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Comparison of Eukaryotic Phytobenthic Community Composition in a Polluted River by Partial 18S rRNA Gene Cloning and Sequencing

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Abstract

We compared the species composition in phytobenthic communities at different sampling sites in a small French river presenting polluted and unpolluted areas. For each sampling point, the total DNA was extracted and used to construct an 18S rRNA gene clone library after PCR amplification of a ca 400 bp fragment. Phytobenthic community composition was estimated by random sequencing of several clones per library. Most of the sequences corresponded to the Bacillariophyceae and Chlorophyceae groups. By combining phylogenetic and correspondence analyses, we showed that our molecular approach is able to estimate and compare the species composition at different sampling sites in order to assess the environmental impact of xenobiotics on phytobenthic communities. Changes in species composition of these communities were found, but no evident decrease in the diversity. We discuss the significance of these changes with regard to the existing level of pollution and their impact on the functionality of the ecosystem. Our findings suggest that it is now possible to use faster molecular methods (DGGE, ARISA…) to test large numbers of samples in the context of ecotoxicological studies, and thus to assess the impact of pollution in an aquatic ecosystem.

Introduction

The intensive use of xenobiotics, such as the use of herbicides in agriculture, has a direct impact on soil and freshwater microalgal communities other than the intended target organisms [1]. Changes in species composition within these communities can therefore be expected, and methods able to detect these changes are needed. Traditional approaches to evaluate microalgal diversity involve species identification based on morphological and morphometrical criteria. However, microscopy is time consuming, and species identification is often difficult because of the phenotype plasticity that characterizes organisms such as microalgae. Furthermore, conventional approaches cannot be used to evaluate intraspecific diversity, and this could be very useful for assessing disturbance of various origins (physical, chemical, or biological origin) in an ecosystem [20].

Many recent studies have demonstrated the effectiveness of molecular methods, based, for example, on

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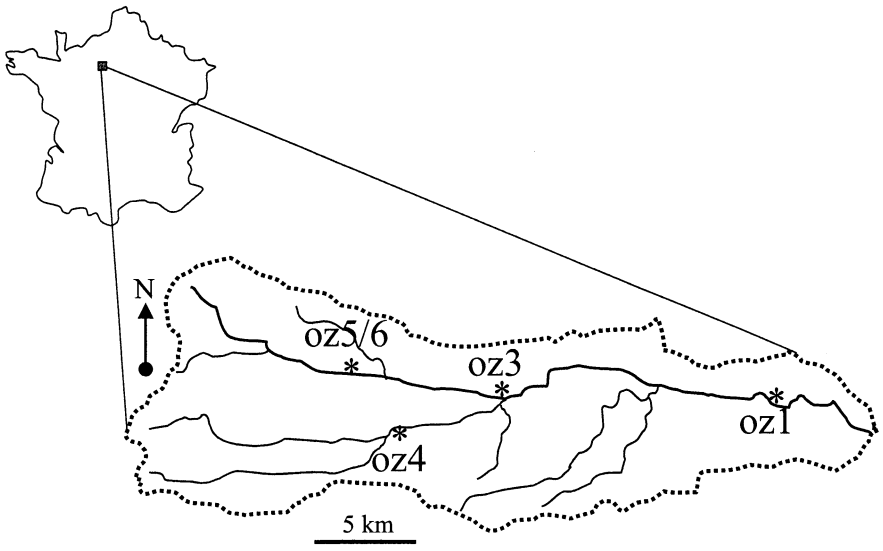

Fig. 1. Location of the river Ozanne in France. Oz1, 3, 4, and 5/6 are the four sampling stations for this study. Dotted line: limit of the catchment area of the river.

screening 16S rRNA gene clone libraries, to estimate the diversity of natural prokaryotic communities in soil [e.g., 22] and in water [e.g., 7, 13]. Similar studies of eukaryotic microorganisms have been successfully applied to marine picoplankton [9, 10, 18, 23, 31], but as far as we are aware, no similar attempt has been made to apply this approach to freshwater microalgal communities. We therefore set out to test the ability of a random sequencing strategy involving 18S rRNA gene libraries to evaluate and compare the species richness of freshwater phytobenthic communities exposed to selection pressure due to herbicide contamination.

