Toxic effects of acetochlor, methamidophos and their combination on nifH gene in soil

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Abstract

Toxic effects of two agrochemicals on nifH gene in agricultural black soil were investigated using denaturing gradient gel electrophoresis (DGGE) and sequencing approaches in a microcosm experiment. Changes of soil nifH gene diversity and composition were examined following the application of acetochlor, methamidophos and their combination. Acetochlor reduced the nifH gene diversity (both in gene richness and diversity index values) and caused changes in the nifH gene composition. The effects of acetochlor on nifH gene were strengthened as the concentration of acetochlor increased. Cluster analysis of DGGE banding patterns showed that nifH gene composition which had been affected by low concentration of acetochlor (50 mg/kg) recovered firstly. Methamidophos reduced nifH gene richness that except at 4 weeks. The medium concentration of methamidophos (150 mg/kg) caused the most apparent changes in nifH gene diversity at the first week while the high concentration of methamidophos (250 mg/kg) produced prominent effects of nifH gene diversity in the following weeks. Cluster analysis showed that minimal changes of nifH gene composition were found at 1 week and maximal changes at 4 weeks. Toxic effects of acetochlor and methamidophos combination on nifH gene were also apparent. Different nifH genes (bands) responded differently to the impact of agrochemicals: four individual bands were eliminated by the application of the agrochemicals, five bands became predominant by the stimulation of the agrochemicals, and four bands showed strong resistance to the influence of the agrochemicals. Fifteen prominent bands were partially sequenced, yielding 15 different nifH sequences, which were used for phylogenetic reconstructions. All sequences were affiliated with the alpha- and beta-proteobacteria, showing higher similarity to eight different diazotrophic genera.

Key words: agrochemicals; acetochlor; methamidophos; toxic effects; nifH gene; PCR-DGGE

Introduction

Soil microorganisms are a fundamental component of soil ecosystem that plays critical roles in metabolism of organic matter and in biogeochemical transformations of elements. As they can respond rapidly to any changes in soil quality, soil microbial communities were regarded as one of bioindicators of soil quality and sustainability, and used for the assessment of soil quality and for the prediction of soil degradation and its potential risks caused by human activities (Kennedy and Smith, 1995; Finlay et al., 1997; Groffman and Bohlen, 1999). The widespread use of pesticides is considered to be a serious threat to the quality of human life. Many studies revealed that the application of pesticides destroyed the original composition and structure of soil microorganism communities to different degrees, resulting in declining of ecological and functional diversities and metabolic activities in soils (Ibekwe et al., 2001; Chang et al., 2001; Säid et al., 1999). Such outcome would affect soil nutrient cycling and element balance and lead to a decline in soil productivity. The agricultural black soil in northeast of China has long been renowned for its fertility and cleanness. In recent years, a varying degree of soil degredation in the black soil has been reported (Scherr, 1999; Chen et al., 2002) and the application of agricultural chemicals was thought to be one of the important factors associated with the soil degradation. Acetochlor and methamidophos were two agrochemicals which were most widely used in agriculture regions both in quantity and in acreage. Recently, studies have been carried out on the effects of acetochlor on microorganisms in black soil, including several culturbale potential plant growth-promoting bacteria (Zhang et al., 2004a, b; Luo et al., 2004) and the effects of methamidophos on soil microorganisms and enzyme activities (Zhu et al., 1999; Zhang et al., 2003). However, the effects of acetochlor and methamidophos on functional genes in the soil are not yet known.

