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# Monitoring Impact of a Pesticide Treatment on Bacterial Soil Communities by Metabolic and Genetic Fingerprinting in Addition to Conventional Testing Procedures

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**Herbogil (dinoterb), a reference herbicide, the mineral oil Oleo (paraffin oil used as an additive to herbicides), and Goltix (metamitron) were taken as model compounds for the study of impacts on microbial soil communities. After the treatment of soil samples, effects on metabolic sum parameters were determined by monitoring substrate-induced respiration (SIR) and dehydrogenase activity, as well as carbon and nitrogen mineralization. These conventional ecotoxicological testing procedures are used in pesticide registration. Inhibition of biomass-related activities and stimulation of nitrogen mineralization were the most significant effects caused by the application of Herbogil. Even though Goltix and Oleo were used at a higher dosage (10 times higher), the application of Goltix resulted in smaller effects and the additive Oleo was the least-active compound, with minor stimulation of test parameters at later observation times. The results served as a background for investigation of the power of "fingerprinting" methods in microbial ecology. Changes in catabolic activities induced by treatments were analyzed by using the 95 carbon sources provided by the BIOLOG system. Variations in the complex metabolic fingerprints demonstrated inhibition of many catabolic pathways after the application of Herbogil. Again, the effects of the other compounds were expressed at much lower levels and comprised stimulations as well as inhibitions. Testing for significance by a multivariate *t* test indicated that the sensitivity of this method was similar to the sensitivities of the conventional testing procedures. The variation of sensitive carbon sources, as determined by factor weights at different observation times, indicated the dynamics of the community shift induced by the Herbogil treatment in more detail. DNA extractions from soil resulted in a collection of molecules representing the genetic composition of total bacterial communities. Distinct and highly reproducible community patterns, or genetic fingerprints, resulting from application of the different herbicides were obtained by the sequence-specific separation of partial 16S rDNA amplification products in temperature gradient gel electrophoresis. Significant pattern variations were quantified. For detailed analysis, application-responsive bands from the Herbogil and Oleo treatments were sequenced and their tentative phylogenetic positions were identified. Data interpretation and the potentials and biases of the additional observation windows on microbial communities are discussed.**

At least once a year about 85% of agriculturally used soils in western Europe are treated with herbicides in order to ensure that high-quality agricultural goods will be produced at levels that cover costs (29). Actual registration procedures include evaluation of the impact of herbicides on the environment by testing for nontarget effects on single species or microbial communities. The impact on soil microbial communities is evaluated in view of their role in sustaining the global cycling of matter and their varied functions in supporting plant growth. In order to observe effects by quantifying changes in microbial biomass or in the general metabolic activities of microbial communities, a variety of laboratory tests have been recommended (12, 24). Internationally, there is wide divergence in the extent to which the performance of such tests is obligatory for registrations. Substrate-induced-respiration (SIR), dehydrogenase activity, and nitrogen turnover are investigated in German registration procedures (2).

The traditional techniques used in our study can be divided into two groups. SIR (1) and dehydrogenase activity are both strictly correlated to biomass, whereas long-term respiration and nitrogen mineralization are not. The immediate respiration of a microbial community following a glucose addition is quantified in a manner avoiding a significant contribution of cell multiplication. Testing for the activity of dehydrogenases, as determined by the accumulation of reduced triphenyl formazane, conveniently monitors the metabolic activities of microbial cells in oxidizing organic substrates. This parameter seems to indicate an impact on microbial activity most significantly. The efficiency of microbial carbon mineralization over an extended period (8 weeks in this report) can be stimulated by the mineralization of dead cells or the degradation of the pesticide itself. Carbon mineralization and nitrogen mineralization serve to complement the interpretation of the results for the biomass-related parameters (23).

Another approach to differentiating bacterial communities by their catabolic potentials is offered by the BIOLOG system (BIOLOG Inc., Hayward, Calif.), originally commercialized for the identification of bacterial strains. The metabolism of 95

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carbon sources in microtiter plates, indicated by the reduction of a tetrazolium dye, affords a detailed view of the catabolic activities of complex bacterial communities (10). These features seem to be suited to monitoring of the impact of stress factors on microbial communities. In contrast to such approaches, testing for effects on a special activity of a single microbial species can be used in some cases of soil contamination (5). Such testing may indicate how the whole community responds to toxic interactions but might be considered to be restricted in its ecological significance.

Analysis of changes in the composition of a community can also be used to characterize its response to stress. Species abundance and diversity measurements are sensitive indicators of environmental conditions (3, 4). However, the conventional microbiological investigation of community diversity has significant pitfalls. First, the time and resources needed for quantification and identification of morphologically similar microbial species preclude an extensive analysis of many samples from different treatments. The counting of colony morphotypes has not been tested as an alternative on a broader scale (14). Second, a significant bias invariably is introduced by the selective process of cultivation. Microbiologists are becoming increasingly aware of the fact that only a small fraction of microorganisms is analyzed by conventional cultivation methods, and frequently these species do not seem to represent the spectrum of organisms active in the habitat of choice (39, 40).

