesis system (Agilent). After the addition of 1 μL of oligo(dT) [1 μM], 1 μL of random primers [1 μM], 0.8 μL of dNTPs [10 mM] and 2 μL of 10× first-strand buffer, the reaction was incubated for 5 min at 65 °C. Then, 1 μL of StrataScript reverse transcriptase [1 U/μL] and 0.5 μL of RNase inhibitor [0.5 U] were added, and the reaction was incubated for 1 h at 42 °C in an Eppendorf Mastercycler. The cDNA mixture was conserved at −20 °C until it was used in a real-time PCR.

Cloning and molecular characterization of the target genes Molecular characterization of the target genes was performed prior to the diuron exposure experiment for *E. minima*, *N. palea* and *P. lanceolatum* cultures. Six genes involved in responses to environmental contamination and/or whose expression could possibly be disturbed by environmental factors were characterized. Three were genes involved in mitochondrial metabolism (*nad5*, 12s, *cox1*), two encoded for major proteins of photosystems I and II (*psaA* and *d1*, respectively) and one was a reference gene (β *act*). Primers were designed by performing ClustalW alignments from corresponding sequences belonging to other phylogenetically related aquatic organisms available in databases. From these alignments, primer pairs were designed in the most conserved regions for amplification of fragments between 280 and 900 bp. PCRs were performed following the manufacturer's instructions. After the addition of 1 μL of dNTP [10 mM], 3 μL of $MgCl_2$ [25 mM], 0.2 μL of *Taq* [5 U/μL], 10 μL of activity buffer 5×, 0.5 μL of each primer (upstream and forward primers) [100 μM], 34 μL of diethylpyrocarbonate (DEPC)-treated water and 1 μL of cDNA, 40 PCR cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min were carried out in an Eppendorf AG thermocycler. PCR products were analysed on a 1 % (*w*/*v*) agarose gel with ethidium bromide. After a purification step using the PCR purification kit (Qiagen) according to the manufacturer's instructions, cDNA was cloned with pGEM®-T (Promega). Successful insertion of the fragments was checked by PCR using T7 and SP6 universal primers following the manufacturer's instructions (40 cycles at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min).

q-RT-PCR primer design and real-time q-PCR From the molecular characterization of the target genes, compatible q-RT-PCR primer pairs for the three species (Table 1) were determined by performing alignments via the ClustalW. Real-time PCRs were performed in a LightCycler (Roche) following the manufacturer's instructions (1 cycle at 95 °C for 1 min and 50 amplification cycles at 95 °C for 5 s, 60 °C for 5 s and 72 °C for 20 s). Each 20 μL reaction contained 1 μL of activity buffer (Syber Green I (Roche), *Taq* Polymerase, dNTP), 3.2 μL of $MgCl_2$ [25 mM], 2 μL of the gene-specific primer pair at a final concentration of 300 nM for each primer, 12.8 μL of DEPC-treated water and 1 μL of cDNA. For negative controls, cDNA was replaced by DEPC-treated water. Specificity was determined for each reaction from the dissociation curve of the PCR product. This dissociation curve was obtained by following the SYBR Green fluorescence level during a gradual heating of the PCR products from 60 to 95 °C. Relative quantification of each gene expression level was normalized according to β-actin gene expression. Relative mRNA expression was generated using the $2^{-\Delta CT}$ method (Livak and Schmittgen 2001).

Table 1 Specific primer pairs for the six genes of *Eolimna minima*, *Nitzschia palea* and *Planothidium lanceolatum* used in our study

