Changes to the structure of *Sphingomonas* spp. communities associated with biodegradation of the herbicide isoproturon in soil

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**Abstract**

The phenyl-urea herbicide isoproturon is a major contaminant of surface and ground-water in agricultural catchments. Earlier work suggested that within-field spatial variation of isoproturon degradation rate resulted from interactions between catabolizing *Sphingomonas* spp. and pH. In the current study, changes to the structure of *Sphingomonas* communities during isoproturon catabolism were investigated using *Sphingomonas*-specific 16S rRNA gene primers. Growth-linked catabolism at high-pH (> 7.5) sites was associated with the appearance of multiple new denaturing gradient gel electrophoresis (DGGE) bands. At low-pH sites, there was no change in DGGE banding at sites in which there was cometabolism, but at sites in which there was growth-linked catabolism, degradation was associated with the appearance of a new band not present at high pH sites. Sequencing of DGGE bands indicated that a strain related to *Sphingomonas mali* proliferated at low pH sites, while strain *Sphingomonas* sp. SRS2, a catabolic strain identified in earlier work, together with several further *Sphingomonas* spp., proliferated at high-pH sites. The data indicate that degradation was associated with complex changes to the structure of *Sphingomonas* spp. communities, the precise nature of which was spatially variable.

**Introduction**

Biodegradation is the principal process controlling pesticide dissipation, and thereby pesticide persistence and susceptibility to leach from soil and contaminate surface and ground water (Aislabie & Lloyd-Jones, 1995). Pesticide biodegradation rates can show considerable within-field spatial variability, with implications for patterns of leaching losses (Walker *et al*., 2001a; Rodriguez-Cruz *et al*., 2006). Spatial variation in pesticide biodegradation rates can sometimes be correlated to variation in gross soil properties; however, the mechanisms underlying such variability are poorly understood (Walker *et al*., 2001a).

The phenyl-urea herbicides are particularly susceptible to within-field spatial variability of biodegradation rates (e.g. Beck *et al*., 1996; Cullington & Walker, 1999; Walker *et al*., 2001a, b). Phenyl-urea compounds, including isoproturon and diuron, are among the most widely used pesticides in Europe, and their slow degradation and moderate motility in soil results in these compounds being found widely as contaminants of agricultural catchments (Sørensen *et al*., 2003).

At one site, Deep-Slade field in Warwickshire, UK, spatial variability in isoproturon degradation rates has been shown to arise from localization in the areas in which organisms had adapted to use the compound as an energy source, which were interspersed with areas in which degradation showed first-order degradation. Such degradation kinetics generally indicate that catabolic organisms do not proliferate (Walker *et al*., 2001a), and has been termed cometabolism, although this could actually reflect very slow growth-linked metabolism masked as first-order degradation. Rapid degradation of isoproturon was associated with high-pH areas, whereas areas with lower pH showed slow degradation (Bending *et al*., 2001). Furthermore, eubacterial community denaturing gradient gel electrophoresis (DGGE) analysis showed proliferation of two *Sphingomonas* bands during isoproturon degradation in rapid-degrading areas (Bending *et al*., 2003). As the DGGE analysis used in the Deep-Slade study profiled dominant eubacterial community members only, it was not clear whether isoproturon degradation was associated with a wider group of sphingomonad strains, as has previously been shown for polychlorophenol degradation in contaminated groundwater (Tiirola *et al*., 2002).

In slow-degrading sites within Deep-Slade, either isoproturon-degrading organisms proliferated slowly, or there was no growth of catabolic organisms (Bending *et al*., 2001).
DGGE analysis at slow-degrading sites showed no change in banding (Bending et al., 2003), so it was not clear whether degradation reflected slow growth of the same organisms responsible for isoproturon degradation at fast-degrading sites, or whether other strains were responsible.

