Linking Diatom Sensitivity to Herbicides to Phylogeny: A Step Forward for Biomonitoring?

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January 24, 2015

Abstract

Phylogeny has not yet been fully accepted in the field of ecotoxicology, despite studies demonstrating its potential for developing environmental biomonitoring tools, as it can provide an a priori assessment of the sensitivity of several indicator organisms. We therefore investigated the relationship between phylogeny and sensitivity to herbicides in freshwater diatom species. This study was performed on four photosystem II inhibitor herbicides (atrazine, terbutryn, diuron, and isoproturon) and 14 diatom species representative of Lake Geneva biofilm diversity. Using recent statistical tools provided by phylogenetics, we observed a strong phylogenetic signal for diatom sensitivity to herbicides. There was a major division in sensitivity to herbicides within the phylogenetic tree. The most sensitive species were mainly centrics and araphid diatoms (in this study, Thalassiosirales and Fragilariales), whereas the most resistant species were mainly pennates (in this study, Cymbellales, Naviculales, and Bacillariales). However, there was considerable variability in diatom sensitivity within the raphid clade, which could be explained by differences in trophic preferences (autotrophy or heterotrophy). These traits appeared to be complementary in explaining the differences in sensitivity observed at a refined phylogenetic level. Using phylogeny together with complementary traits, as trophic preferences, may help to predict the sensitivity of communities with a view to protecting their ecosystem.

Contents

1 Introduction

Over the past 40 years, many methods have been developed for monitoring the eco-
logical quality of water bodies (Lenoir and Coste, 1996; Schaumburg et al., 2004;
Liess et al., 2008). Among these methods, bioindicators constitute a powerful tool to
assess the anthropogenic pressures on biota and ecosystem function. Most bioindi-
cators used in freshwaters, such as fish, macroinvertebrates, and microalgae, address
specific biomonitoring needs (Resh, 2008). Microalgae, and especially diatoms, are
suitable for monitoring the overall health of the environment. They are particularly
used to assess the quality of rivers in terms of organic and nutrient pollution (Rimet,
2012). Their communities respond rapidly to changes in habitat quality (Lowe and
Pan, 1996; Stevenson and Smol, 2003). Diatoms are good biomonitors because they
are found in most aquatic habitats and exhibit a huge diversity in terms of taxonomy
(over 30 000 species, Mann and Vanormelingen, 2013), morphology (Round et al.,
1990), and ecology (Van Dam et al., 1994). Despite these advantages, fewer ecological
assessments are carried out worldwide using diatoms than using macroinvertebrates
or fish (Carter et al., 2006; Gallacher, 2002; Resh, 2007). The high sensitivity of
diatoms to photosystem II inhibitor (PSII) herbicides has been widely observed at
both the single-species (Lockert et al., 2006; Roubeix et al., 2011; Larras, Bouchez,

et al., 2012) and community levels (Roubeix et al., 2011; Pérès et al., 1996; Schmitt-Jansen and Altenburger, 2005). At these two levels of biological organization, some of these herbicides impair diatoms even at concentrations as low as those regularly found in the environment (Larras, Bouchez, et al., 2012; Gilliom, 2007; Loos et al., 2009).

Ecotoxicological studies, based on toxic exposure in laboratory bioassays, provide the basic information required for developing biomonitoring tools. Studies at a higher complexity level, such as microcosms, provide more environmentally realistic data about toxic impacts, but identifying the environmental effects attributable to herbicides is rather complex. As a first step, it is important to determine herbicide toxicity under single-species conditions. We therefore performed single-species bioassays, which are useful tools for assessing the physiological sensitivity of a single diatom species to a single herbicide in which confounding factors were excluded and a high degree of reproducibility can be achieved. However, sensitivity data obtained from these tests are only available for relatively few species because generating these data is time-consuming and labor-intensive. Because diatoms exhibit such a huge diversity, it actually seems inconceivable to assess with bioassays the sensitivity of even a small percentage of these species. Thus, developing methods to predict their sensitivity represents a challenge for using more species sensitivity data to improve their biomonitoring efficiency. The current influx of DNA sequence data allows for establishing robust phylogenies to many species. Connecting sensitivity data to the phylogeny of the tested species is a key tactic that might provide an a priori prediction of the species' sensitivity to contaminants. Recent studies have investigated the potential of phylogeny to assess the sensitivity of amphibians (Hammond et al., 2012), microalgae (Wängberg and Blanck, 1988; Eriksson et al., 2009), fish (Jeffree et al., 2010), macroinvertebrates (Buchwalter et al., 2008), and chironomids and mayflies (Carew et al., 2011) to various pollutants. Many authors have focused on the detection of a phylogenetic signal, which is the tendency for related species to share similar ecological or biological features. For instance, these studies showed that the phylogenetic signals of sensitivity varied in strength depending on the pollutant considered. However, the use of a phylogenetic framework to improve biomonitoring remains an unexplored field, and no data are available for diatoms. Approaches that integrate phylogeny and ecotoxicology could supply information for bioassessment tools operating at a larger taxonomic scale and could thus increase the effectiveness of biomonitoring. The lack of a quality data set based on a set of species presenting high diversity and a wide range of sensitivities (Guénard et al., 2011) has so far prevented this type of study. However, new statistical methods integrating phylogenies offer a promising avenue for exploring the variability of sensitivity within and between taxonomic levels. For example, Blomberg's K statistic (Blomberg et al., 2003) and Pagel's λ (Pagel, 1999) are both well-established statistical tools provided by phylogenetics to detect the phylogenetic signal, to measure its strength, and to test its significance. Blomberg's K statistic has been applied extensively in the context of ecotoxicological studies by Buchwalter et al. (2008) and Carew et al. (2011), whereas Pagel's λ performs well for complex models of trait evolution (Münkemüller et al., 2012). Phylogenetic principal component analysis (pPCA Jombart, Pavoine, et al., 2010) is also a promising new method that allows for working in a multivariate framework to explore the phylogenetic signal and to explore possible correlations

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among the distributions of sensitivities.

