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Influence of herbicide contamination on diversity and ecological guilds of river diatoms

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Abstract – Tools that may be used to characterize the state of aquatic ecosystems and the impacts associated to pesticides are currently requested for the monitoring of ecosystems. Chemical analysis alone is not sufficient for such purposes and biomonitoring is essential. Many bioindicators based on diatoms are commonly used to assess the quality of aquatic environments but have not been developed to specifically address the impacts of pesticides. It is therefore crucial to develop dedicated tools. Our research aimed at performing a field study (analysis of monitoring network data) in order to evaluate the responses of different metrics towards pesticide pressure.

For diatom communities, toxic pressure associated to pesticides, especially herbicides, had a weak effect on species composition and on the various tested metrics. Nutrient and organic matter concentrations (and also typology) are the main influencing factors. When trophic and saprobic levels are controlled, it is possible to identify the effects of herbicides on some metrics. The only responding metric is the abundance of the "high profile" guild, which corresponds to taxa presenting an important contact surface with water flow. Its abundance decreases when herbicide toxic pressure increases. It could therefore be used to assess the impact of herbicides on diatom communities, but in a framework where confounding factors variability (e.g., nutrients, organic matter, river order) is reduced.

Microalgae / taxonomic distinctness / ecological guild / aquatic pollution / bioindication / pesticide / atrazine / biofilm

Résumé – Dans le contexte de la surveillance des milieux naturels il est nécessaire de disposer d'outils qui permettent de caractériser l'état des milieux et les impacts associés à la pression toxique liée aux pesticides. L'analyse chimique ne peut suffire à elle-seule et la mise en œuvre d'outils de bioindication est indispensable. De nombreux outils biologiques basés sur les diatomées ont été développés pour l'évaluation de la qualité des milieux aquatiques mais ne sont pas adaptés pour mettre en évidence de façon spécifique les impacts des pesticides. Le but de cette étude est de tester en milieu naturel (analyse de bases de données régionales) l'intérêt de métriques de diversité de guildes écologiques pour la pression en pesticides.

Pour les communautés de diatomées, il apparaît que la pression toxique liée aux herbicides, exerce dans les conditions naturelles une faible influence sur la composition en espèces et sur les différentes métriques testées. C'est essentiellement la concentration en nutriments et en matières organiques (mais également la typologie) qui vont déterminer leurs compositions et abondances relatives. En se plaçant dans des conditions où ces paramètres sont contrôlés, il est possible d'observer un effet des herbicides sur certaines métriques

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diatomées. La seule métrique qui réponde à la pression toxique en herbicides est la guilde des « profils hauts », qui correspond aux taxons présentant une surface importante de contact avec le courant. Son abondance diminue lorsque la pression toxique associée aux herbicides augmente. Cette métrique pourrait donc être utilisée pour évaluer l'impact des herbicides sur les diatomées, mais dans un cadre où la variabilité des facteurs confondants (nutriments, matière organique, typologie) est réduite.

Microalgues / distinction taxonomique / ecological guild / pollution aquatique / bioindication / pesticide / atrazine / biofilm

INTRODUCTION

The increasing contamination by micropollutants in freshwater systems has become a major problem in modern societies, giving rise to toxicological, sanitary and economical concerns. More than 90% of the rivers in Europe are contaminated by organic persistent micropollutants and herbicides are among the most detected (Loos *et al.*, 2009). In 2010 the French government adopted a plan for a 50% reduction over 10 years in the use of pesticides (plan Ecophyto 2018, INRA, 2010). Until now the existing tools to assess human impact on aquatic biota, and rivers in particular, were not developed for these particular pressures. Five main bio-indicators are routinely used in rivers at present. First, fish which is particularly adapted to warn about interruptions in river connectivity and global pollution. Second, macro-invertebrates are an essential biological element to assess river micro-habitats diversity, organic and nutrient pollutions. Third, macrophytes are good indicators of river eutrophication and to a lesser degree gross organic pollution. Fourth, Phytoplankton is a bio-indicator used in large lentic rivers which enable an assessment of nutrient level. Fifth, diatoms indicate the levels of nutrients and organic matter. Until now, no tools based on diatoms are adapted for the routine assessment of pesticide pressure.

Diatom bio-indication tools have been standardized more than ten years ago (AFNOR, 2003, 2004, 2007.) and are adapted to assess nutrient and organic matter levels in rivers. All European diatom indices routinely used for river quality assessment are based on specific pollution sensitivity (Rimet, 2012) and on the formula of Zelinka & Marvan (1961). However, despite the taxonomic composition of diatom assemblages is known to be sensitive to pesticide pollution (Dorigo *et al.*, 2007; Morin *et al.*, 2009; Guasch *et al.*, 1997, 1998), there is no routine tool base on diatoms able to assess micropollutant impact. Moreover, given their photosynthetic activity, we made the assumption that diatoms should be among the best candidates for the assessment of herbicides impact. The objective of this study is to assess whether two kinds of metrics based on diatom communities could be used to assess the impact of pesticide contamination, especially herbicides, in the French river monitoring network where diatom samplings are carried out every year.