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Soil diazotrophs are the main source of the nitrogen input for the biosphere. Biological nitrogen fixation occurs in a wide range of bacterial phyla, from *Archaebacteria* to *Eubacteria*. All diazotrophs catalyse biological nitrogen fixation using the enzyme nitrogenase, which has been highly conserved throughout evolution. The nitrogenase iron protein gene, *nifH*, is one of the oldest existing functional genes in the history of gene evolution, and the phylogeny among bacteria based on this gene is reportedly in agreement with that inferred from 16S rRNA gene, showing that *nifH* could be considered a good marker for diazotrophic community structure (Borneman et al., 1996; Hennecke et al., 1985; Ueda et al., 1995; Zehr et al., 1995, 2003). Thus the *nifH* gene has been used for determination of the diversity and characterization of nitrogen fixation genes in natural soil microbial communities (Ben-Porath and Zehr, 1994). Traditionally, the diversity of soil microorganisms is studied by cultivating them on synthetic media, a procedure known to select a low percentage of the populations (Ward et al., 1990). *nifH* gene has largely been studied by culture-independent approaches which provide a more complete picture of the diazotrophic community than culture-based approaches. PCR-DGGE (denaturing gradient gel electrophoresis) is one of such techniques and has been used to study the diversity of *nifH* gene in various environments (Muyzer et al., 1993; Rosado et al., 1998; Larocque et al., 2004; Piceno and Lovell, 2000). Environmental parameters are known to affect the activities of soil bacteria, especially nitrogen fixation. It has been reported that diazotrophic activity and community structure can be affected by many environmental factors, such as plant cover (Larocque et al., 2004; Bardgett et al., 1999; Demba et al., 2004), soil texture (Reinhold and Hurek, 1998; Poly et al., 2001), soil management (Poly et al., 2001), N-fertilization (Limmer and Drake, 1998; Tan et al., 2003) and wildfire (Chris et al., 2005). To date, there have been no reports on the influence of herbicides and pesticides on the *nifH* gene in soil.

The objectives of this study were to examine the effects of application of acetochlor and methamidophos on the diversity and composition of *nifH* gene using PCR-DGGE and sequencing methods and to provide useful data for the assessment of soil quality and the prediction of potential changes in the soil ecosystem.

## 1 Materials and methods

### 1.1 Materials

Acetochlor and methamidophos were purchased from the Institute of Chemical Industry, Shenyang, China.

### 1.2 Soil microcosm setup

The soil was collected at 0–20 cm depth from Hailun Agro-ecological Experimental Station (47°27′N, 126°55′E), located in Hailun County, Heilongjiang Province, China. As no agrochemicals had been used since 1989 in the station, the soil was relatively unpolluted. Some physical and chemical characteristics of the soil are listed in Table 1. The soil was homogenized by sieving (2 mm) to remove stones and plant roots and stored at 4°C in the dark until used.

For each treatment, 900 g soil at field moisture content (60% WHC) was used (Table 2). The soil was thoroughly mixed with freshly prepared pesticide solution; 300 g soil (3 replicates in each treatment) was placed in a plastic cup as a microcosm system and sealed with parafilm to minimize water loss. They were incubated in the dark at 25°C and the soil moisture content was maintained by adding sterilized water every week. Samples for bacterial DNA extraction were taken 1 week, 4 weeks, 6 weeks, and 8 weeks after the treatments. At each sampling stage, 10 g soil were taken from each replicate, mixed thoroughly and used for DNA extraction.

### 1.3 DNA extraction and PCR amplification

Total bacterial community DNA was extracted from soil samples using the procedure described by Zhou et al. (1996), which combines both physical and chemical methods to maximize the recovery of DNA from soils of diverse composition. Briefly, 10 g of samples were frozen in liquid nitrogen and thawed at 65°C for three cycles before DNA extraction with proteinase K and extended heating in a high-salt extraction buffer. To remove the humic acid coextracted with the DNA, the crude DNA extracts were purified using the DNase XIA™ kit (OM: Organic matter.

### Table 1 Some physical and chemical characteristics of the tested soil

<table>
<thead>
<tr>
<th>Sampling depth (cm)</th>
<th>pH</th>
<th>OM (g/kg)</th>
<th>Total N (g/kg)</th>
<th>Total P (g/kg)</th>
<th>Total K (g/kg)</th>
<th>Particle size distribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–20</td>
<td>6.58</td>
<td>37.83</td>
<td>2.56</td>
<td>0.61</td>
<td>26.00</td>
<td>Sand (0.01–1.00 mm) 45.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Silt (0.001–0.01 mm) 34.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Clay (&lt;0.001 mm) 20.1</td>
</tr>
</tbody>
</table>

