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Research article

Microalgae community structure analysis based on 18S rDNA amplification from DNA extracted directly from soil as a potential soil bioindicator

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Abstract – Soil algae are photosynthetically active microorganisms showing changeable community structure, depending on the soil type, the agricultural practices and the application of pesticides. To characterise algal community structure, molecular approaches complementary to classical microbiological approaches based on the isolation and the culture of soil algae are required. Our study describes a polymerase chain reaction (PCR) approach targeting algal 18S rDNA sequences of desoxyribonucleic acid (DNA) samples extracted either from unialgal eukaryotic microalgae culture, complex assemblages of microalgae populations or natural soil communities. Our first results showed that microalgae rDNA can be amplified by PCR from soil DNA samples. They also indicated difficulties extracting DNA from diatoms directly from soils, probably because of the presence of robust silicate valves. An 18S rDNA library has been established and preliminary phylogenetic analysis showed the feasibility of applying molecular methods to studying edaphic algae community structure. This is promising for soil algae ecology and for developing soil biological indicators.

algae communities / soil / amplified rDNA

1. INTRODUCTION

The need for preservation and improvement of soil quality is recognised worldwide (Elliott et al., 1996), and surveying it, particularly in relation to agricultural practices, is becoming a major matter of concern (Rivière, 1998). Numerous soil microorganisms are potential bioindicators of soil quality (Pipe and Cullimore, 1980; Roper and Opel-Keller, 1997). Among telluric microorganisms, microalgae, which show cell structure and metabolism (photosynthesis activity) are the only ones similar to higher plants. In consequence, soil algae could constitute an innovative bioindicator which could be used to estimate ecotoxicological impacts of agricultural practices such as herbicide application (Mc Cann and Cullimore, 1979).

Algae are ubiquitous in arable or virgin soils. They are presented in relatively large amounts (150 to 500 kg ha^{-1}) and are phylogenetically diverse (Shtina, 1974). They play an important role in the maintenance of soil fertility and structure (Cullimore, 1971; Thind and Rowell, 1999). These photosynthetically active microorganisms exhibit changeable community structure, depending on soil physico-chemical properties and on anthropogenic activities such as agricultural practices and, in particular, application of pesticides (Fujita and Nakahara, 1999; Pipe, 1992). Methods allowing the detection of microalgae community structure variations are needed. Traditional approaches evaluating microalgal diversity involve isolation and culture previous to species identification based on the study of morphological criteria (Kostikov et al., 2001b). These approaches are time-consuming and direct species identification is often difficult, especially for soil microalgae associated with sediment particles, which present different developmental stages. Their clear-cut identification therefore relies on their isolation from soil and on the development of unialgal culture. Indeed, the poor cultivability of soil microalgae may constitute an important methodological bias. In the last decade, many studies have shown that direct molecular tools revealing the polymorphism of ribosomal DNA (rDNA) sequences are efficient at exploring the composition of complex natural communities of soil bacteria, fungi and nematodes (Engelen et al., 1998; Kuske et al., 2002; Pennanen et al., 2000; Viaud et al., 2000; Waite et al., 2003). Up to now, molecular approaches have not been used to determine soil microalgae communities' structure. The purpose of this study is to develop and evaluate a polymerase chain reaction (PCR) approach based on direct soil desoxyribonucleic acids (DNA) extraction, analyse of rDNA fingerprints and sequences, in order to allow the determination of soil microalgae community structure.

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Table I. Physical and chemical characteristics of the soils used in this study. The soils A and B were sampled in two adjacent cornfields (fluvio-glacial deposit), of the commune of Massongy, Haute Savoie, France. The soil C was sampled in an organic rice field of the commune of Arles, Bouches du Rhône, France. The analyses were performed by conventional methods.

Field	% Clay	% Silt	% Sand	pH (H_2O)
Cornfield A	35	55	10	6.5
Cornfield B	20	57	23	7.7
Rice field C	24.1	67.8	8	8

Table II. Nucleotide sequences of the PCR primers tested for the amplification of 18S rDNA from DNA extracted from soil algal cultures and from cornfield indigenous soil algae.