This study is a preliminary exploration of the potential use of phylogenetic signals to investigate the sensitivity of freshwater diatoms to herbicides.

The first aim was to explore the sensitivity patterns of diatoms to four PSII inhibitor herbicides within the phylogeny. These herbicides were chosen for their high level of phytotoxicity and because they are often detected in French surface waters (Dubois and Lacouture, 2011). We performed single-species laboratory bioassays of a set of fourteen diatom species exposed to four PSII inhibitor herbicides (atrazine, terbutryn, diuron, and isoproturon). This species set is representative of the biofilm diversity in Lake Geneva and was developed in the context of biomonitoring this lake. We also provide a phylogeny of these 14 diatom species reconstructed from the 18S and *rbcL* markers.

Second, we wanted to determine whether the phylogeny of these diatoms was significantly linked to their herbicide sensitivities. We explored the phylogenetic signal for herbicide sensitivity using Blomberg's K statistic and Pagel's λ. We also applied the pPCA to our data as a tool to detect biologically meaningful combinations of herbicide sensitivities that are phylogenetically structured. Because we had sensitivity data for four different herbicides and few possible explanatory factors, the pPCA allowed us to uncover the underlying phylogenetic trends and patterns and to thus reveal the ecotoxicological evolutionary strategies of diatoms. The ability to explore the phylogenetic signal in a multivariate framework is also important in ecotoxicology to consider the multiple environmental stressors. Finally, the results are discussed in light of diatom ecology and provide useful insights into diatom biomonitoring. Traditional and phylogenetically based regression analyses were used to assess the relationships between herbicide sensitivities and commonly used biomonitoring indices. We wanted to determine whether the link between phylogeny and sensitivity should be considered for inclusion in the development of biomonitoring tools intended to protect environmental diatom communities against these herbicides.

2 Materials and Methods

2.1 Diatom Species

Fourteen freshwater benthic diatom species were selected to represent the diversity found in the Lake Geneva biofilm. Each species was maintained in culture and is registered at the Thonon Culture Collection [1]. The selected species included *Achnanthidium minutissimum* (Kützing) Czarnecki (TCC-746), *Craticula accomoda* (Hustedt) D. G. Mann (TCC-107), *Cyclotella meneghiniana* Kützing (TCC-755), *Encyonema silesiacum* (Bleisch) D. G. Mann (TCC-678), *Fistulifera saprophila* (Lange-Bertalot & Bonik) Lange-Bertalot (TCC-535), *Fragilaria capucina* var. *vaucheriae* (Grunow) Lange-Bertalot (TCC-752), *Fragilaria crotonensis* Kitton (TCC-301), *Fragilaria rumpens* (Kützing) G. W .F. Carlson (TCC-666), *Gomphonema parvulum* (Kützing) Kützing (TCC-653), *Gomphonema clavatum* Ehrenberg (TCC-527), *Mayamaea fossalis* (Krasske) Lange-Bertalot (TCC-366), *Nitzschia palea* (Kützing) W. Smith (TCC-139-2), *Sellaphora minima* (Grunow) Mann (TCC-524), and *Ulnaria*

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[1]http://www.inra.fr/carrtel-collection

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ulna (C. L. Nitzsch) Compère (TCC-635). Photographs of the strains are available on the barcoding Web site of the INRA institute [2]. The cultures were maintained in DV culture medium [3] that had been filtered at 0.22 μm (Millipore, Germany) and were grown in a 300 mL Erlenmeyer flask at 21 ± 2 ℃ with a 16 h/8 h light/dark cycle at 66 µmol.m^{-2}.s^{-1}.

2.2 Herbicide Solutions

We tested four herbicides, namely, atrazine, terbutryn, diuron, and isoproturon, from Sigma-Aldrich (St. Louis, MO, purity over 99.5%). For the bioassays, stock solutions were prepared in DV medium. Due to the low solubility of atrazine and diuron, we added a 0.05% concentration of the solvent dimethyl sulfoxide (DMSO) to the stock solutions and sonicated the mixture for 30 min. Previous research (unpublished data) has confirmed that this concentration has no adverse effects on benthic diatoms. Moreover, no interaction between DMSO and photosystem II inhibitor herbicide was observed below 0.5% DMSO (El Jay, 1996).

2.3 Sensitivity Data Set

The effective concentrations that reduce the population growth rate by 10% (EC_{10}, Supporting Information, Table S1) and 50% (EC_{50}, Supporting Information, Table S2) were available from Larras, Montuelle, et al. (2013) for *Fr. capucina* var. *vaucheriae*, *Fr. rumpens*, *U. ulna*, *Cr. accomoda*, *M. fossalis*, *S. minima*, *N. palea*, *A. minutissimum*, *Cy. meneghiniana*, *E. silesiacum*, and *G. parvulum*. To increase the diversity of the tested species, we performed additional single-species bioassays on *G. clavatum*, *Fi. saprophila*, and *Fr. crotonensis* for these four herbicides as described by Larras, Montuelle, et al. (2013). We worked at two levels of sensitivity so that our data could be generalized; EC_{50} and EC_{10} are both relevant in the environmental regulatory framework (European Community, 2003).