Species	Gene name	Primer (5′–3′)
All	*psaA*	GGTCAAGAAATTTTAAATGGTGA[a]
		TAGTGGAACCAACCAGCAAATA[b]
All	*12s*	AGGATGCAAGTGTTATCCGGA[a]
		CAATATCTACGCATTTCACCGCT[b]
All	*d1*	TCTGCAGTATTCTTAGTATACC[a]
		CAGCCATGTGGAATGGGTGC[b]
E. minima	*cox1*	ATAGAAGCAGCACCTGAAAGA[a]
		GTTTTTGGTTATTACCACCTTC[b]
N. palea and *P. lanceolatum*	*cox1*	CAGTAATTCTCACTGCCCAGC[a]
		GTTTTTGGTTATTACCACCTTC[b]
E. minima	*nad5*	TGCTATGGAAGGTCCTACTCC[a]
		CTAATCCTGTTGTGGAAGCAAA[b]
N. palea and *P. lanceolatum*	*nad5*	TCAACTTGGTTTGCATACATGGC[a]
		TTGAACTAATCCTGTTGTGGAAGC[b]
E. minima	*act*	CCAACCGCGAGCGCATGACG[a]
		CGACAGGACGGCCTGGATGTT[b]
N. palea and *P. lanceolatum*	*act*	GGCTCCACAAAACCCCAAG[a]
		GGCGTACCCCTCGTAGAT[b]

psaA psaA protein, *12s* ribosomal RNA 12s, *d1* D1 protein, *cox1* cytochrome C oxidase subunit I, *nad5* NADH dehydrogenase subunit 5, *act* β-actin

[a] Upstream primer

[b] Forward primer

Statistical analysis

All statistical treatments were performed with R software (Ihaka and Gentleman 1996). Prior to analyses, data were