Name	Sequence (5'-3')	Type
P45	ACC TGG TTG ATC CTG CCA GT	Forward primer
P73	AAT CAG TTA TAG TTT ATT TGR TGG TACC	Forward primer
P47	TCT CAG GCT CCC TCT CCG GA	Reverse primer

2. MATERIALS AND METHODS

This study was performed on (i) samples of liquid monoculture of soil algae, (ii) samples of soil algal culture and (iii) soil samples collected from maize fields.

2.1. Algal cultures

Unialgal cultures of the different algal classes, i.e. *Chlorophyceae*: *Stichococcus* sp. (strain number 379-27, CCAP, UK), *Chlamydomonas reinhardii* (strain number CC1010+, *Chlamydomonas* Genetics Center, Duke University, USA) and *Chlorella vulgaris* (strain number 136-1, SHL, France), *Bacillariophyceae*: *Navicula accomoda* and *Nitschia* sp. (strain numbers 107 and 139-3, respectively, SHL, France), and *Chrysophyceae*: *Xanthonema montanum* (strain number 836-3, CCAP, UK) were harvested from liquid batch cultures by centrifugation for 20 minutes at 7000 rpm (Avanti J 301, Beckman). The supernatant was discarded, and the pellet transferred to a 1.5-mL centrifuge tube and stored at –32 °C until use. The same strains were cultivated for two weeks (temperature 20 °C, 8000 lux of light) on sieved (2 mm mesh) and dried (105 °C for 24 hours) soil (Tab. I), and stored in the dark and at a temperature of 4 °C until use.

2.2. Soil algae sampling

Soils sampled during summer 2001 from two adjacent cornfields (Tab. I, Bérard et al., 2004) were investigated. Soil samples were collected in a central area bounded by a five-meter peripheral unsampled zone. Soil surface samples (0–2 cm depth) were collected using a trowel. Twenty small plots were sampled randomly from each cornfield, pooled and placed in sterile plastic bags before being processed in the laboratory for DNA extraction. Soil samples were air-dried (overnight at room temperature) and sieved (< 2-mm mesh). 4-g soil aliquots were weighed, mixed with 50 mL of ultrapure water and shaken for two hours (200 rpm, Heidolph promax 2020) in the dark. The resulting suspensions were sieved (< 500-µm mesh) and stored at –32 °C until use.

2.3. DNA extraction

DNA from liquid algal culture was extracted with the DNAeasy Plant mini-kit (Qiagen) according to the manufacturer's instructions. DNA from soil algal cultures and from cornfield indigenous soil algae (1 g aliquots) was extracted according to a modified procedure originally developed by Martin Laurent et al. (2001): one mL of a solution containing 100 mM Tris-HCl (pH 8), 100 mM EDTA (disodium dihydrogenoethylenediamine tetraacetate, pH 8), 100 mM NaCl, 1% (wt/vol) polyvinylpyrrolidone and 2% (wt/vol) sodium dodecyl sulfate was added to 300-mg aliquots of soil in a 2-mL mini-bead-beater tube containing 0.5 and 0.1 g of 106-µm- and 2-mm-diameter glass beads, respectively. Samples were then homogenised for 30 s at 1600 rpm in a mini-bead-beater cell disruptor (Mikro-Dismembrator S. B. Braun Biotech International). Homogenised samples were incubated for 20 min at 70 °C, and centrifuged at 14 000 × *g* for 1 min at 4 °C. The collected supernatants were added to 1/10 volume of 5 M sodium acetate, incubated for 10 min and centrifuged at 14 000 × *g* for 5 min at 4 °C. After adding 1 volume of ice-cold isopropanol (incubation for 30 min at –20 °C), nucleic acids were pelleted by centrifugation (13 000 rpm for 30 min, 4 °C). The nucleic acid pellet was washed with 70% ethanol and purified using a Sepharose 4B spin column. The quality of soil DNA was checked by electrophoresis on a 1% (wt/vol) agarose gel and quantified using a BioPhotometer (Eppendorf, Hamburg, Germany). Three replicates were used for DNA extraction from each soil sample. Soil DNA extracts were diluted to 30 ng/µL for further PCR analysis.