Our study area was a river located in central France that is known to be subject to variable pollution by two agricultural herbicides, atrazine and isoproturon (data not published). We first defined a new set of universal primers for amplifying an 18S rRNA gene fragment in the main algal groups. Then we constructed a gene library and randomly sequenced clones at three times of year (May, June, September) from all four sampling sites. Finally, we used multivariate analysis to evaluate the changes in species composition within the studied periphytic communities to see whether the pesticides had any impact on these communities.

Methods

Algal Cultures

Monoclonal cultures of the most common algal classes, i.e., Chlorophyceae (*Selenastrum capricornutum* and *Scenedesmus acutus* with strain numbers 81 and 141-1 in our algae collection), Bacillariophyceae (*Synedra delicatissima* and *Nitzchia* sp., with strain numbers 133 and 139-3, respectively), Cryptophyceae (*Cryptomonas* sp. with strain numbers 26 and 62), and Chrysophyceae (*Synura petersenii* with strain number 1c), were harvested from chemostats and batch cultures by filtering a sufficient volume (ca 50 mL) through 1 μm membranes (Whatman Nuclepore), or directly by centrifuging them for 20 min at 7000 rpm (Avanti J 301, Beckman). In this latter case, the supernatant was discarded, and the pellet transferred to a 1.5 mL centrifuge tube. This was stored at −32°C until use.

Study Site

The Ozanne river is located within a region of intensive agriculture (latitude 48°12′40″; longitude 1°9′49″) and is mainly contaminated by atrazine and, to a lesser extent, by isoproturon. Four sampling sites were studied: oz1, oz3, oz4, and oz5/6 (Fig. 1). The stations are listed in order of their relative positions from downstream to upstream. The most upstream point, oz5, was the initial reference station, but when it dried out it was replaced by oz6. oz1, oz3, and oz4 were located in cultivated areas while oz5/6 was located in the upstream of the river and far away from any herbicide applications. All these sites could be classified as "open site," i.e., with a comparatively and relatively good exposure to light and a trivial presence of riparian vegetation. Sampling was performed in May, June (after herbicide application to the fields), and September. To evaluate the reproducibility of our approach, two samples collected separately in May were analyzed from each sampling station. In oz1, oz3, and oz4 sampling sites, atrazine concentrations ranged from 0.1 to 17 μg/L (mean value: 2.5 μg/L) and isoproturon concentrations ranged from 0.11 to 0.48 μg/L. In the oz5/6 sampling site, atrazine concentrations were always less than 0.1 μg/L and isoproturon was only found in May (0.27 μg/L) [12].

Periphyton Sampling

Natural periphytic communities were collected during the year 2000 on 1.5 cm^2 glass disks, which were glued onto 18 × 22 cm

Table 1. Nucleotide sequences of the PCR primers tested for the amplification of a ca. 400 bp fragment in the 18S rRNA gene (primers used in this study shown in bold)

Name	Sequence (5′-3′)	Type
P45	**ACC TGG TTG ATC CTG CCA GT**	**Forward primer**
P46	TCC GGA GAG GGA GCC TGA GA	Forward primer
P47	**TCT CAG GCT CCC TCT CCG GA**	**Reverse primer**
P48	AGA GTG TTC AAA GCA GGC TT	Forward primer
P49	AAG CCT GCT TTG AAC ACT CT	Reverse primer
P50	GAA ATT GAC GGA AGG GCA CC	Forward primer
P51	GGT GCC CTT CCG TCA ATT TC	Reverse primer
P52	GCG GTG TGT ACA AAG GGC AG	Reverse primer