First, biological traits of diatoms, such as ecological guilds, can provide useful information about the structure and architecture of biofilms. An ecological guild is a group of taxa which live in the same environment, but may have adapted differently to abiotic factors. Ecological guilds of Passy (2007) were developed on diatoms and their resistance to water turbulence and nutrients preferenda. Ecological guilds as most of the biological traits are characteristics of whole genera of diatoms. One advantage of their use is that taxonomic identification at the genus level would be easier than species identification and bio-indicators would be more robust and more cost-efficient. Studies carried out on freshwater biofilms in mesocosms and *in situ* rivers concluded that metrics of diatom life forms and ecological guilds are relevant to detect nutrient and organic matter enrichment (Berthon *et al.*, 2011) and pesticide contaminations (Rimet & Bouchez, 2011). Therefore, we chose to test such metrics. Diatom taxa were grouped into ecological guilds (Rimet & Bouchez, 2012) according to their growth form, potential to use resources and resistance to physical disturbances. We hypothesized that increasing herbicide concentration would modify the relative abundances of ecological guilds as observed in mesocosms studies (Rimet & Bouchez, 2011), with, on the one hand, a reduction of the abundance of the high-profile guild and on the other hand an increase of the abundance of the low-profile and motile guilds when herbicide contamination increase.

Second, Morin *et al.* (2009) and Ricciardi *et al*. (2009) showed in *in-situ* studies of a French and a Spanish river basins that species diversity decreased when pesticide contamination increased. Clarke & Warwick (1998) introduced new measures of diversity integrating the taxonomic dimension, the taxonomic distinctness index. For instance a community containing 10 species belonging to the same genus will present a lower taxonomic distinctness than a community containing 10 species belonging to different genera or families. The taxonomic distinctness index measures the average taxonomic distance between two randomly chosen individuals of a community. We made the assumption that such kind of index should respond to pesticide contaminations as classical diversity indices (e.g. Shannon or Simpson index).

Both hypotheses were tested on a large scale data set encompassing several hundred of river basins in eastern France which are monitored for their ecological quality with diatom and physical and chemical samplings.

MATERIAL AND METHODS

Study area

Samplings were carried-out in two large hydrographical basins in France (Fig. 1). The Rhône-Mediterranean catchment in the south, encompasses two major rivers, the Rhône and the Saône. The Rhine-Meuse basin in the north, encompasses three major rivers, the Moselle, the Meuse and the Rhine. 1002 samples were taken from the Rhône-Mediterranean catchment between 2005 and 2008 and 996 samples from the Rhine-Meuse catchment between 2000 and 2005.

Diatom sampling and analyses

Diatoms were sampled as part of the national biomonitoring program for rivers. The sampling procedure followed the French standard (Afnor, 2007). Diatoms were collected once per year, during the low-flow period. Benthic diatoms were collected from at least five stones from the lotic parts of the sampling sites. The upper surfaces of the stones were scrubbed with a toothbrush to collect the biofilms in which diatoms live. Then the sample was fixed in 4%