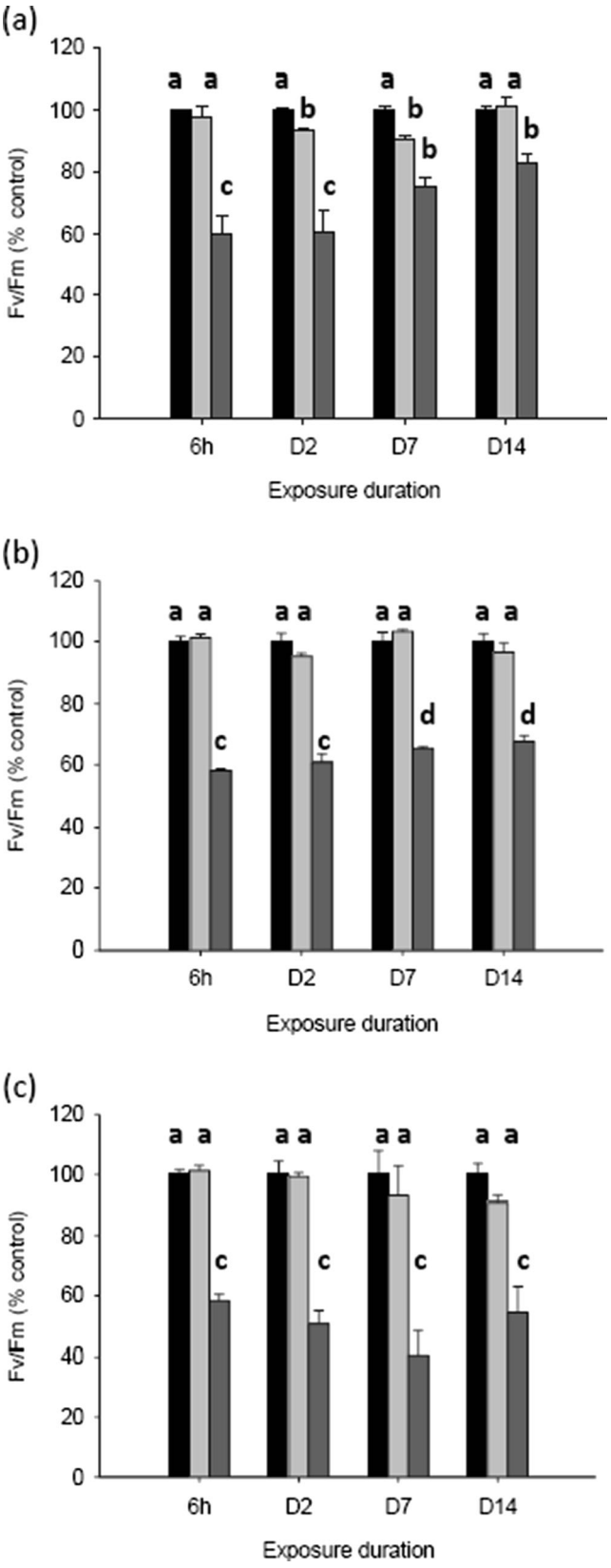

Fig. 1 Optimal photosynthetic quantum yield of *Eolimna minima* (**a**), *Nitzschia palea* (**b**) and *Planothidium lanceolatum* (**c**) after dark adaptation (30 min), for the four sampling times and three exposure conditions. C_0=0 μg/L (*black bars*), C_1=1 μg/L (*light grey bars*) and C_2=10 μg/L (*dark grey bars*) of diuron. Differences between times and contamination are marked with *different letters* at level $p<0.01$. Mean± SE are shown

checked for normality and variance equality. As the data were non-normal, differences in diuron concentration (n=3), effects of diuron exposure on the optimal quantum yield of photosystem II (n=3), cell quantification (n=3) and the genetic response (n=3) were tested by the non-parametric Kruskal-Wallis test (kruskal.test) to evidence between-treatment differences. Values are given in average±standard error.

Results and discussion

Effects of diuron on *E. minima*, *N. palea* and *P. lanceolatum* during laboratory contamination

Diuron water concentrations

During the 14 days of exposure, diuron water concentrations were stable and close to starting concentrations. Indeed, mean (±standard error) diuron concentrations over the experiment were 0, 1±0.2 and 10.9±1.3 μg/L for starting concentrations of 0, 1 and 10 μg/L, respectively.

Hartgers et al. (1998) and Jones et al. (2003) argued that between 1 and 4.5 μg diuron/L are necessary for an effect. No more than slight effects were thus expected in C_1, at the low limit of this range. Moreover, in some cases, diuron concentrations in C_1 were below the starting concentration (e.g. for *P. lanceolatum* and for *N. palea* on D7). This phenomenon could be attributed to absorption and/or adsorption by the diatoms, adsorption to the flask walls (even if Erlenmeyer glass was used) (Pesce et al. 2006) or higher bioaccumulation of the pesticide either due to differences in specific uptake capacity (*P. lanceolatum*, compared to the two other diatoms) or linked to growth kinetics (peak of *N. palea* on D7).

Pulse-amplitude-modulated fluorescence measurements

The optimal quantum yield of PSII during 14 days of direct exposure to diuron is plotted in Fig. 1 as percent of control; thus for C_0, the optimal quantum yield measurement always equals 100 %.

PhytoPAM is a method based on the quantitative relationship between chlorophyll fluorescence and the efficiency of photosynthetic energy conversion (H. Walz GMBH, Effeltrich, Allemagne) and may therefore be used to evaluate the physiological state of algae. Under stress conditions, reactive centres are saturated faster and incoming light is used less efficiently (Laviale 2008). Thus, variations in the optimal

quantum yield reflect the physiological state of and potential damage to an organism. Under non-exposed conditions, the optimal quantum yield of our strains ranged from 0.11±0.01 for *E. minima* to 0.15±0.01 for *P. lanceolatum* and 0.23±0.01 for *N. palea* (n=18 for each culture, data not shown).

Mean optimal quantum yield measurements and overall kinetics at C_1 for the three species were 99.1±4.6 % of C_0, indicating that, at 1±0.2 μg/L of diuron, there was no important impact on PSII functioning. However, for *E. minima* at C_1, the mean optimal quantum yield was significantly lower than at C_0 on D2 and D7 (on average 92.2±1.1 % of C_0); this suggests a possible impact of diuron on photosynthetic capacities at environmentally realistic concentrations in this species.

For strains from the three species at all times, the optimal quantum yields were significantly lower at C_2 (10.9±1.3 μg/L diuron) than at C_0 and C_1. *E. minima*, *N. palea* and *P. lanceolatum* showed similar responses: 6 h after contamination, the optimal quantum yield decreased 45.5±6.9 %, highlighting the fast mode of action of diuron (Legrand et al. 2006). From D7, the optimal quantum yield of *E. minima* and *N. palea* increased, reaching 73±8 % of the control at D14, an increase of 17.5 %. Thus, adaptive mechanisms seem to significantly reduce the diuron impact in these organisms. According to Ricart et al. (2009), diuron contamination induces the formation of shade-type chloroplasts which allow compensation for the reduction in photosynthetic efficiency. Conversely, *P. lanceolatum* optimal quantum yield at C_2 on D7 and D14 stayed at a low mean value of 51±7.6 %.