2.4 Mixture Toxicity Prediction

We predicted the sensitivity of each species at both the EC_{10} and EC_{50} levels to equitoxic mixtures of (1) all four selected herbicides (EC_{10}-Mix and EC_{50}-Mix), (2) the two herbicides belonging to the phenylurea family (EC_{10}-Mix$_p$ and EC_{50}-Mix$_p$), and (3) the two herbicides belonging to the triazine family (EC_{10}-Mix$_t$ and EC_{50}-Mix$_t$). The compositions of the equitoxic mixtures were determined using the concentration addition (CA) model (Equation 1, Faust et al., 2001) because many studies have already shown that this model can predict the toxicity of mixtures of photosystem II inhibitors (Faust et al., 2001; Arrhenius et al., 2004; Porsbring et al., 2010). $EC_{x,mix}$ is the total concentration of the mixture that elicits a total effect of $x\%$, P_i is the relative proportion of each substance within the mixture, and $EC_{x,i}$ is the individual concentration of each substance that induces the effect of $x\%$.

$$EC_{x,mix} = \left(\sum_{i=1}^{n} \frac{P_i}{EC_{x,i}} \right)^{-1} \tag{1}$$

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[2]http://www.rsyst.inra.fr/?q=fr/content/micro-algues
[3]http://www6.inra.fr/carrtel-collection_eng/Culture-media/Composition-of-the-culture-media

2.5   Phylogenetic Analyses

The diatom strains used in this study were genetically characterized by Sanger sequencing using two markers (18S and *rbcL*) as described in Kermarrec, Franc, et al. (2013). Genbank accession numbers are provided for each sequence as Supporting Information (Table S3). The sequences were aligned using the Muscle algorithm (Edgar, 2004) provided in the SeaView graphical user interface (Gouy et al., 2010). The 18S and *rbcL* markers were first aligned separately and then combined. The resulting contig spanned 2457 bp. We used the maximum likelihood (ML) and Bayesian inference (BI) methods in parallel to compute and assess the tree topology from the aligned contig. Each tree was rooted using *Bolidomonas pacifica* L. Guillou & M.-J. Chrétiennot-Dinet, an algal species closely related to diatoms (Guillou et al., 1999). We used the MrAIC software (Nylander, 2004) to select the best substitution model. Because *rbcL* is a plastid gene and 18S is a nuclear gene, these genes may be subject to different evolutionary constraints. We carried out partitioned analyses to independently estimate the model parameters for each gene. Moreover, we constructed trees for the 18S and the *rbcL* genes separately to ensure that these trees both reflected the same evolutionary history. We used RAxML 7.2.8 (Stamatakis, 2006) to identify the most likely tree topology for the ML method. The branch support was assessed by 1000 bootstrap replicates. Finally, we ran an analysis for the BI method with MRBAYES 3.1.2. (Ronquist et al., 2012). The analysis consisted of two runs, three heated chains, 1 000 000 generations, and 2000 samplings from the posterior probability distribution.

2.6   Sensitivity-Phylogeny Relationships

The phylogenetic data (i.e., tree topology with branch lengths) and trait-related data (sensitivity to herbicides) were jointly analyzed to assess the phylogenetic signal of diatom sensitivity. All of the analyses were performed after a square-root transformation of EC_{10} and EC_{50} values for single substances and mixtures to stabilize the variances. To ensure that our results were not an artifact of a particular sensitivity level, we performed phylogenetic signal analyses on both EC_{10} and EC_{50}. However, only the EC_{50} values were used for the complementary analyses because there is less uncertainty for these values.

2.7   Phylogenetic Signal for Herbicide Sensitivity

To measure the phylogenetic signal (i.e., the tendency for related species to be similar to each other) of the herbicide sensitivity of a species, we used the K statistic to quantify the strength of the phylogenetic signal for a given trait (EC values for a given herbicide or mixture). We calculated a K value for each trait and used a randomization test based on the phylogenetically independent contrast (PIC) method (Blomberg et al., 2003) with 10 000 repetitions. Because our sample size was low (14 species), this test is slightly underpowered, and we may expect type II errors to be inflated. As an alternative to the K statistic, intercept-only GLS models that assume no covariance among species (i.e., a star phylogeny) can be fitted and compared with intercept-only GLS models that assume a Pagel's λ correlation structure (Pagel, 1999). Pagel's λ is a coefficient estimated from trait-related data

that weights the Brownian model correlation structure. The models can be compared directly using the Akaike information criterion (AIC) (Paradis, 2011). As a rule of thumb, if the difference between the AIC values of two models exceeds 2 units, then the model with the lowest AIC is considered to best fit the data.

2.8 Phylogenetic Principal Component Analysis

pPCA is a multivariate method constraining traditional PCA to exhibit phylogenetic autocorrelation (Jombart, Pavoine, et al., 2010). This approach reveals the main phylogenetic structures of a set of traits. We used pPCA to highlight patterns among pesticide sensitivities in the diatom phylogeny. Jombart, Pavoine, et al. (2010) defined two types of phylogenetic structures that can occur in biological features. First, global structures are strongly linked to the idea of a phylogenetic signal and result from global patterns of sensitivity similarity in phylogenetically related taxa. Second, local structures express the overdispersion of sensitivity values that occurs for closely related species in specific parts of the phylogenetic tree. Both global and local structures are detected and extracted by pPCA in the form of synthetic variables known as the global principal component and the local principal component, respectively.

We performed pPCA on the EC_{50} values of each species for each herbicide and for mixtures of these herbicides and retained only the first global principal component (GPC1) and the first local principal component (LPC1), which correspond to the highest positive and the highest negative eigenvalues, respectively. Scree plot inspection allowed us visually to check that the selected components were sufficient to summarize our data.

2.9 Ancestral Character Estimations

We estimated ancestral character (sensitivity) values for the EC_{50}-Mix to visualize the order of sensitivity of several diatom species to a mixture of herbicides within the phylogenetic tree. We estimated the ancestral characters for each node assuming a Brownian motion model of evolution for the examined traits using a restricted maximum likelihood method (Paradis, 2011; Schluter et al., 1997). Finally, we transformed our quantitative trait values (i.e., ancestral and current) into a categorical variable with two levels, which can be interpreted as a sensitive/resistant classification. The threshold between these two levels was selected as the mean of the square roots of the current EC_{50}-Mix values (34.78). The values for sensitive species ranged between 8.18 and 20.20 (mean 16.14), while those for resistant species ranged between 36.46 and 79.27 (mean 53.42).