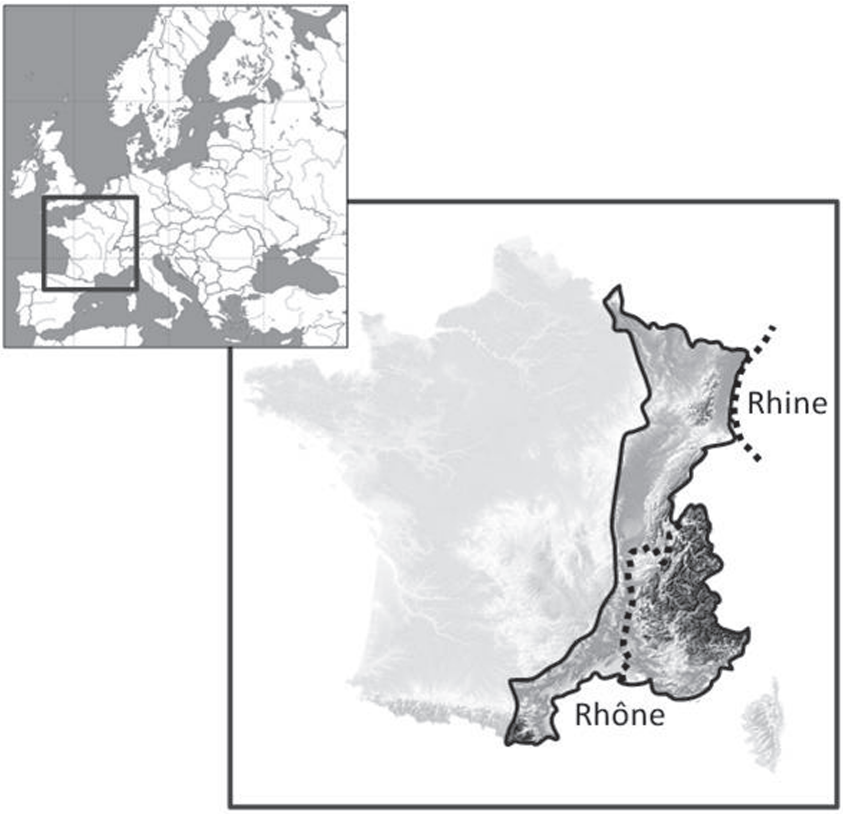

Fig. 1. Study area and its major rivers.

formaldehyde. In laboratory, the diatom valves were cleaned using 40% H_2O_2 and HCl. Clean valves were mounted in a resin (Naphrax©). At least 400 valves from each sample were counted and identified using a light microscope (1000 × magnification) according to European (European Committee for Standardization, 2004) and French (Afnor, 2007) standards. The abundances of all observed taxa were expressed as relative counts. Identifications were carried out to species and sub-species level using Krammer & Lange-Bertalot (1986, 1988, 1991a, b) floras and additional taxonomical bibliography.

Physical, chemical and micropollutants sample analyses

Physical and chemical analyses were carried-out at the same sampling sites every month. Dissolved oxygen and conductivity were measured in the field. For NO_3^-, NH_4^+, Kjeldahl nitrogen (TKN), PO_4^{3-}, Na^+, Ca^{2+}, Cl^-, K^+, Mg^{2+}, SO_4^{2-}, Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), water samples were collected and analyzed in the laboratory according to standard procedures (APHA, 1995). Micropollutants were also measured at the same sampling sites, but less frequently (once a year to each month depending on the water monitoring agency).

Database construction

We combined four kinds of data for each site: (a) taxa abundances; (b) physical and chemical characterization; (c) river typology (d) micropollutants concentrations. For physical and chemical data, we selected the chemical analyses carried out before (maximum interval is one month), and closest in time to each diatom sampling date. For river typology, each sampling site was classified according to the French Typological System used in the Water Framework Directive – WFD – (European commission, 2000). Two typological elements were considered. First, the ecoregions which were defined by Wasson *et al.* (2002) and Chandesris *et al.* (2006) on the basis of geology, climate and relief. Our study area covers 17 ecoregions. Second, the Strahler rank (Strahler, 1963) calculated by Chandesris *et al.* (2006), which was used to classify sampling sites into homogenous river sizes. Rivers within the area studied held ranks ranging from 1 (very small) to 8 (very large rivers). For micropollutants, a yearly average was calculated for each sample.

Diatom metrics

Diatom diversity was calculated by means of two classic indices: Shannon's H' (Shannon, 1948) and Simpson's D (Simpson, 1949). Another kind of indices, which take into account taxonomical relationships between taxa of a sample, was used: the taxonomic distinctness indices (Clarke and Warwick, 1998). Taxonomic distinctness indices are based on measures of the similarity on the taxonomy tree between individuals in a sample. For instance a sample with species which all belong to a single genus have a lower taxonomic distinctness than a sample with species belonging to different genera. The taxonomic distinctness would be even higher if these genera belonged to different families or orders. Clarke and Warwick (1998) proposed three indices based on this concept. We selected the Delta star index (D*) which represents the mean distance between two random individuals that do not belong to the same species.

Ecological guilds are groups of taxa co-existing in the same environment but differentiated by their adaptations to abiotic factors (Devito *et al.*, 2004). They are based on the species ability to use nutrient resources and resist to physical disturbance. The four ecological guilds we tested are based on the ones described by Rimet & Bouchez (2012) and modified from Passy (2007). Ecological guilds can provide useful information about the structure and architecture of biofilms. Four ecological guilds were thus distinguished:

<u>" *Low-profile* " species</u> can grow in nutrient-poor waters and resist physical disturbances, such as those caused by currents. This group is formed by small species, directly attached to the substrate by the whole valve area ("*prostrate*"), attached parallel to the substrate by the apex ("*adnate*") or attached perpendicularly ("*erect*") and slow-moving species. Genera such as *Achnanthes, Achnanthidium, Amphora, Cocconeis, Cymbella, Opephora* and *Remeria* pertain to this group.