pieces of 4 mm thick Plexiglas, using aquarium silicon sealant, assumed to be free of toxic chemicals. Colonization on these artificial substrates does not mimic colonization on natural substrate but reduces the heterogeneity occurring on natural substrate [3, 4, 21, 28] and permits us to obtain high numbers of replicates with the same bias in colonization in all the sampling sites studied. The plates (bearing about 120 glass disks) were then fixed to concrete blocks and placed in the running part of the stream (width varying from 0.5 to 5 m), to avoid sediment covering the disks, and exposed to sunlight to minimize the side effects due to differences in light exposition. All sampling sites were chosen as far as possible to be similar in term of water velocity, nutrient status, and light exposure, as these parameters can affect the composition and the growth of periphytic communities [3, 5, 17]. Glass disks were covered by 10 to 30 cm of running water at the beginning of colonization. After a colonization period of 2 to 3 weeks, the communities were harvested, transported to the laboratory in plastic bags containing river water and inside a cool box, and then stored at a temperature of −32°C until used. Five to 10 glass disks were scraped using a scalpel and analyzed using our molecular method.

DNA Extraction, PCR, and Cloning

DNA was extracted with the DNAeasy Plant mini kit (Qiagen) according to the manufacturer's instructions. The DNA obtained was diluted for further PCR amplification, so that the final concentration was about 30 ng/μL. Several different primer sets (Table 1), defined after alignment of 18S rRNA gene sequences of eukaryotic microalgae, were initially tested on monocultures, then on specific mixes. The 25-μL PCR mixtures contained 60 ng of template DNA, a 120 μM concentration of each of the four dNTPs, 10× PCR reaction buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HC; pH 9), 1 μM of each primer, and 1.5 U of *Taq* DNA polymerase (AmershamPharmaciaBiotech). The DNA templates and a negative control were subjected to an initial denaturing step at 94°C for 1 min. The following 37 cycles consisted of a 50 sec denaturing step at 92°C, a 50 sec annealing step at 57°C, and a 50 sec extension step at 72°C. A final 10 min extension step was carried out at 72°C.

Amplifications of the target region were checked by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.Positive PCR products were cloned in pGEM-T vector (Promega).

Sequence Analysis

At least 10 white colonies were randomly picked from each clone library, and 18S rRNA gene sequences flanking the inserted region were PCR amplified using the commercial primers T7 and SP6 primers. The amplified fragments were sequenced on an Applied Biosystems 373 automated sequencer (PerkinElmer, Foster City, CA) according to the supplier's instructions. The sequences were aligned using the Pileup module of the GCG package (Genetics Computer Group, Inc., Madison, WI) and GeneDoc [24]. The sequences were identified by searching for homologous sequences in GenBank using the Fasta module of the GCG package. Sequences presenting less than 85% homology with plant and fungal sequences contained in GeneBank were eliminated from the alignment.

Comparison of Species Richness in the Sampling Areas

A phylogenetic tree was constructed for the whole data set by neighbor-joining (NJ) on Jukes–Cantor distances using the PHYLIP software package [14]. The bootstrap option was used to run 500 replicates. The tree was drawn using TreeView [25], and several operational taxonomic units (OTUs) were defined on the basis of their bootstrap proportion. The number of sequences belonging to these different OTUs was determined for each sampling station (oz1 to oz5/6) and each sampling date, and used to construct a contingency table. Sequences which were not contained in a subcluster supported by a bootstrap proportion larger or equal to 70% were assigned to two subcategories ("other Viridiplantae" and "other Stramenopiles") and reported as such in the contingency table. All these data were then analyzed by correspondence analysis (CA) using the ADE-4 software package [30].