Thus, the three diatom strains studied can be separated into two groups: *E. minima*/*N. palea* which better resist diuron contamination and *P. lanceolatum* which is more sensitive. So far, few laboratory studies have assessed responses of isolated diatom species to diuron contamination (Larras et al. 2012). However, diatom responses to other pesticides have been assessed in field studies and the sensitivity/tolerance to these compounds has been well described. In a field study conducted by Morin et al. (2009), *E. minima* and *N. palea* were found in areas low in pesticides whereas *P. lanceolatum* was found in areas with higher pesticide levels, including photosystem II inhibitors. These results are contrary to the laboratory results obtained here, underlining the need for combined and complementary laboratory and field research. Indeed, field contaminations are generally composed of mixtures of substances, making the assessment of the toxicity of single substances difficult. Moreover pesticide contamination is often found in conjunction with metallic and/or nutrient contamination (Montuelle et al. 2010), making it difficult to clearly identify the particular effects due to pesticides on diatom assemblages.

Indeed, the complexity of natural environments can modulate the effects of a pollutant, by synergism (e.g. between light and pesticides; Guasch and Sabater 1998) or antagonism (e.g. nutrients stimulating growth; Lozano and Pratt 1994).

Effect of diuron on the growth of E. minima, N. palea and P. lanceolatum

The densities of *E. minima*, *N. palea and P. lanceolatum* strains over the 14 days of direct exposure to diuron are shown in Table 2. Statistical analysis revealed clear growth for the three species of diatoms over the 14 days except for *P. lanceolatum* at C_2.

Globally, *E. minima* and *N. palea* had the same pattern of growth kinetics. Statistical analysis revealed that growth was not significantly different between controls (C_0) and diuron-contaminated conditions of either 1±0.2 or 10.9±1.3 μg/L over the whole duration of the experiment. Thus, diuron, even at a high concentration, does not impact the growth kinetics of *E. minima* and *N. palea*.

On the contrary, for *P. lanceolatum*, the control and C_1 conditions showed positive growth, with classical exponential growth curves. However, at C_2, no growth was observed; the cell number remained low and stable over the 14 days of exposure. Furthermore, the number of dead cells was significantly higher at C_2 (17±2 %) compared to those of C_0 and C_1 (11.4±4 %). As observed for photosynthetic measurements, the growth kinetics were impacted negatively by a high concentration of diuron. Similar results have been reported; the diatom *Navicula accomoda* showed decreased growth when exposed to atrazine (Leboulanger et al. 2001).

The number of dead *N. palea* and *P. lanceolatum* increased significantly over the 14 days of diuron exposure, underlining classical mortality of an algal culture. In contrast, at all concentrations, the number of dead *E. minima* was significantly higher than those of the two other species, with an average of 21±3 % dead cells at 6 h. This culture was the oldest of the three, but also the most concentrated. It is not surprising then, as it was subjected to limiting growth conditions, that it contained the highest proportion of dead individuals. Furthermore, a significant decrease in the number of dead cells was observed after D2. This phenomenon was likely linked to revived growth of this species due to dilution of the strain under experimental conditions.

To conclude, growth kinetics of the three diatom species was not impacted by 1 or 10 μg/L of diuron, except for *P. lanceolatum* at the higher concentration. This parameter, then, appeared less sensitive than optimal photosynthetic yield to determine pesticide impact.