A measurement more representative of the structure of communities in situ may be obtained by isolating and analyzing macromolecules of microbial origin from a habitat, as in other methods of microbial-community characterization (18, 28). A community's composition and diversity are most significantly represented by the complexity of information in nucleic acid molecules. A great variety of methods of isolating DNA and RNA from different habitats, either by direct extraction or following a prior separation of microbial cells, have been published. The information in about 4,000 different bacterial chromosomes, equivalent to more than 10,000 species, seems to represent the complexity of microbial communities in healthy agricultural soils, as estimated by DNA reassociation kinetics (38). Wide divergence in levels of diversity among different soils can be demonstrated, but the technique does not seem to be adaptable to a measurement of induced changes, particularly with respect to its sensitivity.

A more detailed view of the composition of a community can be obtained by sampling the nucleic acid population from a habitat in order to determine the abundance of phylogenetically meaningful sequences, particularly the 16S rRNA and 16S rDNA. The origins of individual sequences are then classified by comparison to large databases and their present interpretation (19). The frequency of sequences within the sample represents the community composition in some detail, depending on the number of sequences analyzed. Such approaches have become an established method of molecular ecology (27, 34). Unfortunately, these techniques are labor intensive and time-consuming, a major drawback for any investigation involving a large number of differently treated samples.

The sequence-dependent separation of 16S rDNA fragments in a denaturing gradient gel (denaturing gradient gel electrophoresis [DGGE]) in order to analyze the abundance of divergent sequences from particular communities was introduced by Muyzer et al. (25). By superimposing a temperature gradient instead of a chemical gradient on the electric field, temperature gradient gel electrophoresis (TGGE) applies the identical principle of separating double-stranded DNA fragments. Minor sequence variations, including point mutations, result in modified migration and distinct bands (26, 31).

The technique of generating a pattern from sequence variants with well-defined phylogenetic meaning is attractive from different points of views. The pattern yields a representation of the community as a whole or of a section of it, defined by the selected primers. The bands of the pattern are not directly interpretable in phylogenetic terms. Their "fingerprint," however, represents the frequency distribution of different sequences in a sample. Variations in such fingerprints due to induced variations in community composition may be easily observed. The degrees of pattern change may be quantified to some extent to allow for ranking. Finally, band isolation and sequencing is one straightforward possibility to investigate induced changes in detail with respect to responsive community members.

We have used both the BIOLOG system and pattern generation by amplified ribosomal sequences to investigate the suitability of these approaches for the analysis of the impacts of herbicide applications on the function and structure of soil bacterial communities. The more traditional methods for analysis of such impacts serve as a well-defined background of ecotoxicological testing.

## MATERIALS AND METHODS

**Soil type and sampling preparation.** Soil samples were obtained from a field that, for many years, was not treated with pesticides. The soil texture is loamy sand with 12.9% clay, 37.7% silt, and 49.4% sand in the mineral fraction. It contains 1.16% organic carbon and has a maximum water capacity of 23.7 g/100 g of soil. The pH is 5.6 to 6.8 in 0.1 N KCl.

Samples from the upper layer (0 to 10 cm) were collected immediately after the frost period and were dried at ambient temperature to allow sieving (mesh, 2 mm). They were adapted to test conditions at a maximum water capacity of 45% for 2 weeks.

**Experimental approach.** Three replicates of experimental treatments were performed by mixing 5 ml of aqueous pesticide solution or water (used as the control) to 1,000 g (dry weight) of soil. Experimental soil-water and soil-pesticide mixtures were incubated in the dark at 20°C. They were kept at 60% of maximum water capacity by weekly controls and compensation for water loss. Samples for testing of microbial activity were taken after 1, 2, 5, and 8 weeks. Table 1 shows chemical characteristics and experimental test ranges of the pesticides used.

**SIR.** As described by Malkomes (21), the soil samples (100 g [dry weight]) were mixed with glucose (0.5 g) plus talcum (0.5 g) and placed in glass tubes connected to an infrared gas analyzer (UNOR). $CO_2$ formation, continually measured for 6 h, was integrated.

**Dehydrogenase activity (reduction of TTC to triphenyl formazan).** As described by Malkomes (22), the soil samples (2 g [dry weight] were incubated for 24 h at 30°C in 2 ml of a triphenyl tetrazolium chloride (TTC) solution (0.5% in 0.1 M Tris-HCl buffer, pH 7.6). The samples were amended with acetone and centrifuged (for 15 min at 4°C and at 4,500 × *g*). The extinction of the supernatant was measured spectrophotometrically (by the optical density at 546 nm [$OD_{546}$]).

**Nitrogen mineralization (including nitrification).** Mineral nitrogen ($N_{min}$) was extracted from samples (10 g [dry weight]) by shaking for 60 min in 50 ml of a KCl solution (2 M). After filtration, extracts were colorimetrically analyzed for nitrate and ammonium ($N_{min}$) by an autoanalyzer (TRAACS 800).