2.4. PCR (Polymerase Chain Reaction) amplification

Several primer pairs (Tab. II), designed from multiple alignment of known 18S rRNA gene sequences of eukaryotic microalgae were used for PCR (Dorigo et al., 2002). PCR amplification from 25 ng of DNA was conducted in a total volume of 25 µL by using 0.8 µM of dNTPs, 1.5 mM $MgCl_2$, 0.5 µM of each primer and 0.625 units of *Taq* DNA polymerase (Pharmacia). Under the following conditions: 1 min at 94 °C, 37 cycles of 50 s at 92 °C, 50 s at 57 °C and a 50-s extension step at 72 °C plus an additional 10-min cycle at 72 °C (Dorigo et al., 2002). The PCR products were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide.

2.5. Restriction fragment length polymorphism (RFLP) of 18S rDNA PCR products

PCR products were then separated by electrophoresis on a 1% agarose gel. Bands were extracted and purified from the gel

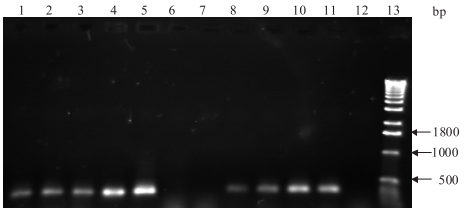


Figure 1. Electrophoresis separation (1% (wt/vol) agarose gel) of the PCR products (about 400 bp) amplified from DNA directly extracted from unialgal culture in soils using the primers (P73-P47) targeting 18S rDNA of eukaryotic algae. Lanes 1 and 2 = *Stichococcus* sp. (*Chlorophyceae),* lanes 4–6 *Chlorella vulgari* (*Chlorophyceae*), lanes 7–9 *Nitschia* sp. (*Bacillariophyceae*), lanes 10–12 *Navicula accomoda* (*Bacillariophyceae*), lane 13 negative control, lane 14 1kb ladder (Boehringer Mannheim).

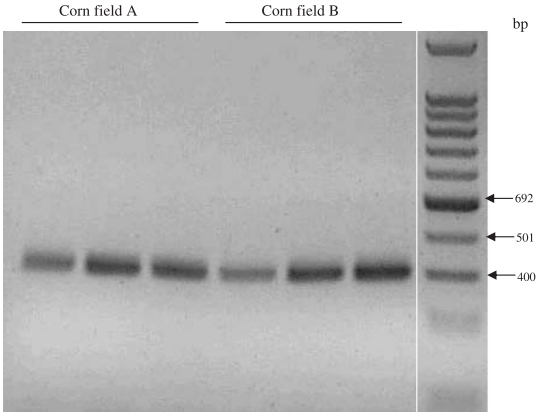


Figure 2. Electrophoresis separation (1% (wt/vol) agarose gel) of the PCR products (about 400 bp) amplified from DNA extracted directly from soil samples collected in two different cornfields.

using the Qiaex II kit (Qiagen, Germany) as recommended by the manufacturers. Ten µL of purified 18S rDNA from each sample were digested with 15 U of the enzyme *Msp*I in 15-µL reaction mixtures. Digestion products were separated by electrophoresis on a native 3% agarose gel run for 6 h at 100 V. Gels were stained with ethidium bromide following the recommendations of the manufacturers (Molecular Probe, France). RFLP profiles were analysed using the Bio-Profil 1D V6.0 program (Vilber Lourmat, France), allowing the construction of a dendrogram relating samples using the similarity "Neighbour-Joining" method. The statistical test of Jaccard at 5.0% of similarity was applied.

2.6. Cloning of 18S rDNA PCR products

18S rDNA PCR products were ligated into the pGEM®-T Easy II vector (Promega®) and then transformed into *Escherichia coli* JM109 competent cells (Promega®) in accordance with the manufacturer's instructions. At least the insert of 50 randomly chosen recombinant clones was amplified by PCR using the primers T7 and SP6. The amplified fragments were sequenced with the kit DTCS-1 (Beckman Coulter®) using a CEQ-2000 XL automatic sequencer (Beckman Coulter®) according to the manufacturer's instructions (Beckman Coulter®).