Gene expression levels

Five target genes were cloned and sequenced. In order to get Q-PCR primers compatible with the three species, alignments were performed with ClustalW software. For the target genes *psaA*, 12s and *d1*, the nucleotide sequences of the three diatoms were used. For *cox1* and *act*, only the nucleotide

Table 2 Total density of *Eolimna minima*, *Nitzschia palea* and *Planothidium lanceolatum* (×1,000 cells)±standard errors and corresponding cell mortality (in parentheses), for the four sampling times and three exposure conditions

Species	Exposure conditions	Time exposure			
		6 h	2 days	7 days	14 days
Eolimna minima	C_0	166±16a (24±2 %)	310±48b (26±1 %)	1.182±170c (14±2 %)	1.610±474c (13±2 %)
	C_1	275±46b (21±4 %)	521±54b (26±2 %)	540±150c (21±8 %)	1.436±613c (12±0 %)
	C_2	275±33b (19±1 %)	367±10b (21±4 %)	1.279±481c (15±1 %)	1.470±179c (13±3 %)
Nitzschia palea	C_0	160±17a (5±1 %)	697±103b (9±3 %)	1.847±441c (13±3 %)	1.230±135c (12±2 %)
	C_1	180±18b (5±1 %)	636±49b (11±6 %)	2.172±464c (11±1 %)	1.496±213c (10±2 %)
	C_2	198±26b (4±2 %)	354±42b (11±3 %)	1.685±181c (11±4 %)	1.517±144c (12±4 %)
Planothidium lanceolatum	C_0	98±15a (1±0 %)	141±37a (8±1 %)	534±160b (9±2 %)	515±94b (11±5 %)
	C_1	86±6a (1±0 %)	636±49a (11±6 %)	440±248b (6±1 %)	660±213b (10±2 %)
	C_2	158±49a (0±0 %)	124±9a (7±1 %)	138±42a (14±3 %)	148±54a (17±2 %)

Significant differences between the different times and conditions of contamination are marked with different letters at level $p<0.05$

C_0 0 μg/L, C_1 1 μg/L, C_2 10.9 μg/L of diuron

sequence of *E. minima* was available. The sequences of *N. palea* and *E. minima* were used for the gene *nad5*. The q-RT-PCR primer pairs are shown (Table 1).

Gene expression levels of *E. minima*, *N. palea* and *P. lanceolatum* during the 14 days of diuron exposure are shown in Table 3. The differential gene expression indicates diuron effects on photosynthetic and mitochondrial metabolism at both concentrations after only 6 h of exposure.

Gene expression levels in E. minima

At 1±0.2 μg/L of diuron, downregulation of the five target genes was detected at 6 h. From D2 to D7, gene expression levels of *E. minima* were equal to that of the control; except for *d1* (PSII) and 12s (downregulations of 0.5 for both). Finally, on D14, induction of *psaA*, *d1*, *cox1* and 12s was detected (upregulations, respectively, of 13, 16, 5.5 and 2.5).

At 10.9±1.3 μg/L of diuron (C_2), inhibition of *psaA* (PSI), *cox1* and 12s expression was observed at 6 h (same level of downregulation as for C_1). At D2, all the studied genes are upregulated, on average by 4.2. Conversely, at D7, gene expression levels were back to normal, the same level as those of the controls. Finally, at D14, upregulations of the genes implicated in photosystem metabolism (*psaA* and *d1*) and the respiratory chain (*cox1*) appeared.

Gene expression levels in N. palea

At 1 μg/L of diuron (C_1), there was a downregulation of the genes implicated in photosynthesis processes 6 h after exposure began. By D2, the expression of those genes was back to normal, i.e. control levels, and a downregulation of the genes implicated in mitochondrial metabolism appeared (inhibition of *cox1* and *nad5*). At D7, the two mitochondrial genes were strongly upregulated (inductions, respectively, of 29 and 9), and at D14, only the *psaA* gene was upregulated.

At 10 μg/L of diuron, as for C_1, there was also an inhibition of the photosynthetic genes at 6 h, as well as an induction of *nad5*. This upregulation of the mitochondrial genes was amplified from D2 to D7, to reach levels of 26 and 4.5, respectively, for *cox1* and *nad5*. Finally, at D14, the genes *d1*, 12s and *psaA* were upregulated.