<u>"*High-profile*" species</u> can grow in polluted waters (nutrient and organic matter-rich) but are sensitive to physical disturbance: longer erect species, filamentous, "*branched*" (arborescent), chain or tube-forming and pedunculate. Genera such as *Diatoma*, *Eunotia, Fragilaria, Gomphoneis, Gomphonema* and *Ulnaria* pertain to this group. The species' ability to form colonies enable them

to exploit resources unavailable for "*low-profile*" but make them more vulnerable to current turbulences and grazing pressure.

Planktic species grow in the water column. These taxa are adapted to maximize their floatability in the most energy-efficient way: thinner frustules, increased area, colonial organization, mucilaginous filaments. Genera such as *Asterionella, Stephanodiscus, Cyclotella, Discotella* and *Aulacoseira* pertain to this group.

Motile species proliferate in nutrient-rich waters and are capable of fast motion. Genera such as *Navicula, Nitzschia, Sellaphora* and *Surirella* pertain to this group. Passy (2007) drew the hypothesis that these taxa should be more resistant to physical disturbances, but showed that they are not.

Pesticides selection and statistical analyses

Descriptive statistical analyses and scattergrams of the pesticide data were performed to select the molecules best suitable for testing their effect on the diatom metrics. The most often detected molecules were selected. Correlation tests (Spearman correlation coefficient) between diatom metrics (ecological guilds and diversity indices) and micropollutants were performed on data concerning the atrazine concentrations on one side and on sum of Toxic units of atrazine, isoproturon and diuron on the other side because these herbicides were the three most detected micropollutants. We used the HC50 (Hazardous concentration) (Posthuma *et al.*, 2002) to calculate Toxic Units (TU). TU allow to evaluate a priori the toxic pressure on algae. To calculate TU, micropollutant concentrations are weighted by their HC50's (hazardous concentration):

$$\text{Toxic Units} = C_{atrazine}/HC50_{atrazine} + C_{isoproturon}/HC50_{isoproturon} + C_{diuron}/HC50_{diuron}$$

The HC50 values used were those of Larras *et al.* (2012) who performed series of ecotoxicological tests on cultures of diatoms representative of river biofilms communities to determine the HC50 for each of the 3 herbicides (Table 1).

Diatom metrics are highly correlated to several important parameters: nutrients concentration (Berthon *et al.,* 2011), river size (Potapova *et al.,* 2002) and ecoregion (Rimet *et al.,* 2007). Moreover, pesticides show important auto-correlations with nutrients. Therefore, in order to fix these environmental parameters and to observe the influence of pesticides on diatom metrics, we fixed these different environmental parameters by selecting comparable rivers. First, we selected a single ecoregion, second we selected inside this ecoregion a particular river size, presenting a given nutrient level. It appeared that the calcareous plains ecoregion was the ecoregion presenting the highest number of available data, this ecoregion was therefore selected. Inside this ecoregion, small rivers (Strahler rank below 3) were the most numerous rivers presenting micropollutant measurements; this river size was therefore selected. We then choose the rivers presenting low nutrient level (in the sense of the SEQeau system, the French system for water quality assessment), that is presenting concentration of nitrate below 10 mg $NO_3^{-}\cdot L^{-1}$ and phosphate concentrations below 0.5 mg $PO_4^{3-}\cdot L^{-1}$.

Table 1. HC50 values from Larras *et al.* (2012) used to calculate Toxic Units (TU)

Molecule	*HC50 µg/L*
Atrazine	283.52
Diuron	24.83
Isoproturon	73.46

Finally, correlations were first tested on 1/ the whole database to show the auto-correlations between herbicides and nutrients, 2/ on samples coming from a single ecoregion (calcareous plains), with strahler rank below 3 (small rivers), and presenting low nutrient concentration.

Additionally, the influence of herbicides on diatoms was compared to the influence of classical environmental parameters such as nutrients, organic matter and mineral content. Canonical Correspondence Analyses and variance partition analysis were performed. Two groups of parameters were defined to run these analyses as either variables or covariables: one group with the herbicides (sum of TU of atrazine, diuron and isoproturon) the other group with physical and chemical parameters (NH_4^+, Cl^-, COD, Conductivity, NO_3^-, NO_2^-, PO_4^{3-}, Ptot, Na^+). These analyses were carried out on diatom assemblages expressed by species abundances, and on ecological guild abundances. The Canoco software (v 4.5 for windows) was used to compute these analyses.

RESULTS

Herbicide selection

Only 896 of the 2170 diatom samples in the database had corresponding pesticide measures. A total of 521 different pesticides were measured, the majority of them being scarcely ever present. Six molecules were measured in all of the 896 pesticide samples: atrazine (herbicide), diuron (herbicide), HCH gamma (insecticide), isoproturon (herbicide), simazine (herbicide) and trifluraline (herbicide).