Table 2 Treatments of acetochlor and methamidophos amendment to soil

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Acetochlor (mg/kg soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Methamidophos (mg/kg soil)</td>
<td>CK</td>
</tr>
<tr>
<td>0</td>
<td>Ma</td>
</tr>
<tr>
<td>50</td>
<td>Mb</td>
</tr>
<tr>
<td>150</td>
<td>Mc</td>
</tr>
<tr>
<td>250</td>
<td></td>
</tr>
</tbody>
</table>

CK: control; A: acetochlor; M: methamidophos; a, b, c: 50, 150, 250 mg/kg soil.
were resuspended in distilled water (dH₂O) and purified on a Wizard column (Promega) as recommended by the manufacturer. The nifH gene sequences from nitrogen-fixing microorganisms were amplified using a nested PCR to increase the sensitivity. The primers and amplification procedure were adopted from Demba et al. (2004a). The first PCR was performed with the forward primer FGPH119 and the reverse primer PolR. The forward and reverse primers were 24× and 8× degenerated, respectively. The amplification yielded 429 bp fragments. The second PCR was performed with the forward primer PolF containing the GC clamp and the reverse primer AQER. The forward and reverse primers for the second PCR were 24× and 2× degenerated respectively. The amplification product had 320 bp including the GC clamp sequence. The final PCR cocktails contained 5 ml of 10× PCR buffer 1.5 mmol/L MgCl₂, 0.5 mmol/L each degenerate oligonucleotide primer, 200 mmol/L each deoxynucleoside triphosphate, 2.5 U of Taq DNA polymerase (Takara). For the first PCR, 50 ml volume of the purified (using the Wizard® DNA CleanUp kit (Promega)) DNA was amplified in a MJ cycler. For the second PCR, 3 ml of the first PCR product was used as a template. Each mixture was adjusted to a final volume of 50 ml with sterile water. The cycling conditions were 30 cycles consisting of denaturation at 94°C for 1 min, annealing for 1 min at 55°C for the first and at 48°C for the second PCR, extension at 72°C for 2 min, with a final extension at 72°C for 5 min. The presence of PCR products and their concentration were determined by analysing 5 ml of product on 2% agarose gels, staining with ethidium bromide and comparing with a molecular weight marker (Takara). A PTC-200 programmable thermal controller (MJ Research Inc., Reno, NV, USA) was used for all amplifications.

1.4 DGGE analysis

The DGGE was carried out using the D-code system from Bio-Rad Laboratories. PCR products were loaded onto 8% (w/v) polyacrylamide gels, 1 mm thick, in 1× TAE buffer (20 mmol/L tris-acetate, pH 7.4, 10 mmol/L acetate, 0.5 mmol/L disodium EDTA). The denaturing gradient contained 40%–65% denaturant (100% denaturant corresponded to 7 mol/L urea and 40% (v/v) deionized formamide). Electrophoresis was performed at a constant voltage of 180 V for 6 h. Approximately 400–450 ng of PCR product was loaded in each lane and the temperature was set at 60°C. After electrophoresis, gels were soaked for 30 min in 1× TAE containing SYBR Green I (1:10000 dilution, FMC BioProducts, Rockland, ME, USA). The stained gel was immediately photographed using Gel Documentation Systems (Bio-Rad, USA). The bands were visualized on a UV transillumination table equipped with a digital CCD camera.

Dendrogram analysis of DGGE banding patterns was performed using Quantity one 4.2.3 software (Bio-Rad, USA). The dendrogram was created automatically using UPGMA (unweighted pair-group method with arithmetic averages) based on Dice coefficient (Cs). The Shannon diversity index was estimated using the number of bands present (as an indicator of richness) (Demba et al., 2004a) and the relative intensities of the bands. The Shannon index was calculated as:

\[ H = - \sum (P_i) \ln(P_i) \]  

where, \( P_i \) is the importance probability of the \( i \)th band in a lane (Eichner et al., 1999). The importance probability \( P_i \) was calculated as:

\[ P_i = n_i / N \]  

where, \( n_i \) is the intensity of the \( i \)th band and \( N \) is the sum of the intensities of all the bands in the lane. The intensity was measured automatically using Quantity one 4.2.3 software (Bio-Rad, USA). The inhibition rate (\( R_1 \)) was calculated as

\[ R_1 = (N_{ck} - N_i) / N_{ck} \times 100\% \]  

where, \( N_{ck} \) is the number of bands in control samples and \( N_i \) that in the samples treated with the agrochemicals.