Traditionally, enrichment techniques have been used to isolate and identify organisms responsible for the degradation of pesticides and other xenobiotics in the environment (Aislabie & Lloyd-Jones, 1995). However, degradation of xenobiotics is commonly associated with mobile plasmids that can be transferred between strains (Van der Meer et al., 1992), particularly under the selective pressures associated with enrichment (Newby et al., 2000). The extent to which catabolic strains were obtained by enrichment techniques accurately reflect in situ catabolic communities is therefore unclear. In the Deep-Slade field studies, two different Sphingomonas isolates were obtained from rapid-degrading soil by enrichment (Sørensen et al., 2001; Bending et al., 2003). DGGE analysis suggested that one of these isolates (SRS2) proliferated during isoproturon degradation (Bending et al., 2003), but it was unclear what role the other strain (F35) played in degradation in situ.

The aim of the current study was to resolve questions relating to the role and spatial diversity of Sphingomonas communities associated with isoproturon catabolism, which were raised in earlier studies in Deep-Slade field. Using Sphingomonas selective rRNA gene primers, DGGE and quantitative PCR were used to investigate changes in the Sphingomonas community during isoproturon degradation in fast- and slow-degrading areas of Deep-Slade field.

Materials and methods

Soil samples

The soil samples described in Bending et al. (2003) were used in this study. Briefly, samples of soil were taken from two transects within Deep-Slade field, on the farm at Warwick HRI, Wellesbourne, Warwickshire, UK. The soil is a sandy-loam of the Wick series (Whitfield, 1974). The field had received regular applications of isoproturon for 20 years before the study. Soil samples were collected from five sites at 20-m intervals (labelled B, C, D, E, F) along two transects separated by 50 m. Transect 1 soil had pH of 7.1–7.4 (Table 1) and degraded isoproturon rapidly, while transect 2 soil had pH of 5.6–6.1.

Isoproturon addition and analysis

Description of the procedure for isoproturon addition and characterization are given in Bending et al. (2003). Briefly, an aqueous suspension of commercial formulation of isoproturon was added to soils to give 15 mg isoproturon kg⁻¹ soil, and −33 kPa. Control samples were set up using distilled H₂O instead of formulation. Over 65 days, isoproturon residues were extracted from subsamples of soil using acetonitrile, and concentrations measured by HPLC, as described in Bending et al. (2006).

Using data presented in Bending et al. (2003), the Gompertz model provided the best fit to the degradation data and was used to derive time to 50% degradation (DT50) and the length of the lag phase prior to exponential degradation. Calculations were performed using GenStat (7th edition, VSN International Ltd).

DNA extraction, PCR and DGGE analysis

At 90% (DT90) degradation of isoproturon, and after 9 months, DNA was extracted from control and isoproturon-treated soil according to Cullen & Hirsch (1998). Sphingomonas community 16S rRNA genes were amplified using the primers Sphingo108f/GC40-Sphingo420r (Leys et al., 2000). The extent to which xenobiotics is commonly associated with mobile plasmids (Aislabie & Lloyd-Jones, 1995). However, degradation of xenobiotics (7th edition, VSN International Ltd).

| Table 1. pH and isoproturon degradation characteristics [calculated from data presented in Bending et al. (2003)] in soil from transects 1 and 2 across Deep-Slade field |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Site            | Transect 1      | Transect 2      |                  |                  |                  |                  |                  |                  |                  |
|                 | B               | C               | D               | E               | F               | B               | C               | D               | E               | F               |
| pH              | 7.4             | 7.4             | 7.3             | 7.2             | 7.1             | 6.0             | 5.6             | 5.9             | 6.1             | 5.8             |
| Lag phase (days)| 5.0             | 5.6             | 5.2             | 4.9             | 5.2             | > 65            | > 65            | 11.1            | 6.3             | 38.8            |
| DT50 (days)     | 6.1             | 6.7             | 6.2             | 6.1             | 6.2             | 25.7            | 25.1            | 18.0            | 8.4             | 21.3            |

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migration was tested against the original sample using DGGE. Clones containing the correct insert DNA were sequenced using M13 forward and reverse primers (Invitrogen), while Sphingo108f/GC40Sphingo420r were used in sequencing reactions directly from DGGE bands. The PRISM BigDye Terminator Cycle Sequence reaction kit (Applied Biosystems, Warrington, UK) was used for sequencing with products analysed on an Applied Biosystems 377 DNA sequencer.