Results

Primer Evaluation

Different primer sets were separately tested on monocultures of freshwater microalgal species belonging to the most common algae classes: Bacillariophyceae, Chlorophyceae, Cryptophyceae, and Chrysophyceae. The combination of the P45 and P47 primers gave good amplification of a ca 400-bp 18S rRNA gene fragment for all the species tested (Table 2). This set of primers was then successfully evaluated on DNA mixes containing variable amounts of DNA from each species. After cloning

Table 2. Results of the amplifications performed with all the primers tested (Table 1), on the main algal classes[a]

	Bacillariophycea		Chlorophyceae		Cryptophyceae		Chrysophyceae
	133	139-3	81	141-1	62	26	1c
p45–p47	+	+	+	+	+	+	+
p46–p49	+	−	+	+	−	−	+
p48–p51	+	−	+	+	−	−	+
p50–p52	−	+	−	+	+	−	+

(+) 139means that a ca 400 bp fragment was amplified, and (−) that amplification was either nonexistent or not reproducible. 133, *Synedra delicatissima*; 139-3, *Nitzschia* sp.; 81, *141-1, Scenedesmus acutus*; 62 and 26, *Cryptomonas* sp. 1c, *Synura petersenii*.

and sequencing, the proportion of each species in each of these DNA mixes was globally retrieved (data not shown).

Phylogenetic Analysis of the Results

The NJ tree of all the sequences revealed two main clusters, which were strongly supported by their bootstrap proportions. The first cluster consisted of 43 Chlorophyceae sequences (C1), and the second of 81 Bacillariophyceae sequences (C2) (Fig. 2). Nine sequences were also observed that belonged to none of these two clusters. Each of the C1 and C2 clusters was reanalyzed separately, to define several subclusters (G), which were validated by having a bootstrap value of at least 70%. For each of these subclusters and for the nine other sequences, we found sequences homologous to sequences in the GenBank database (Table 3). In cluster 1 (Chlorophyceae), very strong homologies (>99%) were found between consensus sequences of the subclusters G1 and G3, and species belonging to the *Characium* and *Monostroma* genera, respectively. In contrast, for the subcluster G11, which contains most of the sequences of cluster 2 (Bacillariophyceae), no homology greater than 95.5% was found in the GenBank database with the consensus sequence of this subcluster. However, numerous sequences belonging to several Bacillariophyceae genera matched with 93–95% homology.

Seasonal and Anthropogenic Effects on Phytobenthic Community Composition

To estimate the effect of the seasonality and herbicide pollution on the biodiversity of phytobenthic communities, we compared the proportion of the different OTUs at each sampling site and each sampling date. This revealed that both seasonal and anthropogenic effects on the species richness can be clearly identified. For example, the subcluster G3 only included sequences from samples isolated in May. Similarly, the subcluster G7 only contained sequences from samples isolated in September. The effect of herbicide pollution (anthropogenic effect) was reflected, for example, in the fact that no sample isolated at the unpolluted station oz5/6 was present in the large G11 subcluster whereas samples from the unpolluted oz5/6 station were overrepresented in subcluster G3. Generally speaking, it appears that the proportion of Bacillariophyceae sequences was lower at the unpolluted station oz5/6 than at polluted areas oz1, 3, and 4.

To obtain a graphical representation of these observations, we performed a correspondence analysis of these data (Fig. 3). The projection of the stations in the planes defined by the three major axes accounting for most of the variance (sum of the inertia >66%) showed, first, that the replicates from each station were grouped together (oz1m/1 with oz1m/2; oz3m/1 with oz3m/2…), confirming the reproducibility of our approach, and second, that the unpolluted station (oz5/6) stood out clearly from all the other stations at all sampling dates but particularly in June and September, when herbicide loads were found to be high. The first axis allows us to distinguish between the oz6s sample and the other September samples, whereas axis 2 distinguishes oz6j from the other June samples and axis 3, the oz5m samples from the other May samples.