1.5 DGGE fragment isolation and sequencing

Fifteen bands were excised from DGGE gels with a surgical knife and placed into sterile Eppendorf vials. To elute the DNA, 50 ml TE (10 mmol/L Tris, pH 7.6, 1 mmol/L EDTA) was added and the vials were incubated at 4°C overnight. Three microliters of the supernatant was used as template DNA in a PCR with the primers PolF (without GC clamp) and AQER as previously described (Demba et al., 2004a). The PCR products were purified using a purification kit (Takara) according to the manufacturer’s instructions and sequenced by a DNA sequencing service provider (Shanghai Sangon Biological Engineering Technology and Services Co., Ltd) using the primer AQER.

Environmental nifH sequences from the NCBI GenBank database were selected on the basis of their similarity to those sequenced in this study. Sequences from relevant known, formally described diazotrophs were also used for phylogenetic reconstruction. The nifH sequence segments corresponding to the primers were removed from all the sequences prior to phylogenetic analysis. Sequences were aligned with clustalX1.83 and then corrected by manual inspection. A phylogenetic tree was constructed with Mega 3.1 (Kumar et al., 2004) using the neighbour-joining method with a gap penalty of 100% and was bootstrapped 500 times to provide a confident estimate of its topology.

2 Results

2.1 DGGE fingerprinting analysis

In the second PCR, about 320 bp fragment from the nifH gene was amplified using primers with GC clamps, and the resulting products were separated on DGGE gels. Various DGGE banding patterns were presented in the different soil samples (Figs.1 and 2). The total of bandtypes detected in each gel were of 36, 37 and 41 respectively. Using the software, the bands in different gels were matched. The results indicated that at least 41 nifH genes were present in the soil samples in this study.
Subsequently, the $R_I$ values were gradually reduced and the average inhibition rate of three treated samples decreased from 37.9% in the first week to 8.7% in 6 weeks and 15.0% in 8 weeks. This may be explained by the fact that the concentration of acetochlor in the artificial soil decreased due to the degradation of acetochlor occurred naturally and by microorganisms (Xiao et al., 2006; Ye, 2003), therefore, the effects weakened. However, the toxic effects on $nifH$ gene remained 8 weeks after the treatments with acetochlor.

Different $nifH$ gene bands in DGGE gel responded differently to the toxic effects of acetochlor (Fig.1a). The predominant band A6 was detected at 1 and 4 weeks of treatments in the samples treated with the medium (150 mg/kg) and the low (50 mg/kg) concentrations of acetochlor, but not found at 6 and 8 weeks. However, in the high concentration (250 mg/kg), no A6 band appeared in all the four sampling times. This result showed that $nifH$ gene A6 endured the medium and the low concentrations in the first a few weeks after the treatments, but not the late stage. In control, A6 was detected in all the four sampling times. At the medium and the high concentrations, Bands A3 and A8 were not found at 1 and 4 weeks but detected at 6 and 8 weeks of treatments. Band A5 was not found at 1 week but was detected at 4, 6, and 8 weeks at the high concentration. The results suggested that the three $nifH$ genes (A3, A5, and A8) were inhibited by the medium and the high concentrations in early time of treatment. With the degradation of acetochlor and the toxic effect weakened, the three $nifH$ genes appeared gradually. Band A2 were present in all samples, suggesting that this $nifH$ gene was resistant to acetochlor. Band A1 and A4 gradually faded over four sampling times in the controls. However, at the low concentration, band A1 and A4 were dominant at four sampling times except A1 at 6 weeks. The two bands could also be detected at all levels of acetochlor treatment at 8 weeks, showing that acetochlor had a stimulative effect on the two bands. At 8 weeks, due to the degradation of acetochlor, the level of acetochlor residue of the medium and the high concentrations became similar to the low concentration and lead to the appearance of A1 and A4. Band A7 could only be found at the low concentrations and not even in controls indicating band A7 may be a
characteristic band of the low concentration of acetochlor. The analysis using Shannon diversity index (Table 3) showed that nifH gene diversity was markedly influenced by the acetochlor treatments. Compared to the control, the diversity index values decreased as the concentration of acetochlor increased. nifH gene diversity was not restored to the control level until 8 weeks.