Cloning and sequencing

Sphingo108f/GC40-Sphingo420r PCR products were generated using DNA from isoproturon-treated and untreated samples of soil B from transect 1, at 90% isoproturon degradation. The products were cloned and sequenced using the methods described above. A total of 71 and 37 clones were sequenced for the isoproturon-treated and control samples, respectively.

The sequence data were edited and assembled using the DNASTAR II sequence analysis package (Lasergene Inc., Wisconsin), and the sequences were compared with those on the EMBL nucleotide database using the program BLAST. Using the environmental sequences, and reference sequences from the EMBL database, phylogenetic trees were constructed using the PHYLIP (version 3.5c) packages SEQBOOT, DNADIST and NEIGHBOR. The dendrogram was generated using neighbour-joining analysis, and the results were viewed using DRAWTREE. Clone sequences have been deposited in the EMBL nucleotide accession database under the accession numbers AM412676–AM412706.

Real-time PCR

Real-time PCR was performed on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) using a Platinum® SYBR® Green qPCR SuperMix UDG Kit (Invitrogen). Amplification of Sphingomonas spp. 16S rRNA gene was carried out using Sphingo108f/GC40Sphingo420r and general eubacterial (Muyzer et al., 1993) primers. PCR reactions contained 2 μL DNA template, 1 × Platinum® SYBR® Green qPCR SuperMix-UDG, 0.4 μL ROX reference Dye, 4.0 mM MgCl₂ and 300 nM Sphingo420r primer or 200 nM eubacterial primer. The Sphingomonas spp. reaction conditions were 2 min at 50°C, 10 min at 95°C, followed by 50 cycles of 95°C for 15 s, 62°C for 30 s and 74°C for 30 s. For eubacteria, the programme was 2 min at 50°C, 10 min at 95°C, followed by 50 cycles 92°C for 45 s and 55°C for 30 s, 68°C for 45 s.

DNA from Sphingomonas strain SRS2 (Sorensen et al., 2001) was used to prepare standard curves. Reactions were duplicated. Real-time PCR data were analysed by SDS 2.1 application software (Applied Biosystems). Copy numbers of Sphingomonas spp. and eubacterial 16S rRNA genes were calculated according to Park & Crowley (2005). The size and specificity of the PCR product were confirmed using a 1% agarose gel stained with ethidium bromide.

Results

Isoproturon degradation

Samples from transect 1 had a lag phase of 4.9 to 5.6 days before exponential decay (Table 1). In transect 2, for two samples (B and C), there was no phase of exponential decay. For the remaining three samples, the lag phase ranged from 6.3 to 38.8 days. Time to 50% degradation (DT50) varied between 6.1 and 6.7 days in transect 1, and between 8.4 and 25.7 days in transect 2.

DGGE analysis

Strain Sphingomonas SRS2 gave a single dominant DGGE band, together with a number of faint minor bands (Fig. 1). At DT90, new bands appeared in isoproturon-treated samples from transect 1, which were not present in control soils. The major band that appeared matched migration of the dominant band seen in SRS2 (S6), and the faint bands seen in SRS2 also appeared (S1–5, S7). Three further bands (H1, H2 and H3) appeared in all five samples, while H4 appeared in samples C, D and E only, and band H5 and H6 appeared only in samples C and F, respectively. None of these bands matched banding of isolate F35 or SRS2.

At DT90 in transect 2, treatment with isoproturon had induced no effect on banding in samples B and C. In samples D and F, one new band (L1) appeared on treatment with isoproturon, while the same band plus one matching the position of the major band generated by SRS2 (L3), together with one of the faint bands produced by SRS2 (L2), appeared in sample E. After 9 months, the differences in banding between isoproturon-treated and untreated samples in transect 1, but not transect 2, were still evident (data not shown).