Figure 2 shows the scatter of their concentrations in the two river basins and underlines several biases related to the data. The first bias, which is well observable on Figure 2 with the horizontal lines of points: these horizontal lines of points correspond to measures with exactly the same values (the measures are aligned on the same line and make these lines of points). This means that most of the pesticides were measured with different quantification limits. It is evident for trifluraline for which three quantification limits are present in the Rhine basin and only one in the Rhone basin. Moreover the two river basins (Rhin-Meuse *vs* Rhone-Mediterranean basin) pesticides analyses were conducted by different laboratories which had for a given micropollutant different quantification limits. It is the case for isoproturon or trifluraline, which quantification limits are inferior in the Rhine-Meuse basin than in the Rhône-Mediterranean basin. The differences of quantification limits were also observed and discussed in a report of the French Ministry of Ecology and sustainable development (Commissariat général au développement durable. Service de l'observation et des statistiques, 2011) and concluded on the difficulty to compare different regions monitored by different laboratories.

Therefore, given these biases, the herbicide best suitable for correlation analysis with the diatom metrics is atrazine, because it showed the greatest number of measurements (896 samples), the greatest number of detection over the quantification limits and the largest range of concentration. Diuron and isoproturon were selected in addition to atrazine because they were measured as many times than atrazine and showed good ranges of concentration. Test of correlation between these three herbicides expressed in TU, and the diatom metrics were carried out.

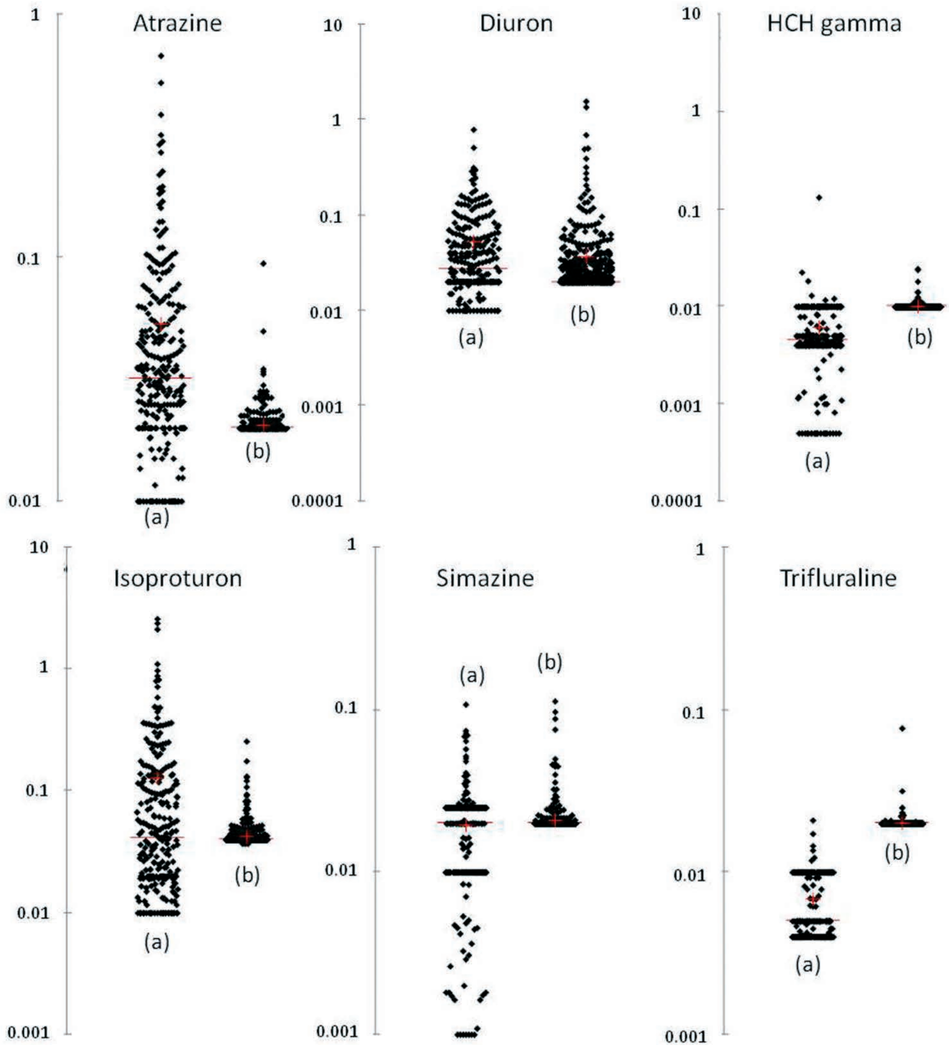

Fig. 2. Scattergrams of concentrations in the six pesticides most often detected in the Rhine (a) and Rhône (b) river basins.