2.7. Sequence analysis

Sequences were compared with those available in the GenBank database using Blast. Multiple alignments were carried out with the CLUSTALX program and phylogenetic analyses were conducted with the NJ-plot program using the "Neighbour-Joining" method. 1000 iterations were realised to calculate bootstrap values.

3. RESULTS AND DISCUSSION

3.1. Extraction and primer evaluation

The primer pair P45-P47 was previously designed to specifically amplify the major photosynthetic groups among fresh-water periphytic Eukaryotes (Dorigo et al., 2002). It has also been used to characterise genetic diversity of fresh-water phytoplankton within a mesocosm. In order to prevent amplification of zooplankton and fungi, a new forward primer (P73) was defined by multiple alignment of known 18S rDNA of Chlorophyceae and diatoms, within the region delimited by P45 and P47 (Tab. II). These two primer pairs (i.e. P45/P47 and P73/P47) were then tested on DNA samples prepared from monocultures. These preliminary PCR studies revealed that only the P73/P47 primer pair allowed the successful amplification of all the algae monocultures tested (data not shown). This set of primers was therefore chosen for further PCR analysis carried out on DNA extracted from soil algae cultures and from soil collected in corn- and ricefields.

18S rDNA PCR products obtained from DNA samples extracted from unialgal cultures displayed the expected size of about 400 bp. This result was confirmed by PCR products generated from DNA samples extracted from unialgal and complex assemblages of algae directly cultivated on soil (Fig. 1). PCR fragments generated from soil DNA extracted from the two sampled maize fields were similar to those obtained from DNA extracted from liquid and soil algal cultures (Fig. 2).

PCR-RFLP fingerprints obtained with the restriction enzyme *MspI* revealed that the fingerprints of the fields A and B were similar to that of *Xanthonema* sp., the algae of reference (Fig. 3A). However, on the basis of a finer analysis of 18S rDNA PCR-RFLP fingerprints realised by comparing the percentage of similarity of each fingerprint generated, these 3 samples could be separated on a dendrogram deduced from similitude analysis matrices (Fig. 3B).

These results indicated that algal 18S rDNA could be successfully amplified from soil DNA extracts and that PCR products could be further analysed by RFLP in order to assess the structure of the soil algal community.

3.2. Phylogenetic analysis of the 18S rDNA sequences

The 18S rDNA PCR products obtained were cloned in *E. coli* using p-GemT, a cloning vector adapted for PCR fragment

cloning. 50 clones were randomly chosen from the bank of clones and sequenced. All the sequences showed high similarities with *Chlorophiceae* sequences (Fig. 4). The NJ tree of all these sequences revealed two main clusters which were strongly supported by their bootstrap values. One cluster presented strong homologies with Chlorococcales algae sequences (homologies of 95 to 98%, a2-16, t2-14, t2-6, a2-11, a1-6, a2-7, a1-5, t2-18 and t2-11). The second one presented strong homologies with *Trebouxiophyceae* algae sequences (95 to 99%, a1-34 and a1-2). Among the sequences related to those of Chlorococcales, four sub-groups (G2.1, G2.2, G2.3 and G2.4) were identified, whereas the sequences related to *Trebouxiophyceae* formed a single group.

3.3. DNA evidence of soil algae

Recently, algae and cyanobacteria isolated from biological crust were characterised using molecular tools starting from DNA extracted from liquid culture of individual isolates (Lewis and Flechtner, 2002; Boyer et al., 2002). To our knowledge our work is the first report of application of direct molecular approaches based on soil DNA extraction, amplification and analysis to study of edaphic algae communities. It showed that 18S rDNA sequences can be amplified from DNA samples extracted directly from soil and that the structure of soil algal communities could be revealed by either the analysis of PCR-RFLP fingerprints or of the 18S rDNA bank of clones. These first results are promising and offer new perspectives in the study of the ecology of soil algae. This kind of work will contribute to estimating soil-algae diversity and describe their phylogeny. Better knowledge of telluric algae will allow their use in routine molecular ecology to notably study the impact of soil contamination and cultural practices on soil algae community structure, which could be used as an early indicator of soil quality.