Phylogenetic analysis of 16S rRNA gene from clones, isolates and DGGE bands

Of the 108 clones sequenced, 97 showed homology with Sphingomonas spp., with the remainder showing the closest homology to related Alphaproteobacteria, including Stella sp., Skermanella sp. and Azospirillum sp. The clone libraries generated 31 unique Sphingomonas spp. sequences. All Sphingomonas spp. clones showed 95–100% similarity to Sphingomonas sp. 16S rRNA gene sequences from the EMBL database, with clones showing homology with Sphingomonas sensu stricto, Sphingobium, Novosphingobium and Sphingopyxis (Takeuchi et al., 2001). Relative to control soil, isoproturon-treated soil showed increased numbers of
sequences related to *Sphingomonas* strain SRS2, which represented 27% of the clone library in isoproturon-treated soil, compared with 5% in the control soil library (Fig. 2).

DGGE band S6, which was the dominant band appearing in all samples from transect 1 at DT90, showed 100% similarity to *Sphingomonas* SRS2 (Table 2). DGGE band H4 showed 96% homology to *Sphingomonas* D12 (accession number AB015809), and DGGE band H3 showed 99% homology to *Sphingomonas* CFDS-1 (accession number AY702969). DGGE band L1, which appeared in transect 2 only, showed 98% homology to *Sphingomonas mali* (accession number SM16SR).

**Real-time PCR**

The PCR efficiencies were 100% ($R^2 = 0.995$) and 97% ($R^2 = 0.985$) for the *Sphingomonas* and eubacterial primer sets, respectively. The *Sphingomonas* spp. 16S rRNA gene copy numbers in the samples ranged from $6.41 \times 10^9$ to $2.30 \times 10^{10}$ g$^{-1}$ soil while the total eubacterial 16S rRNA gene copy numbers ranged from $6.51 \times 10^{12}$ to $1.47 \times 10^{13}$ g$^{-1}$ soil. The percentage of *Sphingomonas* spp. within the bacterial community did not change significantly during isoproturon degradation (data not shown).

**Discussion**

While some studies using general 16S rRNA gene primers have shown a change in the structure of bacterial communities following the application of herbicides or other pesticides to soil, others have reported no change (Engelen *et al.*, 1998; Sigler & Turco, 2002). The data in this study in combination with that of an earlier study (Bending *et al.*, 2003), demonstrate that when group-specific primers are used to provide a finer scale of resolution, the complexity of changes to community structure can be far greater than those indicated using general primers.

Strains of *Sphingomonas* spp. capable of degrading a wide range of xenobiotics have been isolated, suggesting that this genus is extremely effective at adapting to degrade new compounds (Basta *et al.*, 2004). The DGGE data show that growth-linked degradation of isoproturon was associated with proliferation of a variety of *Sphingomonas* spp. Strain *Sphingomonas* SRS2, which was isolated from Deep-Slade field using enrichment methods (Sørensen *et al.*, 2001), clearly had a role in degradation *in situ*. However, strain *Sphingomonas* F35, which was isolated from the isoproturon-amended soil described in this paper (Bending *et al.*, 2003), did not appear to proliferate in soil during isoproturon catabolism. This could suggest that the enrichment
Fig. 2. Distance tree constructed with partial (303 bp) 16S rRNA gene sequences, showing relationships of clones from isoproturon-treated and untreated (C) soil with members of the genus Sphingomonas spp. Figures in italics show the number of clones matching the sequence with > 98% homology. Scale bar represents the number of changes per nucleotide position.
process facilitated degradative gene transfer from pesticide degraders to a background organism that was not involved in degradation in situ (Newby et al., 2000).

The role of the other *Sphingomonas* spp. that appeared during degradation of isoproturon at high-pH sites is uncertain. Horizontal gene transfer can occur readily between *Sphingomonas* spp. (Basta et al., 2004). Tiirola et al. (2002) showed evidence that natural horizontal transfer of the *pcpB* gene between sphingomonads facilitated the evolution of polychlorophenol-degrading sphingomonads within a contaminated groundwater. Given the potential for horizontal gene transfer between *Sphingomonas* spp., gene transfer from SRS2 could provide a mechanism to explain the proliferation of other *Sphingomonas* spp. during isoproturon degradation. However, it is possible that the other strains could have proliferated via indirect mechanisms, such as through altered competitive interactions resulting from the activities of isoproturon-catabolizing communities. Further work using stable isotope probing (Mahmood et al., 2005) approaches would be required to provide unequivocal evidence for the contribution of such *Sphingomonas* spp. strains to biodegradation.