### 2.2.2 Effects of methamidophos on nifH gene diversity

Different from acetochlor, methamidophos enhanced the nifH gene richness at 4 weeks, while reduced the values at 1, 6, and 8 weeks. At 4 weeks, there were more bands in all the three methamidophos treatments compared with the control, showing the promotive effect on nifH gene. The number of bands at 1, 6 and 8 weeks were fewer than that in the control (Table 3), with the average inhibition rates of 28.7%, 17.4% and 25.4%, respectively.

The dominant band B2 (Fig.1b) was not detected at 1 week but it appeared in the following three sampling stages at the medium (150 mg/kg) and the high (250 mg/kg) concentrations of methamidophos. B2 was present in all the treatments at the low concentration and in controls. This indicates that the B2 nifH gene was inhibited by methamidophos of higher concentrations at the early stage of experiment and recovered gradually with the degradation of methamidophos. Band B1 was detected in all samples, showing that it was resistant to methamidophos.

The diversities of nifH gene (Table 3) fluctuated in methamidophos treated samples. At 1 week, the diversities in the three methamidophos treatments were lower than that in the control, lowest at the medium concentration. At 4 weeks, the diversities in all the three methamidophos treatments were higher than that in the control, highest at the high concentration. At 6 and 8 weeks, the index values in methamidophos treatments were lower than that in the control, lowest at the high concentration.

It is not clear why the medium concentration of methamidophos had the strongest cute toxic effects on nifH gene and not the high concentration at 1 week. In the late period, it was the high concentration that resulted in the most notable toxic effects. The half-life of methamidophos is about 1 week. With the breakdown of methamidophos, its effective concentration in soil may decrease to the middle level and demonstrate results similar to that of the medium concentration of methamidophos.

### 2.2.3 Cluster analysis

Fig.2 shows the results from cluster analysis of DGGE patterns of nifH gene. The treatments with acetochlor and the 4 controls were distributed in four groups (Fig.2a). The three treatments at 1 week (Aa1, Ab1, Ac1) were placed in three groups (I, II, IV), the treatments at 4 (Aa4, Ab4, Ac4) and 6 (Aa6, Ab6, Ac6) weeks in II and III groups and the treatments at 8 weeks (Aa8, Ab8, Ac8) in cluster IV. The results indicated that there were changes in nifH gene composition at 1 week after the treatments with or without the agrochemicals. The level of changes became less at 4 and 6 weeks and minimal at 8 weeks. The treatments of the low concentration of acetochlor (Aa4 and Aa6) and controls (CK4 and CK6) at 4 and 6 weeks were grouped together in cluster III, while the other two higher concentrations (Ab4, Ac4, Ab6, and Ac6) were placed in group II. This may indicate that the nifH gene compositions at low concentration recovered after 4 weeks and became similar to the corresponding controls and that the compositions at the medium and the high concentrations were relatively similar. The results were in agreement with that obtained from the analysis of diversity index.

As shown in Fig.2b, the methamidophos treatments and the control were placed in one cluster (I) at 1 week, in three clusters (I, II, IV) at week 4, in two clusters (III, IV) at week 6 and in two clusters (II, III) at week 8. The results indicate that the most drastic effects occurred at 4 weeks and the structure of nifH gene was not recovered at 6 and 8 weeks.