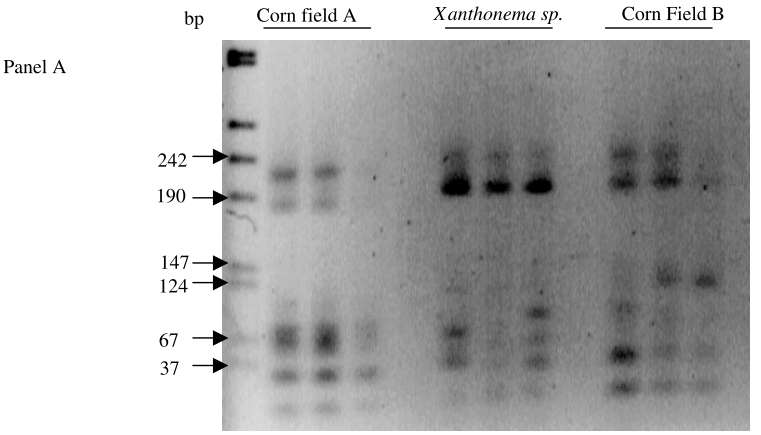
18S rDNA sequences recovered from the two sampled maize fields showed high similarities to *Chlorophyceae* sequences belonging to two taxonomic groups. The first group of sequences was related to sequences belonging to the order of Chlorococcales: Coccoid green algae, and the genera *Dimorphococcu*s and *Coelastrum. Dimorphococcu*s *lunatus, Coelastrum sphaericum, Coelastrum asteroideum* and *Coelastrum reticulatum* are ubiquitous aquatic plankton species (Komàrek and Fott, 1983). Up to now, they have not been reported in soil (Ettl and Gärtner, 1995; Kostikov et al., 2001b). The genus *Coelastrum* was reported by Shubert and Stark (1995) in reference sites used to study soil algae in the reclaimed surface of a mined area. To our knowledge, only two species of *Coelastrum* were observed in soil samples – *Coelastrum printzii* Rayss – in arctic and subarctic soils (Ettl and Gärtner, 1995), and *Coelastrum microporum* Naeg. – in Ukrainian coniferous forest (Kostikov et al., 2001a). It has to be noticed that the last was classified as a hydrophilic species growing from diaspores. *Dimorphococcus lunatus* is a planktonic species known only from aquatic habitats (Komàrek and Fott, 1983).

The second group of sequences was related to sequences belonging to the Ulotrichales, which are widely dispersed in soils (Metting, 1981). The genus *Stichococcus* was isolated from soils and cultivated for taxonomic studies by Chodat (1909). *Stichococcus chodatii* (Bialosuknia) Heering 1909 – belongs to Trebouxiophyceae. It is an ubiquitous soil algae, typical of temperate and humid regions (Kostikov et al., 2001b). In this study, one of the strains used for unialgal culture was a *Stichococcus* which was previously shown to exhibit a transitory sensitivity to herbicides (Cullimore and Mc Cann, 1977). The genus *Raphidonema* has already been reported in soil studies (Metting, 1981), while *Raphidonema nivale*, a snow algae according to Starmach (1972), has not yet been observed in soil (Ettl and Gärtner, 1995; Kostikov et al., 2001a).

It was a surprise to mainly characterise 18S rDNA sequences similar to aquatic algal species since the samples analysed were collected from maize fields. A first explanation of this observation could be that survival forms of algae from the lake of Geneva (which is 1 km from the maize fields) may have been disseminated from the lake by the wind onto the surrounding landscape (Broady, 1979; Brown et al., 1964). However, the *Coelastrum* species is very rare in the lake (Anneville, pers. comm.). The sampled cornfields are quite humid but they are drained and not irrigated. Hence, the hypothesis of the inoculation of aquatic algae by means of the irrigation water of the cornfields is excluded. However, we have to consider that the phylogeny of edaphic algae has up to now not been well described since very few sequences of edaphic algae are available in the GenBank for similarity analysis. It is therefore possible that soil algae related to aquatic *Chlorophyceae* effectively exist, but have not yet been described by classical phylogeny. This hypothesis is reinforced by the recent study of Lewis and Flechtner (2002) conducted on desert microbiotic crust green algae, showing that these different taxa derived from common freshwater algae ancestors. This observation highlights that the use of molecular approaches to study soil algae will certainly contribute to the description of an unexpected diversity of telluric algae.