2.10 Phylogenetic Regressions

Phylogenetic generalized least squares (PGLS) and traditional regressions (with a star phylogeny) were used to assess and rank the explanatory power of two biomonitoring indices (VDAM-NH, Van Dam et al., 1994 and IPSS, Coste, 1982; Table 1) for herbicide sensitivities. For each herbicide, we compared the set of PGLS and traditional regressions for both indices using AIC as an indicator of the relative support. Ecological guilds (Rimet and Bouchez, 2012; Table 1) and multiple regressions

Metric	Value/Code	Class
Specific pollution sensitivity index (IPSS, Coste, 1982)	From 1 to 5	From bad toward best water quality media
Nitrogen uptake metabolism	1	N-autotrophic, tolerate very low organic [N]
(VDAM-NH defined here as trophic	2	N-autotrophic, tolerate high organic [N]
mode, Van Dam et al., 1994)	3	Facultative N-heterotrophic, need periodically high organic [N]
	4	Obligatory N-heterotrophic, need continuously high organic [N]
Ecological guild (Rimet and Bouchez, 2012)	LP (low profile)	Small species that can tolerate physical disturbance and low levels of resources
	HP (high profile)	Taller species that do not resist physical disturbance and do tolerate high nutrient levels
	M (motile)	Species that can move and tolerate high nutrient levels
	P (planktonic)	Species that evolve freely in the water column

Table 1: Ecological and Biological Metrics of Diatoms Considered for Regression Analysis

including both VDAM-NH and IPSS as explanatory variables were not tested here because these models require much more data. Our data set would have led to convergence errors or misleading results. The PGLS models were fitted by log-likelihood maximization.

2.11 Statistical Packages

All of the statistical analyses were performed using R 3.0.0 software (R Development Core Team, 2013). The phylogenetic data handling and Pagel's λ correlation structure and ancestral character estimations were performed using the ape package (Paradis et al., 2004). The PGLS were adjusted using the nlme package (Pinheiro et al., 2013). The phylogenetic multivariate analyses were performed using the adephylo package (Jombart, Balloux, et al., 2010), and Blomberg's K statistics were computed using the picante package (Kembel et al., 2010).

3 Results

3.1 Phylogenetic Tree Analysis

The two methods (ML and BI) used to reconstruct the phylogeny produced very similar topologies. Similarly, using 18S, $rbcL$, or $18S + rbcL$ contigs produced similar topologies with minor variations in the branch lengths. These small topological differences did not affect the methods used here (results not shown). Phylogenetic trees computed with every combination of methods and markers are provided as Supporting Information (Figure S1). The topologies were well supported overall by bootstraps and Bayesian posterior probabilities. The tree computed from the $18S +$ $rbcL$ contig using the ML method with branch support values for each method and its agreement with other references are presented in Figure 1. The diatom species clearly fell into three different groups: the centric, araphid, and pennate raphid diatoms. The centric diatom (*Cy. meneghiniana*) was placed near the pennate diatoms. The araphid pennate diatoms (*U. ulna* and Fragilariales species) were sister species to the nine raphid pennate diatom species in our data set. The raphid diatoms were divided into two clades: one clade has a raphe canal (*N. palea*), and the other does not (Naviculales, Achnanthales, and Cymbellales). Subsequent clades included taxa belonging to the Cymbellales, freshwater Achnanthales, and Naviculales species, which were well supported. The nodes that separate these important groups were robust with Bayesian support values close to 100 (Figure 1). The support values computed by ML were lower, especially in the case of the node that separates the raphid and araphid diatoms (67). The deepest node of Naviculales was poorly supported by ML and was unresolved by BI. This convergent tree was used in all of the subsequent phylogenetic analyses.

3.2 Diatom Sensitivity to Herbicides

An overview of diatom sensitivity to herbicides is presented in Figure 2. Species sensitivities show similar patterns for both EC_{10} and EC_{50} (Figure 2). The complete data set of untransformed EC_{10} and EC_{50} values for each herbicide and each species is provided as Supporting Information (Tables S1 and S2). *Cy. meneghiniana* and Fragilariales species were the most sensitive to the four herbicides under both single and mixture conditions. More resistant species, such as *N. palea* and *S. minima*, showed different patterns depending on the herbicide family tested. *N. palea* was more resistant to the triazines, while *S. minima* was more resistant to the phenylurea herbicides, especially at the EC_{10} level. *Fi. saprophila* tended to be more resistant to the triazines, especially at the EC_{50} level. The opposite was observed for all of the herbicides for *G. clavatum*. As a general trend, *E. silesiacum*, *A. minutissimum*, and *M. fossalis* tended to have medium sensitivities compared to those of the other species.