There was spatial diversity in the response of the *Sphingomonas* spp. community during isoproturon degradation. Within transect 1, several bands did not appear at all sites, although this was not related to differences in degradation rate or gross soil characteristics. In transect 2, SRS2 proliferated at only one of the sites. At sites in which SRS2 did not proliferate, growth-linked degradation was associated with the appearance of a strain related to *S. mali*, which did not occur in transect 1. In sites B and C in transect 2, degradation followed first-order kinetics, suggesting that there had been no proliferation of degraders. In these sites, there was no change in the *Sphingomonas* spp. community DGGE profile, providing some evidence that those strains which did appear on the DGGE profile at locations at that there was growth-linked metabolism could have been involved in isoproturon degradation.

Most studies in which sphingomonad strains capable of xenobiotic degradation have been identified have used enrichment procedures from environmental samples collected from a single sampling location (e.g. Schmidt et al., 1992; Wittich et al., 1992; Tanghe et al., 1999). Clearly, sphingomonad communities acting in situ may be diverse. Single strains isolated using enrichment techniques may not provide accurate models with which to understand degradation processes in the environment. The presence of diverse communities of phylogenetically related catabolic strains, together with strict nutritional or environmental requirements, such as pH, could also help to explain why sphingomonad strains shown to degrade xenobiotics in the laboratory may not show degradative abilities when inoculated into environmental samples, including systems from which they were isolated (Shi et al., 2001).

Although the *Sphingomonas* spp. community profile changed during isoproturon degradation, there was no change in the number of *Sphingomonas* spp. or the proportion of *Sphingomonas* spp. within the bacterial community. This could indicate that the change in *Sphingomonas* spp. was small relative to the overall size of the *Sphingomonas* spp. community. As all sequenced bands that appeared in the DGGE profiles during isoproturon degradation belonged to *Sphingomonas sensu stricto* (Takeuchi et al., 2001), the use of primers specific to this group may have provided more useful data on population dynamics.

*Sphingomonas* spp. strains F35 and SRS2 were found to yield multiple DGGE bands. Similarly, Leys et al. (2004) showed that a number of pure *Sphingomonas* strains give multiple banding patterns using the 16S rRNA gene primers used in the current study. In prokaryotes, possession of more than one 16S rRNA gene copies with sequence divergence in the genome is a common phenomenon (Klappenbach et al., 2001).

To conclude, strain SRS2 was required for rapid isoproturon catabolism, SRS2 was not involved in isoproturon degradation at most low-pH sites, strain F35 had no involvement in isoproturon degradation at high- or low-pH locations and isoproturon degradation was associated with complex changes to *Sphingomonas* spp. community structure that varied according to soil pH and whether degradation occurred by growth-linked or cometabolic processes.

### Acknowledgements

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**Table 2. Closest match of DNA sequenced from excised DGGE bands to sequences from the EMBL database**

<table>
<thead>
<tr>
<th>Band</th>
<th>EMBL accession number</th>
<th>Closest match and accession number</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>AM412812</td>
<td><em>Sphingomonas</em> sp. CFDS-1 AY702969</td>
<td>99</td>
</tr>
<tr>
<td>H4</td>
<td>AM412813</td>
<td><em>Sphingomonas</em> sp. D12 A8105809</td>
<td>96</td>
</tr>
<tr>
<td>S6</td>
<td></td>
<td><em>Sphingomonas</em> sp. SRS2 SSP251638</td>
<td>100</td>
</tr>
<tr>
<td>L1</td>
<td>AM412814</td>
<td><em>Sphingomonas mali</em> SM16SR</td>
<td>98</td>
</tr>
</tbody>
</table>

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References