Correlations between atrazine concentrations and diatom metrics

When the gradient of nutrient concentrations (NO3–, $PO4^{3-}$) is important, or when typology is very diverse in terms of river sizes (Strahler rank) as well as in terms of ecoregions, the Atrazine concentrations are significantly correlated to nutrients and mineralization parameters (Cl–, Na+) (Table 2). No correlation is observed between atrazine and nutrients or mineralization parameters when the analysis is restricted to a selection of rivers of a given ecoregion, of a given river size and of a low nutrient level. The only significant correlation observed with regard to diatom metrics is a negative correlation between the “high-profile” guild

Table 2. Results of the correlation tests between atrazine concentration and physical and chemical parameters, biodiversity metrics, taxonomic distinctness and ecological guilds. Values in the table correspond to p-values associated to the test of the Spearman correlation coefficient. Significant values are given in bold

Correlation with Atrazine		
Parameters	*All data*	*Single ecoregion Small rivers Low nutrients*
Cl–	***< 0,0001***	0,980
NO_3^-	***0,029***	0,955
PO_4^{2-}	***0,018***	0,106
Na+	***< 0,0001***	0,984
D*	***0,006***	0,249
Simpson	0,253	0,330
Shannon	0,435	0,470
High profle	***< 0,0001***	***0,003***
Low profile	0,125	0,382
Motile	0,142	0,901
Euplanctonic	***< 0,0001***	0,643

abundance which can be modelized by a second order polynomial equation (Fig. 3). It shows a significant decrease of their relative abundance when Atrazine concentration increases.

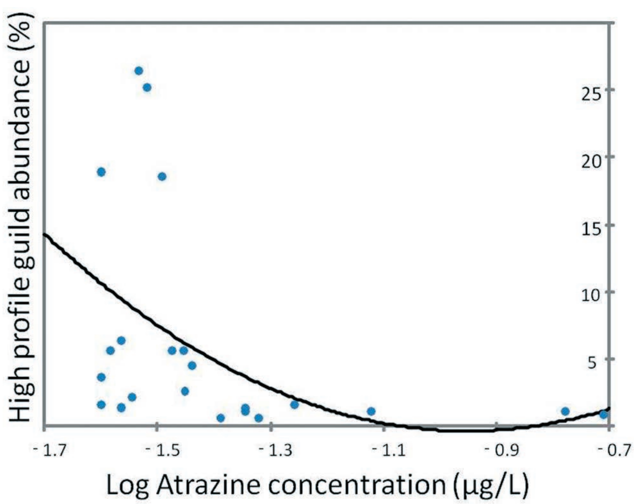

Fig. 3. Second order polynomial regression between the “high profile” guild abundance and log transformed atrazine concentration.

Correlations between Toxic Units and diatom metrics

When the nutrient gradient is strong (NO_3^-, PO_4^{3-}) or typology is diverse in terms of river size (Strahler rank) as well as ecoregion, TU are correlated to nitrate concentrations and mineralization parameters (Cl^-, Na^+) (Table 3). No correlation is observed when the analysis is performed only in the case of a selection of river size in a given ecoregion and a low nutrient level. TU show no significant correlation with the diatom metrics.

Table 3. Results of the correlation tests between Toxic Units (TU) and physical and chemical parameters, biodiversity metrics, taxonomic distinctness and ecological guilds. Values in the table correspond to p-values associated to the test of the Spearman correlation coefficient. Significant values are given in bold. TU were calculated with Atrazine, Diuron and Linuron concentrations and their HC50 (Table 1)

Correlation with Toxic Units		
Parameters	*All data*	*Single ecoregion Small rivers Low nutrients*
Cl–	**< 0,0001**	0,734
NO_3^-	0,557	0,839
PO_4^{2-}	**< 0,0001**	0,280
Na+	**< 0,0001**	0,734
D*	0,447	0,242
Simpson	0,828	0,734
Shannon	0,455	0,402
High profle	**0,020**	0,162
Low profile	0,697	0,745
Motile	0,668	0,576
Euplanctonic	0,935	0,502

Variance partition of the diatom metric data between herbicide concentration and physical and chemical parameters

To assess the amounts of variance in the diatom communities that can be attributed to the herbicides and the physical and chemical parameters (nutrients, organic matter and mineralization) two variance partition analyses were performed (Fig. 4). The first one is carried out with the specific composition