3.3 Phylogenetic Signal for Herbicide Sensitivity

The phylogenetic signal tested with Blomberg's K was significant ($\alpha < 5\%$) at the EC_{10} and EC_{50} levels for atrazine, Mix_t, and Mix (Table 2). Mix_p was found to be significant only at the EC_{10} level. We found that Pagel's λ was closely correlated to Blomberg's K (Pearson correlation 0.76, p-value 0.002) and that both values led
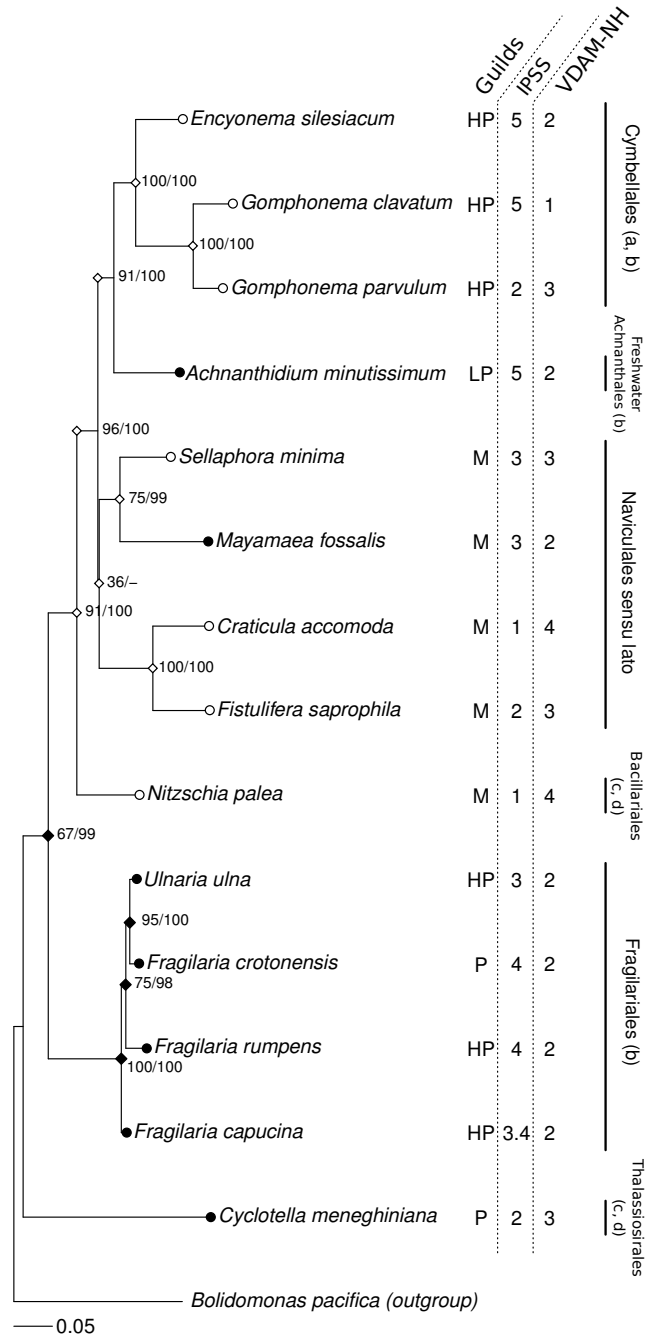
Figure 1: Phylogenetic tree of 14 diatom species inferred from the 18S and *rbcL* DNA sequences and their related ecological guild,50 global pollution sensitivity (IPSS, Coste, 1982), and trophic mode (VDAM-NH, Van Dam et al., 1994). The branch lengths were computed using the maximum likelihood method. Statistical support (as a percentage) is provided at each node for the two methods (ML bootstraps/BI posterior probabilities). The clades are replaced in their bibliographic context with a = Kermarrec, Ector, et al. (2011); b = Medlin (2011); c = Medlin and Kaczmarska (2004); d = Theriot, E. Ruck, et al. (2011). For the ancestral characters, the herbicide sensitivity of the diatoms was based on EC_{50}-Mix data. Black and white symbols represent sensitive and nonsensitive species, respectively. Circles represent current characteristics, and tilted squares represent inferred ancestral characters. Ecological guilds: P = planktonic, LP = low profile, HP = high profile, M = motile. IPSS: from 1 (species in poor-quality water) to 5 (species in good-quality water). VDAM-NH: 1 = autotrophic, sensitive to low organic N concentrations; 2 = autotrophic, tolerant to high organic N concentrations; 3 = facultative heterotrophic; 4 = obligatory heterotrophic.
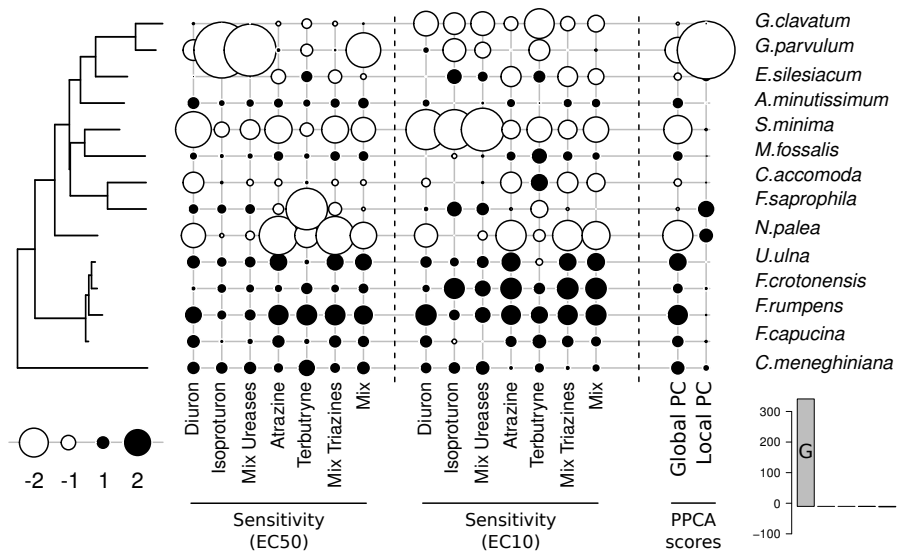
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Figure 2: EC_{50}, EC_{10}, and pPCA species scores of the 14 diatom species mapped onto the phylogenetic tree. The EC values are centered and scaled by their standard deviation within the treatment. The scores are centered and scaled by their standard deviation for each component. The larger the circle, the higher (black circle) or the lower (white circle) the value. The bar plots represent the eigenvalues for the four principal components of the pPCA. The first global and local principal component eigenvalues are indicated by the letters "G" and "L", respectively.