### Table 3 Changes of the nifH gene richness and diversity index affected by acetochlor and methamidophos

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>Sample No.</th>
<th>Acet</th>
<th>CK1</th>
<th>Aa1</th>
<th>Ab1</th>
<th>Ac1</th>
<th>CK4</th>
<th>Aa4</th>
<th>Ab4</th>
<th>Ac4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 week</td>
<td>Meth</td>
<td>CK1</td>
<td>Ma1</td>
<td>Mb1</td>
<td>Mc1</td>
<td>CK4</td>
<td>Ma4</td>
<td>Mb4</td>
<td>Mc4</td>
<td></td>
</tr>
<tr>
<td>4 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>Sample No.</th>
<th>Acet</th>
<th>CK6</th>
<th>Aa6</th>
<th>Ab6</th>
<th>Ac6</th>
<th>CK8</th>
<th>Aa8</th>
<th>Ab8</th>
<th>Ac8</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 weeks</td>
<td>Meth</td>
<td>CK6</td>
<td>Ma6</td>
<td>Mb6</td>
<td>Mc6</td>
<td>CK8</td>
<td>Ma8</td>
<td>Mb8</td>
<td>Mc8</td>
<td></td>
</tr>
<tr>
<td>8 weeks</td>
<td></td>
<td></td>
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</tbody>
</table>

**Table 3** Changes of the nifH gene richness and diversity index affected by acetochlor and methamidophos.
2.3 Effects on the \textit{nifH} gene diversity by the combination of acetochlor and methamidophos

The number of bands and the diversity indices of all samples treated with the combination of acetochlor and methamidophos were less than the corresponding controls (Table 4), indicating that the combination of two agrochemicals also had toxic effects on \textit{nifH} genes. Moreover, interactions between the two factors may have led to much more complex results. At 1 week, the combination of the low concentration acetochlor and the high concentration methamidophos (AaMc1) markedly reduced the number of bands while the least effect was observed in the combinations AbMa1 and AbMc1. At 8 weeks, the most significant changes were detected in AbMc8 and AcMa8. All three treatments containing medium concentration of methodimidophos (A-Mb8) had little effect on the diversity.

The bands C7, C8, and C9 (Fig.3) in the control disappeared in AaMc and AcMa at two sampling times (week 1 and week 8). The dominant bands C2, C3, and C5, which did not occur in any samples at 1 week, but appeared in AaMc8. It seems that the above six bands were sensitive to the combination of the low concentration of acetochlor and the high concentration of methamidophos and the combination of high concentration of acetochlor with the low concentration methamidophos. Band C1 and C6 could be detected in all samples and band C4 disappeared only in two treatments (AcMb1, AcMb8).

2.4 Sequence analysis of DGGE bands

Lane CK1 in Figs.1 and 3 derived from the same control sample, and the banding pattern in the three tracks were identical. Therefore, using the band profile of CK as a reference, band A2, B1 in Fig.1 and band C1 in Fig.3 were identified as the same band and A5, B2 and C6 as identical. A total of 15 interesting bands in three DGGE gels were excised for sequencing and corresponding sequences of 270 bp were obtained from all the isolated bands. All the isolated bands were sequenced with relative ease and minimal background interference, suggesting that each band represented a unique \textit{nifH} gene. The closest match for each of the 15 sequences, obtained by BLAST, is shown in Table 5. The percentage similarities between our sequences and the reference sequences from the databases varied from 86% (A1) to 96% (A6, C7, and C8).

Apart from the 8 \textit{nifH} sequences from GenBank which showed a high level of similarities to our sequences (Table 5), a further 17 \textit{nifH} sequences from GenBank which were relatively similar to ours were selected to construct a phylogenetic tree. Of the 25 \textit{nifH} sequences from GenBank, 19 were from known and formally described diazotrophs and 6 were from uncultured diazotrophs. The phylogenetic tree (Fig.4), constructed using 40 \textit{nifH} sequences, contained 6 clusters. The results showed a high degree of \textit{nifH} gene diversity in the test soil. The sequences obtained in this study belonged to Proteobacteria and were distributed between the \(\beta\) (73.3%) and \(\alpha\) (26.7%) subdivision (Table 5, Fig.4).