Discussion

Our approach to estimate the species richness of natural phytobenthic communities is already in use for the study of bacterial communities. This kind of method has been shown to be effective in these model organisms, although several sources of biases have been detected in the estimation of species richness. Most of them are linked to artifacts generated by PCR (replication errors or chimeric sequences, for example) as demonstrated by Qiu et al. [26]

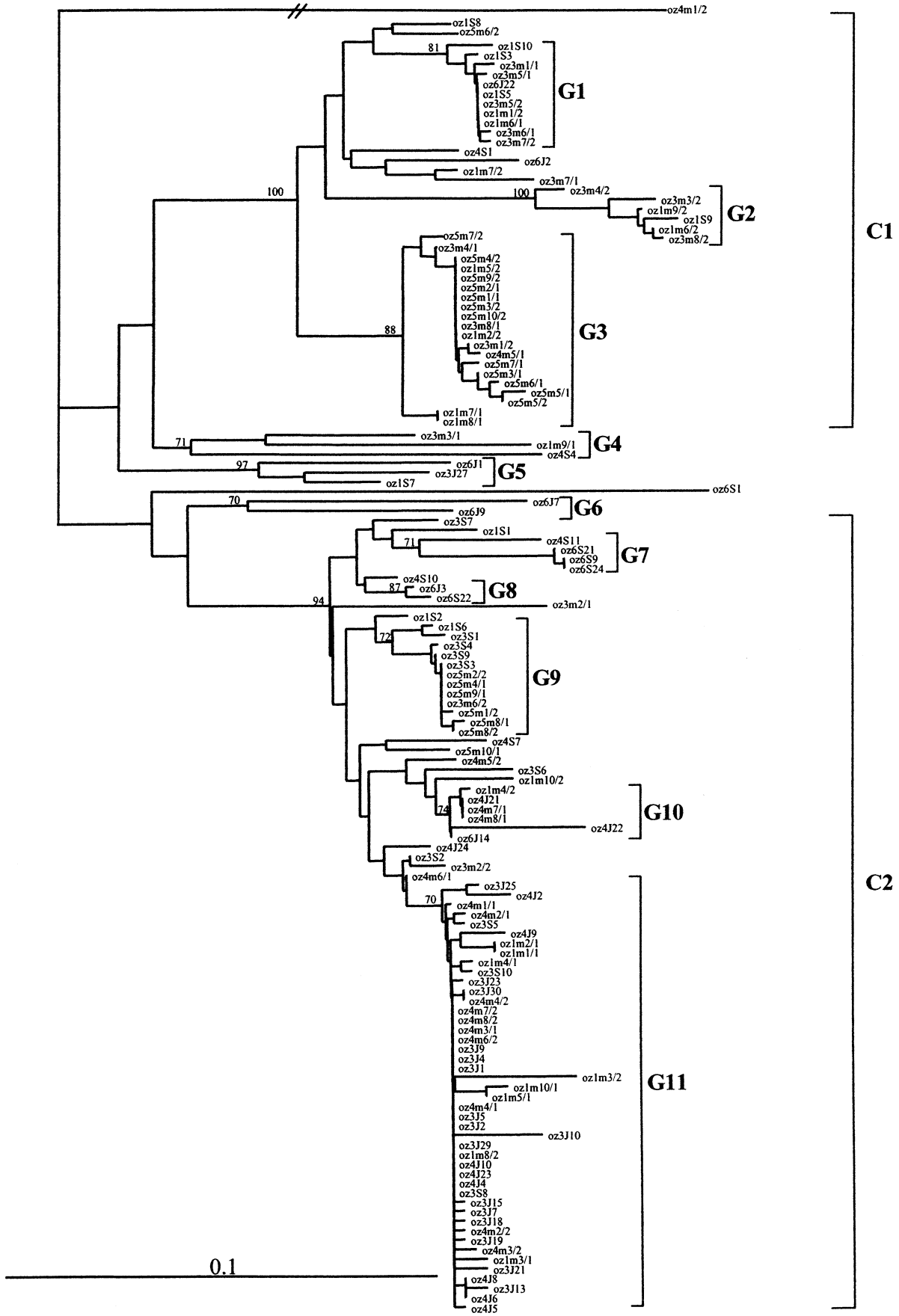

Fig. 2. NJ tree showing the species composition of the periphyton samples. m, May; j, June; s, September. For example, oz1m4/2 means sampling station 1, month of sampling May, fourth sequence foundin the sample, second set of extractions. Bootstrap values greater than 70% are indicated at the nodes of the tree. oz4m1/2, which belongs to Rhodophyta, was used as outgroup.