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		K	K p-value	Pagel's λ	ΔAIC
EC$_{50}$	Diuron	0.602	0.103	1.018	0.150
	Isoproturon	0.517	0.399	0.233	-1.708
	Mix$_p$	0.535	0.258	0.279	-1.561
	Atrazine	0.691	0.032	1.000	3.578
	Terbutryn	0.638	0.062	1.030	0.452
	Mix$_t$	0.717	0.027	1.011	4.041
	Mix	0.680	0.031	0.844	1.146
EC$_{10}$	Diuron	0.624	0.083	1.057	0.867
	Isoproturon	0.460	0.195	0.675	-1.022
	Mix$_p$	0.627	0.054	NA	NA
	Atrazine	0.860	0.007	1.062	7.448
	Terbutryn	0.401	0.178	0.290	-1.655
	Mix$_t$	0.862	0.008	1.053	5.882
	Mix	0.834	0.008	1.058	5.768

Table 2: Results of Statistical Analysis for the Phylogenetic Signal of the Toxicity of Herbicides Alone and in Mixtures. ΔAIC corresponds to the difference between the AIC of the "star" model and that of the "Pagel's λ" model. ΔAIC > 2 indicates the presence of a phylogenetic signal. NA values are produced if the PGLS values are not convergent.

to similar conclusions. Interestingly, EC$_{10}$ appeared to reveal a stronger phylogenetic signal than did EC$_{50}$ for the phenylurea compounds, especially for diuron. Blomberg's K statistic ranged from 0.401 for EC$_{10}$-terbutryn to 0.862 for EC$_{10}$-Mix$_t$. We found no statistically significant differences in K values between the EC$_{10}$ or EC$_{50}$ groups of traits (Wilcoxon paired test, p-value 0.438).

To illustrate the phylogenetic signal, we mapped the EC$_{50}$-Mix values and the estimated ancestral character values for EC$_{50}$-Mix onto the phylogenetic tree (Figure 1). The ancestral character estimation showed a clear phylogenetic segregation between the sensitive (Thalassiosirales and Fragilariales) and resistant (Cymbellales, Naviculales, and Bacillariales) species. Moreover, the trophic mode index VDAM-NH (Van Dam et al., 1994), the ecological guild (Rimet and Bouchez, 2012), and the values of the specific pollution sensitivity index of the IPSS biotic diatom index (Coste, 1982) of each species are given (Figure 1). From traditional and phylogenetic regressions (Supporting Information, Table S4), it was clear that VDAM-NH offers better explanatory power for herbicide sensitivities than does IPSS. Terbutryn sensitivity was an exception, as it was equally well explained by both IPSS and VDAM-NH. The trophic mode appeared to match both the sensitivity and phylogeny, as demonstrated statistically (p-value < 0.05). It is also interesting to note that, for isoproturon, the Mix$_p$ and Mix phylogenetic models performed substantially better than did the traditional models. For diuron, atrazine, and Mix$_t$, the traditional and phylogenetic models offered similar performances. Terbutryn is the exception for which traditional models performed better than did phylogenetic models.

The ecological guilds were closely related to the phylogeny but were not clearly

linked to species' sensitivity to herbicides. Most of the sensitive species (centrics/araphids) belonged to the high-profile guild, which also included some resistant species in the raphid group. Moreover, species belonging to the motile guild displayed various levels of sensitivity, similar to or lower than those of many of the high-profile species.

The pPCA on EC_{50} highlighted a strong global structure and a weak local structure (Figure 2, scree plot), which support the presence of a phylogenetic signal as demonstrated by statistical analyses. Species scores on the first global axis (Figure 2, global PC) highlight groups of closely related species that share the same range of herbicide sensitivities. These scores also reveal contrast between sensitive species, such as the Fragilariales and the centric species (*Cy. meneghiniana*), and resistant species, such as *N. palea* and *S. minima*. Species scores on the LPC1 reveal a marked contrast between *N. palea* and both *Fi. saprophila* and G. parvulum (Figure 2, local PC). These species are closely related, but their responses to the different pesticide families are different: *G. parvulum* is sensitive to triazine and resistant to phenylurea, whereas *Fi. saprophila* and *N. palea* are sensitive to phenylurea and resistant to triazine (Figure 2, EC values).

All four herbicides were negatively correlated with the first global component (Figure 3, global PC), suggesting the existence of a general pattern of sensitivities within the phylogeny that is independent of the herbicide. However, the first local component (Figure 3, local PC) seemed to oppose the two groups of herbicides, even if it involved only three species (Figure 2, local PC, *N. palea*, *Fi. saprophila*, and *G. parvulum*).