Sequences of bands A7, A8, and C9 fell into a large group containing \(\alpha\)-proteobacteria \textit{Methylosinus} and \textit{Pelomonas}. A7 and A8 belonged to the same phylotype, and with C3, subclustered with a \textit{nifH} sequence from an uncultured ditzotroph, which was obtained from forest soil (or agricultural soil) of Switzerland (Burgmann et al., 2004). The sequence of band C3 having \textit{Methylosinus trichosporum} OB3b (a methanotrophic bacteria) (Dedysh et al., 2004) as its closest known relative (Table 5), showed 98% and 97% similarities to the \textit{nifH} sequences of an uncultured bacterium and the clone AO12 which was detected in Oligotrophic oceanic waters (Zehr et al., 1998).

The sequence of band C2 showed 87% similarity to the \textit{nifH} sequence of \textit{Herbaspirillum} sp. B501, which was derived from shoot (leaf and stem) of wild rice (You et al., 2005). Sequences of bands C4, C8, A6, and C7 were placed within a large group possessed either \(\beta\)-proteobacteria or \(\alpha\)-proteobacteria. C4 showed 90% similarity to an uncultured bacterial clone b1-AB4 which

![Fig. 3 nifH-DGGE banding patterns from soil under the stress of the combination of acetochlor and methamidophos. CK: control; A: acetochlor; M: methamidophos; a, b, c: 50, 150, 250 mg/kg soil; 1, 8: treated for 1, 8 weeks; C1–C9: excise and sequenced.]

**Table 4 Effect of the combination of acetochlor and methamidophos on the \textit{nifH} gene diversity**

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>Week 1</th>
<th></th>
<th>Week 8</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample No.</td>
<td>CK1</td>
<td>AaMa</td>
<td>AaMb</td>
<td>AaMc</td>
</tr>
<tr>
<td>Number of bands</td>
<td>25</td>
<td>20</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>(H) value</td>
<td>1.346</td>
<td>1.241</td>
<td>1.254</td>
<td>0.958</td>
</tr>
<tr>
<td>Sample No.</td>
<td>CK8</td>
<td>AaMa</td>
<td>AaMb</td>
<td>AaMc</td>
</tr>
<tr>
<td>Number of bands</td>
<td>19</td>
<td>15</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td>(H) value</td>
<td>1.216</td>
<td>1.129</td>
<td>1.184</td>
<td>1.075</td>
</tr>
</tbody>
</table>
Fig. 4 Phylogenetic tree among the nifH sequences conducted by neighbour-joining method. Bootstrap values above 50 are shown representing the percentage support for the cluster out of 500. Species or strain names are preceded by GenBank accession numbers. DGGE bands detected in this study are given in bold. Bar, 2% sequence divergence.

Table 5 Result of BLAST analysis of the 15 nifH/DGGE excised and sequenced bands

<table>
<thead>
<tr>
<th>Band No.</th>
<th>Accession No.</th>
<th>Closest match in NCBI search</th>
<th>Bacterial group</th>
<th>Homology (%)</th>
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</table>

was often found in the PCR primers supplied by some manufacturers contained nifH gene and nifH-like DNA (Goto et al., 2005). C8, A6, and C7 subclustered together with Azohydromonas australica (Xie and Yokota, 2005). A6 and C7 belonged to the same phylotype, and possessed 96% similarity to Azohydromonas australica. Sequences of bands A2, C5, and A5 grouped together in a large cluster. C5 and A5 had Dechloromonas sp. as their closest relative (92% similarity). A2 subclustered with Azonexus fungiphilus isolated from sludge of a main aerobic treatment tanks (Quan et al., 2006). Sequences of band A1, A3, and A4 were quite similar and clustered away from
relevant known diazotrophs and showed high similarities ranging from 86% to 89% to the closest match *Ideonella* sp. Long 7.