Table 3. Closest similarities, found using the Fasta module of GCG, between our sequences and those available in GenBank

Code in the tree	Taxonomic position	Similarity
G1	Chlorophyta, Chlorophyceae, Chlorococcales (GenBank accession number: M63001)	99.2%
G2	Chlorophyta, Chlorophyceae, Chaetophorales (GenBank a.n.: D86499)	97.9%
G3	Chlorophyta, Ulvophyceae, Ulotrichales (Genbank a.n.: AF015279)	99.2%
G4	Fungi, Zygomycota (GenBank a.n.: AF277013)	92.5%
G5	Fungi, Ascomycota (GenBank a.n.: AF163295)	89.0%
G6	Stramenopiles, Eustigmatophyceae, Pseudocharaciopsis (GenBank a.n.: U41052)	98.0%
G7 to G11	Stramenopiles, Bacillariophyta, Bacillariophyceae (GenBank a.n.: X85402, AJ243065, AJ243062 …)	>95%

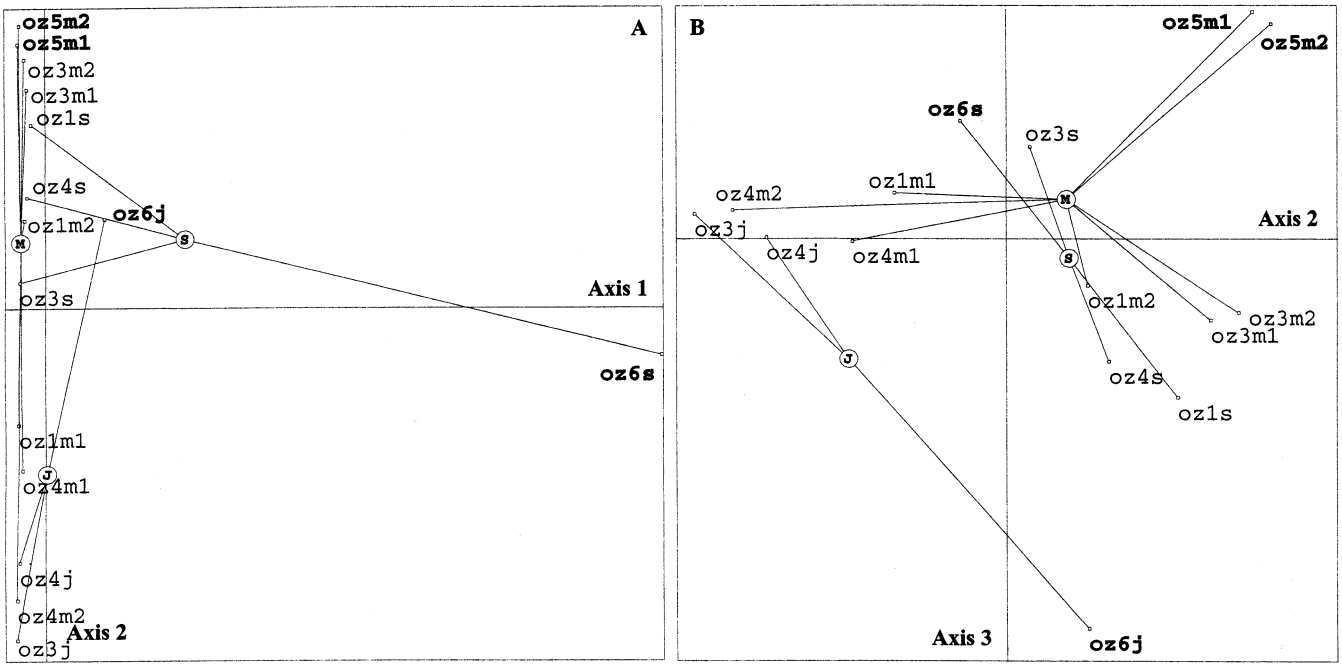

Fig. 3. Correspondence analysis of the distribution of the different OTUs defined in the NJ tree (Fig. 2), for each sampling site (oz1 to oz5/6) at each sampling month (June, May, and September). For each sampling month, all points are connected by a star representation to their barycenters (M, J, and S). (A) Projection in the plane defined by the axes 1 (29.7% of variance) and 2 (21.7% of variance). (B) Projection in the plane defined by the axes 2 (21.7% of variance) and 3 (15.4% of variance).

and Wang and Wang [32]. For this reason, we excluded from our analyses any sequences displaying insufficient homology (<85%) with known plant and fungi sequences. A second source of bias in the estimation of the diversity is linked to differences in the DNA extraction yield due, for example, to the morphological characteristics of the different species. Our DNA extraction protocol seems to avoid this source of bias because we found a significant correlation between the nucleotide diversity and the number of species identified by microscopic examination [11]. A third source of bias in the estimation of the diversity is linked to a differential amplification yield between sequences. Our results obtained on DNA mixes during the primer evaluation procedure showed that appropriate proportions of each species were retrieved after sequencing. Finally, the repeatability of our approach was demonstrated by the similarity of the results obtained for replicates performed in May at every sampling site.