4 Discussion

4.1 Does Phylogeny Reflect Herbicide Sensitivity?

Interactions between phylogeny and ecology are often studied because they provide complementary information that can help to explain patterns of species occurrence (Pavoine et al., 2011) or extinction (Green et al., 2011) over time. Nevertheless, studies relating the phylogeny of freshwater organisms to their sensitivity to pesticides are less common, even if they could provide very useful data for monitoring purposes. Among these few studies, Hammond et al. (2012) found a phylogenetic signal of sensitivity for many amphibians to the insecticide endosulfan, implying that large ranids were the most sensitive frogs. Similarly, Wängberg and Blanck (1988) found phylogeny and sensitivity to be linked for a wide range of phototrophic organisms. Cyanophyta and Chlorococcales exhibited different levels of sensitivity toward many chemicals. These first studies gave promising results regarding the potential use of phylogeny in ecotoxicology (Hammond et al., 2012; Wängberg and Blanck, 1988; Guénard et al., 2011) for these specific taxonomic groups and chemicals. To enhance the reliability of these results, studies must be conducted on data sets with wide ranges of both species diversity and species sensitivity (Guénard et al., 2011). The lack of such a sensitivity data set in the literature makes it currently impossible to carry out this type of study, especially on organisms of interest for biomonitoring. Diatoms appear to be appropriate organisms due to their high degree of diversity, their varying sensitivities to herbicides, and their critical importance
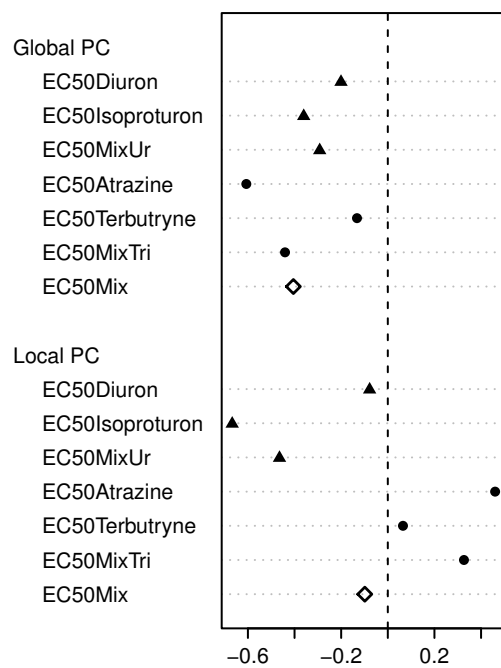
Figure 3: Loadings of herbicide treatments for the first global and local principal components of the EC_{50} pPCA. Black triangles represent single substances and the binary mixture of phenylurea compounds, black circles represent single substances and the binary mixture of triazine compounds, and white tilted squares represent the quaternary mixture.

in river bioassessment. These organisms are already being successfully used as indicators to assess the ecological level of aquatic ecosystems (Lenoir and Coste, 1996; Schaumburg et al., 2004), which is required for regulatory monitoring, such as for the European Water Framework Directory (European Community, 2000). However, it may be difficult to identify diatoms at the species level due to their great diversity and the fact that they require highly qualified personnel and resources (Rimet and Bouchez, 2012; Kelly et al., 1995).

In our study, we worked with a set of 14 taxa. These species were chosen on the basis of their presence in Lake Geneva to work with a wide range of taxonomic diversity in diatoms. Therefore, this species set is representative of biofilm diversity in Lake Geneva; the sensitivity of the species toward various pesticides has already been explored in mesocosm studies in the context of this particular lake (Rimet and Bouchez, 2011). Moreover, the chosen taxa belong to various clades that include the main freshwater orders. Diatom phylogeny has been explored on the basis of various different markers, such as 18S, *rbcL*, *psb*C, *cox1*, ITS, and LSU, by several authors (Medlin and Kaczmarska, 2004; Medlin, Kooistra, et al., 1996; Beszteri et al., 2001; E. C. Ruck and Theriot, 2011; Theriot, Ashworth, et al., 2010; Zechman et al., 1994; Lundholm et al., 2002). The two methods (ML and BI) used to reconstruct the phylogeny produced exactly the same topology, which was statistically well supported and was consistent with previously published results (Kermarrec, Ector, et al., 2011; Medlin, 2011; Theriot, E. Ruck, et al., 2011).

We observed a phylogenetic signal of the sensitivity of benthic diatoms to PSII herbicides at both the EC_{10} and EC_{50} levels. Blomberg's K and Pagel's λ statistics, which were used to detect this signal, were underpowered in this study. Simulations showed that, with 14 species, Blomberg's K detects 80% of true positives and Pagel's λ detects 75% of true positives. This result suggests that the phylogenetic signal was most likely more pronounced than is shown here. Therefore, a relationship likely exists between diatom phylogeny and herbicide sensitivity. Possible explanatory hypotheses are discussed in the following section.

4.2 Sensitivity Patterns in Phylogeny: Explanatory Hypotheses

According to the phylogeny and the first axis of the global axis of the pPCA, two groups of sensitivity toward PSII inhibitors were observed: (a) one including the sole centric diatom species (Thalassiosirales) and the araphid pennate diatoms (Fragilariales), which were generally more sensitive than (b) the pennate raphid species (Bacillariales, Naviculales, Achnantales, and Cymbellales). *Cy. meneghiniana* was the only centric diatom we included. Moreover, this species displayed mixotrophic capabilities (Van Dam et al., 1994), which are rare among centric diatoms, indicating that no generalization could be made regarding the sensitivities of centric diatoms. In our study, ancestral character estimation demonstrates that the resistant state emerged very early in the tree. The main difference between these two groups is the presence of the raphe, which is present only in raphid diatoms (Theriot, Ashworth, et al., 2010; Sims et al., 2006; Round et al., 1990). Raphe played an important role in benthic habitat colonization, principally by enabling diatoms to move and to secrete an exopolysaccharide matrix (Sims et al., 2006). Nevertheless, it is hardly conceivable that the advent of the raphe in diatom evolution led directly to the changes in lowered sensitivity to herbicides. Rather, we hypothe-

size that, during evolution, species that developed a raphe structure simultaneously developed other characteristics (genetic, physiological, or cellular traits) that enhanced their resistance to PSII inhibitors. Raphid and araphid pennate diatoms are both characteristic of benthic communities (Schmitt-Jansen and Altenburger, 2005; Rimet and Bouchez, 2011; Brown and Austin, 1973). Under low light conditions, some of these diatoms can evolve heterotrophic capacities and can metabolize other organic substrates, making them less dependent on photosynthesis (Van Dam et al., 1994; Hellebust and Lewin, 1977). In particular, genera such as *Craticula*, *Encyonema*, *Fistulifera*, *Gomphonema*, *Nitzschia*, and *Sellaphora*, most of which display motile and/or raphid characteristics, are known to be better adapted to coping with higher organic matter concentrations than most centrics (e.g., *Cyclotella*) or araphid diatoms (e.g., Fragilariales, Berthon et al., 2011).