Gene expression levels in P. lanceolatum

At C_1, there was a downregulation of the gene implicated in PSI (*psaA*) and the 12s gene after 6 h of exposure. At D2, an induction of almost all genes was observed. There was particularly strong upregulation of *psaA*, *d1 and 12s* (levels were multiplied by 79, 51.5 and 55, respectively, between 6 h and D2). From D7 to D14, gene expression levels were similar to those of controls.

For C_2, there was strong upregulation at 6 h (induction of 109.5 for *cox1*, 22.5 for 12s and 16 for *psaA*) and D2 (induction of 42 for *d1*, 22.5 for *psaA* and 22 for 12s). At D7, gene expression levels were equal to those of controls, and at D14, a downregulation of *psaA*, *d1* and 12s was seen.

Gene expression levels: conclusion

Studies on diatom genetic responses to contaminants are scarce because of the difficulties in accessing their nucleotide sequences (Kim Tiam et al. 2012). Genetic response studies are based on changes in gene expression in the organism facing contamination. Here, genes that are part of photosynthetic and mitochondrial metabolism were chosen, as both are potentially impacted by diuron.

Table 3 Differential gene expression as compared to actin from *Eolimna minima*, *Nitzschia palea* and *Planothidium lanceolatum* after 6 h and 2, 7 and 14 days of exposure to 1 and 10.9 μg/L of diuron by direct route

Functions	Genes	Diuron-contaminated experimental units							
		C_1 (1±0.2 μg/L)				C_2 (10.9±1.3 μg/L)			
		6 h	2 days	7 days	14 days	6 h	2 days	7 days	14 days
Eolimna minima									
Photosynthetic metabolism	*psaA*	0.5	/	/	13	0.5	4.5	/	4
	d1	0	/	0.5	16	/	4	/	8
Mitochondrial metabolism	*cox1*	0.5	/	/	5.5	0	3.5	/	2.5
	nad5	0	/	/	/	/	4.5	/	/
	12s	0.5	/	0.5	2.5	0.5	4.5	/	/
Nitzschia palea									
Photosynthetic metabolism	*psaA*	0.5	/	/	2	0.5	/	4	3.5
	d1	0.5	/	/	/	0.5	/	/	3
Mitochondrial metabolism	*cox1*	/	0	29	/	/	10.5	26	/
	nad5	/	0	9	/	2.5	6	4.5	/
	12s	/	/	/	/	/	/	2	3
Planothidium lanceolatum									
Photosynthetic metabolism	*psaA*	0.5	39.5	/	/	16	22.5	/	0
	d1	/	51.5	/	/	7	42	/	0
Mitochondrial metabolism	*cox1*	/	8.5	/	/	109.5	5	/	/
	nad5	/	/	/	/	4	/	/	/
	12s	0.5	55	/	/	22.5	22	/	0

Significant induction (>2) and repression (<0.5) factors are indicated by values, compared to the respective controls

/ identical to control levels

In the three species studied, there were clear impacts on the expression of *psaA*, *d1*, *cox1*, *nad5 and* 12s at both diuron concentrations. This supports the fact that diuron has a strong effect on photosynthetic and mitochondrial metabolism. The gene expression alterations can be linked to the significant decrease of photosynthetic activity for the three diatoms and the growth inhibition of *P. lanceolatum* at the highest diuron concentration.

E. minima and *N. palea* had a common profile of up- and downregulation of the five studied genes. A decrease in general metabolism appeared after 6 h of exposure to 1 μg/L of diuron, suggesting that the respiratory chain and photosynthesis are rapidly impacted at diuron concentrations as low as 1 μg/L. In fact, diuron is known to have a rapid mode of action: toxic effects can appear after 15 min of exposure (Legrand et al. 2006). By D7, there was some compensation for diuron damage as adaptive mechanisms were put in place. Importantly, there was no diuron effect on photosynthesis nor growth kinetics at 1 μg/L, indicating that genetic responses can reveal the impact of diuron on both mitochondrial and photosynthetic metabolism at a concentration that does not provoke damage to classical endpoints. At the higher diuron concentration (C_2), comparable effects were detected; however, damage appeared sooner—at D2 compared to D7 for C_1—suggesting a dose-dependent effect where at higher concentrations of diuron adaptive mechanisms are activated earlier. *E. minima* and *N. palea* seemed to be strongly impacted shortly after diuron exposure began and then to alter gene expression through adaptive mechanisms to adjust to the contaminant. Here, genetic results are in agreement with the recovery observed through photosynthetic measurements: both diatom species showed a decreased optimal quantum yield followed by an increase, a physiological effect that mirrored the genetic effect.