This means that it is now possible to adapt other molecular approaches based on the study of the same target DNA to our models in order to speed up the comparison of the species composition of phytobenthic communities. Sequencing large numbers of clones is not suitable for the screening of a large number of samples, whereas techniques such as DGGE, SSCP, or ARISA can be used to carry out a comparison of complex communities [e.g., 33].

Diez et al. [10] have demonstrated that T-RFLP, sequencing, and DGGE on 18S rRNA gene fragments all gave similar estimations of diversity in marine picoeukaryotic assemblages. These techniques allow the processing and comparison, in a short time, of a great number of samples, which is necessary to better assess the spatial diversity in species composition of phytobenthic communities. With microscopic examination, the number of studied samples is more limited, because of the long time needed for the identification and counting of species.

One remaining question concerning the evaluation of diversity is the choice of the target sequence. Coding regions of the rRNA operon are useful for estimating species diversity, but noncoding regions (IGS or ITS), which are more variable, can be used to distinguish not only between different species but also between different strains [15, 35]. In our model, these variable sequences could be very useful for comparing diversity within given algal classes (e.g., Bacillariophyceae) in different communities. This should make it possible to avoid problems related to possible differences in the efficiency of the DNA extraction due to the widely differing morphologies of the groups studied.

Other genes could also be very useful in this kind of study. As a result of the problems linked to the use of the 16S rRNA gene, the *rpo*-B gene has been successfully used for the analysis of microbial communities [8]. One of the shortcomings of our approach was linked to the fact that we did not take the cyanobacteria into account when estimating the composition of phytobenthic communities, whereas in fact these species were quite abundant. This is why we are now trying to define another set of primers that will allow us to amplify one of the *psa* genes or the *rubisco* gene that are present in all photosynthetic organisms (both prokaryotes and eukaryotes).