3 Discussion

The relatively unpolluted soil tested in this study was taken from an Agro-ecological Experimental Station where no agrochemicals had been applied since 1989. The fact that at least 41 *nifH* genotypes were detected from the soil (Fig.2) suggests that the test soil is a good *nifH* gene pool. Under the present experimental conditions, treatments with acetochlor, methamidophos and their combinations had marked effects on the diversity and composition of *nifH* genes. The 15 sequences obtained in this study had high similarities (86%–96%) to at least 12 diazotrophs genera, confirming high *nifH* gene diversity in the test soil. The present results suggest that application of agrochemicals may affect the functional gene pool in soil and reduce the soil quality.

In general, the effects of agrochemicals on soil microbial communities have been studied using culture-dependent methods (Wardle and Parkinson, 1990). However, it is well known that more than 90% of the microorganisms existing in nature are refractory to selective enrichment cultures (Ward et al., 1990; Pace, 1997). In recent years, there has been growing interest in using molecular techniques to overcome the drawbacks of these culture-dependent methods. Since the cloning-sequencing strategies and hybridization approaches are rather cumbersome, time-consuming and expensive, the fingerprinting techniques such as PCR-DGGE (Larocque et al., 2004), PCR-RFLP (T-RFLP) (Poly et al., 2001a, b), ERIC-PCR (Bhattacharya et al., 2003; Wong and Lin, 2001), and PCR-SSCP (Schwieger and Tebbe, 1998), have been adapted to the study of bacterial communities and functionally significant taxa in the natural habitats. These fingerprinting methods are rapid and relatively easy to perform and allow the simultaneous analysis of multiple samples, which makes it possible to compare the genetic diversity of microbial communities from different habitats, or to study the behaviour of individual communities over time.

In this study, in order to increase the sensitivity and the specificity of *nifH* amplification, we used nested PCR to investigate the genetic diversity of *nifH* gene in soil samples. Since diazotrophic bacteria are rarely dominant populations in terrestrial ecosystems (Demba et al., 2004b; Mergel et al., 2001), it is difficult to directly amplify *nifH* gene from soil samples. Nested PCR is performed by allowing a first round of amplification with less stringent external primers, followed by a second round with internal primers designed to recognize specific regions within the initial amplicon. The usefulness of the nested PCR approach has been well demonstrated in the studies of functional gene diversity (Rebecca et al., 2005; Webster et al., 2002; More et al., 1994). Karolien et al. (2004) reported that DGGE profiles were similar between 16S rDNA gene amplicons generated by the direct and nested PCR respectively. Moreover, nested PCR could detect some populations that failed to appear in the directe PCR approach.

Biological nitrogen fixation is essential for maintaining fertility in soil ecosystems (Vitousek and Howarth, 1991) and plays an important role in maintaining soil quality. Nitrogenase is the enzyme responsible for catalysis of N₂ to ammonium. *nifH* is the gene that encodes for the iron protein subunit of nitrogenase and, amongst *nifH* genes is best suited for phylogenetic analysis, making it an ideal molecular marker for the study of diversity and stability of diazotrophic communities. Therefore, the number of *nifH* gene sequences in GenBank has expanded rapidly (1000 sequences in 2002 and 10000 in 2006). Furthermore, there are many known cultivable nitrogen-fixing microorganisms available in laboratories. For these reasons, analysis of the *nifH* gene provides a robust, culture-independent way of examining nitrogen-fixing bacterial diversity and community composition in the environment. In conclusion, our data showed that the two agrochemicals could have significant impacts on both the diversity and composition of the *nifH* gene. After application of the agrochemicals, for example, four individual bands (A6, C7, C8, C9) were not present, five bands (A1, A4, C2, C3, C4) were stimulated, and four bands (A5, A2, A7, C4) showed strong resistance to the agrochemicals. Band A6 disappeared in the presence of acetochlor and bands C7, C8, and C9 disappeared in the treatments with the combination of acetochlor and methamidophos. Although the half-time of acetochlor and that of methamidophos were short and no residue of agrochemicals could be found in the microcosm at the end of the experiment (8 weeks), there were changes in the bands richness, diversity index and composition of all the agrochemical-treated samples compared to the controls.

References