P. lanceolatum had a different pattern: as early as 6 h, damage was detected and metabolism was decreased until the end of the experiment. This diatom seemed opposite to *E. minima* and *N. palea*; it reacted almost immediately to contamination, but the genetic response quickly returned to control levels, suggesting that disturbance of photosynthetic and mitochondrial metabolism exceeded critical levels. These results agree with photosynthetic measurements; the optimal quantum yield at C_2 stayed low during the 14 days of exposure.

It clearly appears that photosynthetic metabolism in the three diatoms is strongly impacted by an exposure to diuron through inhibition and then induction of *psaA* and *d1* genes as early as 6 h after exposure. Diuron impact on photosynthetic

activity at the physiological level is known and well documented (Debenest et al. 2010; Hartgers et al. 1998; Idedan et al. 2011; Jones et al. 2003). Here, there was also a strong effect on mitochondrial metabolism. Induction of the *cox1* and *nad5* genes suggests increased ATP production to counteract the deleterious effects of a toxicant (Kim Tiam et al. 2012). Indeed, those two genes are implicated in the functioning of the respiratory chain. Furthermore, the 12s gene was also strongly upregulated, testifying to an increase in the number of mitochondria (increase of the *cox1*/12s ratio).

Scarce literature is available on the genetic impact of diuron on diatoms, and more generally on superior plants. Studies on PAH (organic contamination) or copper (inorganic contamination) exposure of *Thalassiosira pseudonana* highlight a common biomarker response: during a contamination episode, there are up- and/or downregulations of genes implicated in the stress response (Carvalho et al. 2011; Davis et al. 2006).

Results from this study complement those obtained by Kim Tiam et al. (2012) which showed an early upregulation of *E. minima* genes for general and photosynthetic metabolism following a metallic contamination of 100 μg Cd/L. The biomarkers used in our study were impacted as early as 6 h after a diuron contamination of 1 μg/L. Classical descriptors (photosynthetic activity and cell number) did not reveal these impacts, as also observed by Kim Tiam et al. (2012) for metal contamination. It is clear that the improvement of molecular tools, and more precisely biomarkers, is an asset for monitoring organism contamination and water quality.

Conclusions and perspectives

In the present study, molecular tools were developed and successfully used in diuron experimental contamination. The major implications of the work are the following: (i) five genes of interest were sequenced for *E. minima*, *N. palea* and *P. lanceolatum* strains allowing the application of q-PCR tools for these species; (ii) diuron contamination had impacts on growth rate, optimal quantum yield of PSII (ΦPSII) and expression of several genes involved in mitochondrial and photosynthetic mechanisms; (iii) q-PCR tools appeared to be more sensitive than ΦPSII and growth rate analyses to reveal diuron effects; and (iv) the study increased knowledge about relative sensitivity of diatom species to diuron in regard to the different endpoints tested. *E. minima* and *N. palea* strains appeared to be more tolerant than *P. lanceolatum*.

The present study is one of only a few reporting the use of q-PCR on river benthic diatoms, and the results obtained are extremely promising for future applications in the field of water quality assessment. This study also evidenced the usefulness of diatom strains to describe the ecotoxicological impacts of pesticides recovered in the natural environment. The analysis of genetic expression of *nad5*, *cox1*, 12s, *d1* and *psaA* by q-PCR could so constitute an early warning biomarker of metal pollutions. Future studies could also investigate sequences of genes coding for catalase, superoxide dismutases or glutathione peroxidase in order to study response to oxidative stress generated by pollutants. The next application will be to apply these genetic biomarkers on multispecific natural biofilms for impact assessment of toxic pollution and to confirm results obtained in laboratory-controlled conditions.

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