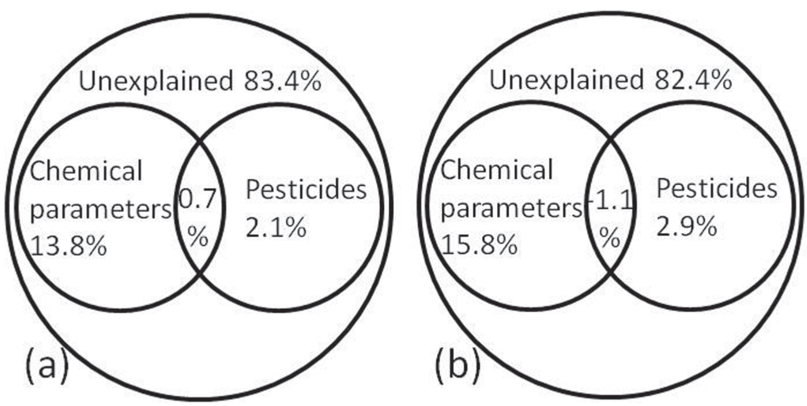

Fig. 4. Partitioning of the total variance in the abundance data of the different diatom species (a) and ecological guild metrics abundances (b) between two categories of explanatory variables (chemical parameters and pesticides).

(abundances) and the second with diatom metrics (diversity indices and ecological guilds). Two explanatory sets of variables were used, first the TU (TU of atrazine, TU of diuron and TU of isoproturon) and second, the chemical parameters (NH_4^+, Cl^-, COD, Conductivity, NO_3^-, NO_2^-, PO_4^{3-}, Ptot, Na^+).

The amount of variance explained by the chemical parameters is much higher than that explained by herbicides, both when considering species and data metrics. We observe an antagonistic effect of the two groups of parameters (negative value) on the metrics and a synergistic effect on the abundance data.

DISCUSSION AND CONCLUSIONS

The present study aimed at highlighting correlations between pesticides and diatom metrics in rivers. We used data from the river monitoring network. The discussion presents the most important limitations that prevent getting groundbreaking results, then some comparisons with former studies are given and finally we conclude about the use of diatom metrics for herbicide monitoring.

Database limitations

Despite the large amount of data collected to build the database (2170 samples), the statistical exploitation was bothered by several limitations.

The first most important limitation for the data analyses is that a large majority of substances were measured only in a few sampling sites. This implies that many substances cannot be used for statistical analyses on a large number of samples. Therefore, in order to have a sufficient number of data, a selection of the most often measured pesticides has to be done. Atrazine, diuron, isoproturon, trifluraline and simazine were the five most measured micropollutants, they are all herbicides, inhibitors of the photosystem. An additional substance, HCH-gamma (or Lindane) was among the six most measured molecules, and is an insecticide. These six pesticides were measured in 41% of the 2170 samples and made a sub-dataset of 896 samples possible to exploit. Nevertheless, a large majority of the measures correspond to the quantification limits of the micropollutants, and therefore, if considering only Atrazine, only 281 samples out of 896 samples presented measures over the quantification limit. Moreover, three of the six most measured pesticides (lindane, simazine and trifluraline) showed such an important proportion of values equal to quantification limits that it was impossible to use them for further analyses. Indeed, such data present the problem of sparse matrices where systems are loosely coupled, or where it is difficult to show connections between systems. These molecules were therefore not selected for correlations analyses and values equals to concentration limits were removed for the three selected pesticides (atrazine, diuron, and isoproturon). These three herbicides were further used to search for correlations.

The second limitation was caused by an important variability in the pesticides laboratory analysis procedures. Indeed, for a given molecule, quantification limits are different from a laboratory to another. This artifact was also observed for a given laboratory over years since lab procedures and detection material changed from a year to another and therefore, quanitification limits changed. This was also observed at a national scale by other authors

(Commissariat général au développement durable. Service de l'observation et des statistiques, 2011). In the case of our database, we brought to light important differences of quantification limits between regions managed by different water agencies (Agence de l'Eau Rhin-Meuse versus Agence de l'Eau Rhone-Méditerrannée). Therefore we chose to select a single region (Rhin-Meuse), which presented the lowest quantification limits. Inside this region, the calcareous plains ecoregion (Wasson *et al.*, 2002) was chosen to limit the ecoregional heterogeneities observable inside the Rhin-Meuse river basin in terms of diatom communities composition (Rimet, 2009).