These results also indicate that, even if our primers are well suited to amplifying 18S rDNA of both *Chlorophyceae* and diatoms in cultures, it seems that only the 18S rDNA of *Chlorophyceae* could be amplified from soil-extracted DNA. It is noteworthy that diatom silicate valves, extracted from the same soil samples, were identified by microscopy (Bérard et al., 2004). In addition, we observed that it was more difficult to extract DNA from diatoms than from green algae and Cyanobacteria cultivated in liquid medium (data not shown). Therefore, the difficulty encountered extracting DNA from Bacillariophyceae may explain why no 18S rDNA of diatoms were observed in the soil PCR products sequenced. The extraction of DNA from Bacillariophyceae algae is difficult, probably because of the presence of robust silicate valves. This methodological difficulty has to be solved in order to allow their study in soil because of the ecological and ecotoxicological interest that they show in aquatic and edaphic environments (Bérard and Pelte, 1999; Gugger et al., 2002; Kelly et al., 1995; Pipe and Cullimore, 1980; Say and Whitton, 1980).

The isolation, cultivation and identification of soil algae by the means of traditional techniques associated with molecular analysis may be of benefit in future investigations of soil algae ecology. This molecular study will be completed by isolation and identification with light microscopy of algae from the same soils (work in process). The 16S rDNA region has been analysed for systematic approaches of soil bacterial communities



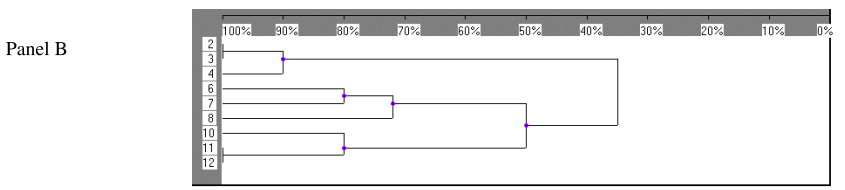


Figure 3. Panel A: RFLP profiles of 18S rDNA PCR products generated from DNA extracted directly from cornfield A (lanes 1–3) and cornfield B (lanes 7–9) or from DNA extracted from the soil monoculture of the Chlorophyceae, *Xanthonema montanum*. Panel B: Dendrogram deduced from the comparison of 18S rDNA-PCR-RFLP profiles generated from samples of DNA extracted from the soil monoculture of the Chlorophyceae, *Xanthonema montanum* (samples 2–4), cornfield A (samples 6–8) and cornfield B (samples 10–12).