The sensitivity levels of the different species are generally constant within the araphid/centric diatom clade, whereas they are variable within the raphid clade, which encompasses a greater diversity of genera. Several species from this latter clade, such as *M. fossalis*, *A. minutissimum*, and *E. silesiacum*, were more sensitive than other raphid species. We observed variability within the raphid clade, but more sensitivity data per genus are required to develop a firm hypothesis regarding the variation of sensitivity within the raphid clade in relation to phylogeny. However, we suggest that the trophic mode may play a role in the greater sensitivity observed for these three autotrophic species within the raphid clade. Our main hypothesis is that the trophic mode of diatoms, defined by Van Dam et al. (1994), is partly related to phylogeny and indirectly influences the sensitivity of species to PSII inhibitors. The results of traditional and phylogenetic regressions both suggest that phylogeny provides a first general level of sensitivity to these herbicides. Moreover, the trophic mode of species may help to refine the level of sensitivity within clades characterized by species with a wide diversity of ecological and physiological characteristics. Diatoms with higher heterotrophic capacities are less susceptible to PSII inhibitors because they are able to shift to organic nutrition to sustain themselves. Moreover, the mixotrophic capacities of diatoms vary greatly between species. Several studies seem to confirm this hypothesis: heterotrophic diatom species are less sensitive to herbicides than are autotrophic diatom species (Roubeix et al., 2011; Larras, Bouchez, et al., 2012; Pérès et al., 1996; Debenest et al., 2009; Berard and Pelte, 1999), such as the highly sensitive araphid diatoms. Within the raphid clade, species exhibit various trophic modes, and some may be heterotrophic, which may explain the different levels of sensitivity found for the small number of species tested in our study.

Finally, we observed another level of variation among the most resistant species of the raphid group. As highlighted by the first local axis of the pPCA, there is greater variation in sensitivity to the different herbicide families (triazine vs phenylurea) for *G. parvulum*, *E. silesiacum*, *Fi. saprophila*, and *N. palea*, especially at the EC_{10} level. Triazine and phenylurea herbicides both inhibit photosynthesis by preventing the electron flow within thylakoid membranes (Moreland and Hill, 1962). However, these two herbicide families are characterized by different chemical structures (Gramatica et al., 2001). Moreover, within each chemical family, specific and different parameters influence herbicide toxicity (Gramatica et al., 2001). The fact that herbicides exhibit a range of different chemical structures may influence their uptake by algae and/or

their binding to the binding site and finally modulate their toxicity (Oettmeier, 1999).

4.3   Use of Phylogeny in the Context of Biomonitoring

Our results suggest that an a priori assessment of the sensitivity of benthic diatoms to PSII inhibitors can be made from both their phylogeny, which discriminates two major patterns of sensitivity, and their trophic mode, which refines the sensitivity level of diatom species, especially in the raphid clade, indicating that (1) phylogeny has a considerable potential for use in ecotoxicological studies involving diatoms and PSII inhibitor herbicides and (2) phylogeny provides promising results to predict bioindicator sensitivity, which may be used for the environmental bioassessment of herbicide impacts. Obviously, this study is a first step, and more studies are needed (e.g., within clades and on other diatom species) before an operational biomonitoring tool for herbicide contamination can be proposed. In our study and as a general trend, araphids and autotrophic species remain the most sensitive. Even *Cy. meneghiniana*, which is defined as mixotrophic and planktonic, was characterized as sensitive. This species is considered as an exception because most of the mixotrophic diatom species present in the environment are pennate (Van Dam et al., 1994). Centric and mixotrophic species are rare. Moreover, centric diatoms are rarely found in benthic habitats, and little information is available from micro-mesocosm studies. Pérès et al. (1996) have shown that centric (*Melosira varians*) and araphid (*Staurosira venter*) diatoms tend to disappear under pressure from isoproturon, whereas raphid species (*Cymbella mesiana* and *S. minima*) tend to be favored. Similarly, Schmitt-Jansen and Altenburger (2005) observed a decrease in the relative abundance of araphid species (*Fragilaria* sp., *Fr. capucina* var. *gracilis*) after exposure to atrazine and isoproturon. In an agricultural watershed, the relative abundance of *Fr. capucina* (araphid) tended to decrease, while that of *Navicula lanceolata*, *Nitzschia lineariz*, and *N. palea* (raphids) tended to increase (Roubeix et al., 2011). For IPSS (used to assess the water quality from diatom species abundance) and for ecological guilds, no relevant link was observed between the ranking of species' sensitivity and their phylogeny. Indices relevant for monitoring a specific stressor cannot easily be transposed to monitor another stressor. In our study, we observed that although the IPSS index is relevant in the context of global pollution, it does not seem to be appropriate for herbicide risk assessment, highlighting the need to develop specific biomonitoring indices for herbicide contamination. In the context of risk assessment and bioassessment, integrating phylogeny into ecotoxicological studies seems to offer a promising way to assess the order of species' sensitivity toward PSII inhibitor herbicides. Moreover, the predicted order of species' sensitivity has been supported by other experiments that integrate higher levels of complexity, such as mesocosm (Rimet and Bouchez, 2011) and in situ studies (Marcel et al., 2013). In such studies, diatoms are exposed to numerous biotic and abiotic environmental factors (turbulence, light, grazing, etc.) where herbicide contamination accounted only for part of the stress on the diatom assemblage. Ecotoxicology is a discipline in which it is necessary to conduct studies of varying complexity, ranging from molecules to ecosystems (Boudou and Ribeyre, 1997), to provide a solid foundation for biomonitoring. Environmental monitoring aims (1) to connect pressures and impacts for a posteriori assessment and (2) to predict the a priori risk of

contaminants. Both approaches rely on basic sensitivity data that are generally too few and heterogeneous and are obtained from model species unrepresentative of the ecosystems. Using the link between phylogeny and sensitivity offers the prospect of extending these data and increasing their environmental representativeness, thereby improving biomonitoring tools.

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