Anyway, we could detect changes in species composition of phytobenthic communities in polluted stations. The most significant difference between unpolluted and polluted stations is that the proportion of Bacillariophyceae sequences was lower at the unpolluted station oz5/6 than at polluted areas oz1, 3, and 4. In the same way, the correspondence analysis performed on the distribution of the different OTUs at each sampling point and each sampling date (Fig. 3) clearly demonstrated that the unpolluted sampling station (oz5/6) was distinct from the other stations. To explain these changes in species composition of phytobenthic communities, two kinds of hypotheses can be proposed. The first is that the differences in species composition were due to natural environmental differences between the sampling sites; the second is that pollution by atrazine and isoproturon is at the origin of these changes. Concerning the first hypothesis, we cannot exclude an impact of environmental conditions on species composition of phytobenthic communities. But the choice of the sites where the artificial substrates were deposited, was made with the goal of minimizing differences in light exposition and hydromorphological parameters. In addition, distances between the unpolluted sampling station (oz5/6) and the three polluted sites (oz1, 3, and 4) were in the same order as distances between these three unpolluted sites. Concerning the second hypothesis (differences in species composition due to atrazine and isoproturon pollution), CA analysis showed that most of the changes in phytobenthic communities in the different sampling sites occur in June and September, after herbicide application to the fields. Secondly, the fact that the proportion of Bacillariophyceae sequences was higher at the polluted stations in comparison with the unpolluted site agrees with observations of Bérard et al. [2] showing that diatoms are more tolerant than other algae to PS II inhibitors such as atrazine and isoproturon. Finally, by measuring the photosynthetic efficiency in all sampling sites with regard to increasing concentrations of atrazine, Dorigo and Leboulanger [12] found that phytobenthic communities at polluted sites (oz1, 3, and 4) were more tolerant to atrazine than the community from the unpolluted site (oz5/6). All these elements suggest that atrazine probably constituted a selective pressure on freshwater periphytic species from the river Ozanne that results in changes in species composition of phytobenthic communities.

These results also showed that herbicide pollution has an effect on the species composition of eukaryotic phytobenthic communities. This effect was not a reduction in the number of species, but rather a change in the species composition of the community. This observation is probably attributable to the fact that the herbicide contamination was not continuous because of agricultural practices and rainfall events, and that the level of this contamination was always relatively moderate in the river studied [11]. According to the intermediate disturbance hypothesis [6, 16], moderate and discontinuous selective pressures help to conserve the diversity. In contrast, a strong selective pressure exerted by a continuous and high level of herbicide contamination would have probably led to a dramatic reduction of the diversity in phytobenthic communities [19]. Thus, changes in species composition

of our studied phytobenthic communities are probably not linked to lethal or sublethal effects of herbicide pollutants but rather to modifications in the relative fitness of the different species of the communities in presence or absence of these molecules.

The consequences of these changes in species composition observed during the three sampling periods on the ecosystem functioning has not been investigated. According to Reynolds [27], species can be classified with regard to their functional properties. Reynolds distinguished three different ecostrategies (C, S, and R) based on functional properties: differential cell or colony size, susceptibility to zooplankton grazing, sedimentation rate, phosphorus uptake, and maximal potential growth rate. Apart from the sedimentation rate, these functional properties that have been defined for pelagic species are also probably available for benthic species. This suggests than changes in species composition without any decrease in diversity may affect the functioning of the whole ecosystem.

In conclusion, our findings imply that changes in the composition of phytobenthic communities can be used to detect herbicide pollution in aquatic ecosystems and, more generally, to assess the state of health of an ecosystem. The level of contamination can be roughly estimated from the nature of the changes in the biodiversity of the phytobenthic community. A loss of diversity in the community studied would reflect a continuous and high level of contamination, whereas changes in the species composition of the communities without any decrease in the diversity would reflect discontinuous, low-level contamination. Single-species toxicity tests and environmental chemistry are not adequate for this purpose, because they do not reflect the complexity of assemblages of organisms interacting with each other and with their abiotic environment [29]. To conduct aquatic ecological risk assessment of pesticides we need ecotoxicological tools useful to investigate both the physiological responses and the structure of biological communities [34]. New tools, based on biomolecular methods, look promising as a complement to conventional taxonomic investigations in ecotoxicological studies of this kind.

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