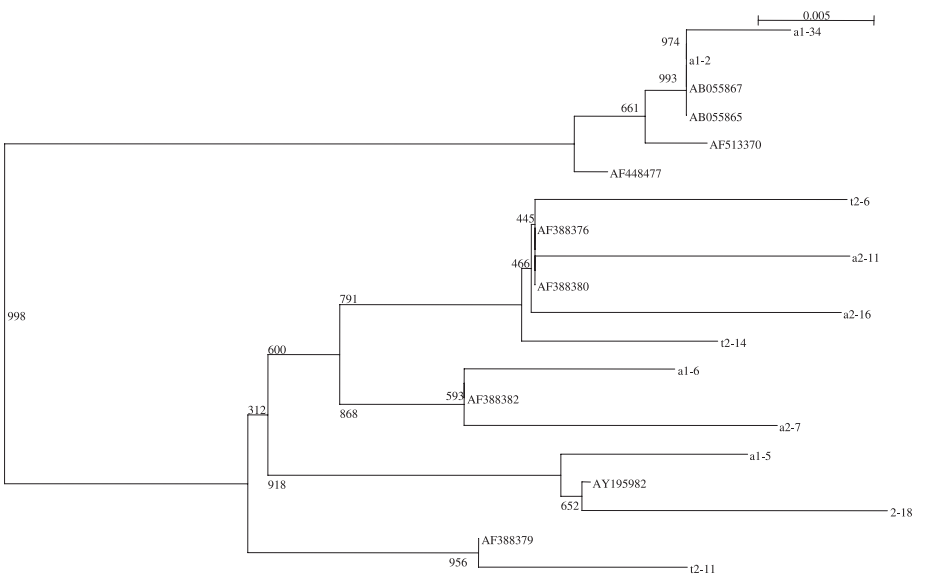


Figure 4. Phylogenetic analysis realised on nucleotide sequences of PCR products from 18S rDNA extracted and purified from cornfield soils (a2-16, t2-14, t2-6, a2-11, a1-6, a2-7, a1-5, t2-18, t2-11, a1-34 and a1-2). Comparison with sequences from Genbank (*Coelastrum sphaericum* AF388376, *Coelastrum astroideum* AF388380, *Coelastrum reticulatum* AF388382, Coccoid green algae AY195982, *Dimorphococcus lunatus* AF388379, *Stichococcus chodati* AB055867, *Raphidonema nivale* AF448477, *Stichococcus sp.* AF513370, *Stichococcus bacillaris* AB055865). Bootstrap values over 1000 iterations are indicated.

(Embley et al., 1994), as well as for the evaluation of changes in bacterial communities submitted to environmental and anthropogenic stresses (Mc Caig et al., 1999). More recently nematode soil communities were investigated with similar methods targeted on 18S rDNA (Blaxter et al., 1998). The use of molecular methods to study eukaryotic algae and Cyanobacteria systematics and diversity is quite recent (Dorigo et al., 2002; Gugger et al., 2002; van Hannen et al., 1998). This kind of study is lacking for edaphic algae and Cyanobacteria communities.

The perspective of our work is to follow up the description of soil algal diversity and based on this knowledge elaborate

fingerprinting techniques such as T-RFLP (terminal-restriction fragment length polymorphism) or DGGE (denaturing gel gradient electrophoresis) allowing the description of the structure of the soil algal community. These molecular fingerprinting approaches help to investigate a large number of soil samples, which is a requirement for community structure studies. In addition, it could constitute a very interesting tool to elaborate soil sampling strategies adapted to estimate microorganism structure. Soil is known to exhibit a great spatio-temporal heterogeneity of its microenvironments, and algae communities are highly influenced by environmental factors specific to the soil niche (Hoffmann, 1989; Metting, 1981; Whitton, 2000). In aquatic environments, it has already been shown that the sensitivity of algae to herbicides depends on environmental factors (Bérard et al., 1999; Bérard and Benninghoff, 2001). Soil sampling strategies seem to be one of the key points which needs to be carefully studied to understand soil algae ecology.

Because of their photosynthetic activity, edaphic algae are concentrated in the top few centimetres of the soil profile. They could therefore be considered to be the first biological crust submitted directly to pollution. In addition, among soil microorganisms, they are of prime interest in ecotoxicological studies dealing with photosynthetic inhibitors (herbicides and some heavy metals). It is thus likely that both the structure and activity of soil algae communities are early indicators of pollution-related disturbances, integrating environmental and seasonal changes over several weeks. We have recently shown that the selection/tolerance community concept (Pollution-Induced Community Tolerance) (Bérard et al., 2002), could be applied to edaphic microalgae and their photosynthetic activity in arable soils (Bérard et al., 2004). Hence, algae might constitute a pertinent bioindicator of soil quality. Soil microorganism communities (nematodes, microarthropods, protozoaires, fungi, bacteria, cyanobacteria, eukaryotic microalgae, etc.) exist in unceasing interaction with each other (Gerson, 1974; Thirup et al., 2000; Yeates et al., 1993). Their interaction is fundamental for the regulation of functional processes of edaphic environments, and may be disturbed by soil pollution (Pankhurst, 1997).

We suggest that algae could constitute a novel bioindicator complementary to those described for bacteria and fungi (Martin-Laurent et al., 2001; Waite et al., 2003). It is most likely that future approaches aiming to study ecological and ecotoxicological impact of agricultural practices on soil quality must be based on a polyphasic approach relying on the use of multi-microbial bioindicators.

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