The third limitation is related to the numerous auto-correlations inherently present when considering the entire dataset. Diatoms metrics such as life-forms and ecological guilds are known to be strongly correlated to nutrients, organic matter concentration (e.g. Passy, 2007; Berthon *et al.,* 2011) but also river size and geology (e.g. Biggs & Gerbeaux, 1993; Cattaneo *et al.*, 1997; Passy, 2007; Song, 2007). This was also observed in our case. Strong correlations were also observed between the three selected herbicides (Atrazine, Diuron and Isoproturon) and nutrients (Tables 2 and 3). This can be explained by the fact that increasing nutrients level in waters is an indicator of increasing human activity (e.g. European commission, 2000), and therefore, to an increase of pesticide concentration in waters. In order to avoid these auto-correlations between pesticides and nutrients and consequently on diatom metrics, and in order to solely observe the effect of herbicides on diatoms we chose to limit the variability of nutrients (nitrate and phosphate), inside a given river size, and a given ecoregion. We therefore selected small rivers (Strahler rank) of the calcareous plains, with very low levels of nutrients in the sense of the French surface waters assessment system (SEQeau v1, Agences de l'Eau, 1999). Only 22 samples were selected.

Impact of herbicide on diatom metrics

When analyzing the sub-set of 22 samples, Atrazine was not correlated anymore with neither nutrients (NO^{3-}, PO_4^{2-}), nor parameters of mineralization (Cl^- and Na^+). The same observation can be given for the three herbicides (Atrazine, Linuron and Diuron) when expressed in Toxic Units. It appeared also that the number of significant correlations between herbicide concentrations and diatom metrics was extremely low compared to the number of correlations when analyzing the all the data (281 samples). Herbicide exposure generally alters the diversity of diatom communities (Debenest *et al.*, 2010). Ricciardi *et al.* (2009) and Morin *et al.* (2009) observed a reduction in diatom diversity when herbicide concentration increased (Atrazine, Linuron and Diuron). Despite their findings, we did not observe such tendencies in our study. The difference between those results could lie in the fact that in both publications (Ricciardi *et al.,* 2009; Morin *et al.,* 2009) the authors did not try to remove the effects of the variations in nutrient levels and typology to assess the effects of pesticides. Our hypothesis is therefore that their results probably show an effect of nutrients rather than pesticides.

We observed only one significant correlation: a decrease of the "high-profile" guild abundance when atrazine concentration increased. This tendency was the same for the three herbicides (Diuron, Isoproturon and Atrazine) expressed as Toxic Untis, but not significant. A similar correlation was obtained in mesocosms experiments (Rimet & Bouchez, 2011) as well as in *in situ* experiments (Bouchez *et al.*, 2012). Concordance of the results obtained in systems of different complexities enlightens the interest of the "high-profile" guild to detect the effects of some pesticides on diatom assemblages. Even if we fixed nutrients and typological parameters by selecting comparable rivers in the dataset, many other environmental parameters affecting diatom communities structures were still varying a lot (e.g. water turbulence, shading …). This is pointed out by the results of the variance partitioning analysis (Fig. 4). Variance explained by the concentrations of the three herbicides is low (2.9%) compared to that of the physical and chemical parameters (15.8%).

CONCLUSIONS

This study demonstrates how difficult it is to relate herbicide contamination to diatom communities, even if they are simplified into diatom metrics. This was mainly due to data limitations (many micropollutants measured in a minority of samples, heterogeneity of quantification limits for a given molecule, problems of numerous auto-correlations present in the database…). But this is also due to the fact that, when considering *in situ* data, many environmental factors (shading, turbulence…) have much more important impact on diatoms than herbicides. This can be explained also by differences in terms of sensitivity that can be observed for a given species which has been adapted to different environments subject to contrasted herbicide contaminations (e.g. Ishihara, 2009; Roubeix *et al.*, 2012). A further possibility could be the use of passive organic chemical integrative samplers (POCIS) on the river monitoring network. POCIS allow to measure an integrated sample over up to a month, which would lessen the occurrence of concentrations lower than the detection limits (Mazzella *et al.*, 2007). Moreover, they could provide valuable information, as pesticides concentrations can show large and rapid variations that are not represented in the occasional samples from the monitoring networks.

Despite the initial size of the database, very few significant results were observed. Nevertheless, the high-profile diatom guild showed a significant decrease when atrazine concentration increased, similarly to the observations in mesocosms and *in situ* experiments, and therefore seems to be a good candidate for herbicide contamination monitoring. Mesocosms studies (Rimet & Bouchez, 2011), which enable fixing important parameters such as water turbulence, shading, nutrient level showed much more clearly an impact of pesticides on ecological guilds than the present database approach. An *in-situ* survey (Bouchez *et al.*, 2012), which enables studying a given river type with a better control of the relevance of the pesticde samples, similarly showed an impact of herbicides on ecological guilds. This strengthen the necessity to use approaches integrating different ecological complexities (Boudou & Ribeyre, 1997) to understand the complex effect of herbicides on diatoms assemblages in natural situation. Such approaches integrating different ecological complexities are of prime importance to develop new tools for biomonitoring.

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