**Long-term respiration (carbon mineralization).** For the analysis of long-term respiration, soil samples (200 g [dry weight]) were separately treated. Aqueous solutions of the herbicides or the mineral oil (4 ml) were added. Samples were placed in 1-liter sealed glass bottles and continually aerated with 1 liter of $CO_2$-free air/h for 8 weeks. Mineralization was quantified weekly by titrating $CO_2$ trapped in a KOH solution.

Variations in data obtained with these tests were analyzed for significance by Dunnett's *t* test ($\alpha = 0.05$).

**Extraction of microorganisms from soil.** Microorganisms were extracted from soil samples by the following mechanical and chemical treatments.

(i) A suspension of 5 g of soil in 45 ml of sterile NaCl solution (0.85%) was homogenized in a stomacher blender (for 60 s at high speed) and transferred into sterile Falcon tubes (50 ml). Soil particles were removed by a low-speed centrifugation step (2 min, 500 × *g*, 20°C), and a pellet of bacteria was harvested from the supernatant (10 min, 5,000 × *g*, 20°C). The supernatant from this centrifugation was discarded.

(ii) The soil pellet from the low-speed centrifugation was resuspended in 10 ml of SDP solution (0.02% sodium deoxycholate and 0.5% polyethylene glycol, sterile filtered) and incubated with 1 g of the ion-exchanging resin Chelex 100 (100/200 mesh; Bio-Rad, Munich, Germany) in a 100-ml Erlenmeyer flask (for 1 h, with shaking, at 20°C) that contained 30 glass beads (diameter, 4 mm). Both the removal of divalent cations by ion-exchange resins and the use of detergent enhance the soil dispersion and detachment of bacteria (15). Glass beads were

TABLE 1. Chemical characteristics of the investigated effectors

| Characteristic | Goltix WG | Flüssig Herbogil[a] | Oleo FC |
|---|---|---|---|
| Manufacturer | Bayer AG | Rhône-Poulenc | Feinchemie Schwebda GmbH |
| Formulation | Water-dispersible granules | Spray emulsion | Emulsifiable concentrate |
| Chemical class | Triazinon derivative | Phenol derivative | Mineral oil |
| Active ingredient | Metamitron (herbicide) | Dinoterb (herbicide) | Paraffin oil (additive) |
| Chemical expression | 3-Methyl-4-amino-6-phenyl-1,2,4-triazin-5-(4H)-one | 2-*tert*-Butyl-4,6-dinitrophenol | ND[c] |
| Sum formula | $C_{10}H_{10}N_4O$ | $C_{10}H_{12}N_2O_5$ | ND |
| Amt of active ingredient | 710 g/kg | 250 g/liter | 803 g/liter |
| Field dose | 5–10 kg/ha | 4–5.5 liters/ha | 5 liters/ha in mixture |
| Concn of the product in soil[b] | 93.3 mg/kg (10×) | 7.3 μl/kg (1×) | 66.7 μl/kg (10×) |

[a] No longer approved as a herbicide in Germany.
[b] Simulated concentration in soil after penetration of field-related dose into the upper 0.5 cm (10×) or 5 cm (1×).
[c] ND, not determined.

removed by sieving in a sterile metal sieve. The soil particles were pelleted (for 2 min at 500 × *g* and at 20°C), and the supernatant was added to the bacteria pellet of step i (stored at 4°C).

(iii) The soil pellet (from step ii) was resuspended again in 10 ml of SDP solution and was treated in the stomacher blender a second time. Soil particles pelleted by centrifugation (for 2 min at 500 × *g* and at 20°C) were discarded.

(iv) The supernatant (from step iii) was added to the bacterial fraction (from step i) and centrifuged (for 10 min at 5,000 × *g* and at 20°C). The bulked pellet of bacteria was suspended in 10 ml of a sterile NaCl solution (0.85%). This suspension served as a common source for BIOLOG GN microplate inoculations and DNA extractions.

**Inoculation of BIOLOG GN microplates.** Six replicates of bacterial suspensions from experimental variants were adjusted to a defined OD ($OD_{546}$ = 2.0) and transferred to BIOLOG GN microplates with a multipipette (0.15 ml per well, corresponding to $1 \times 10^4 \pm 5 \times 10^3$ CFU per well, as determined by plate counts on Luria-Bertani media). The microplates were incubated in the dark at 28°C. $OD_{596}$ values were determined after 32, 36, 44, and 56 h of incubation with an enzyme-linked immunosorbent assay (ELISA) reader (Vmax; Molecular Dynamics, Menlo Park, Calif.).

**Processing of BIOLOG data. (i) Raw data treatment.** OD values were processed by blank subtraction ($OD_0$), and AWCD correction ($OD_0$/AWCD = $OD_{awc}$; AWCD = [sum of $OD_0$ values]/95). Variables with no color development in all observations (mean $OD_0 \leq 0$) were eliminated.

**(ii) Reading time for analysis.** A common (early) reading time for statistical analysis was determined as described by Glimm et al. (13).

**Statistical analysis of BIOLOG data.** Regular variations in the complex patterns between treatments were tested for their significance by a multivariate *t* test (principal component [PC] test [20]). PC analysis (PCA) was used to reduce the dimension of data (95 variables) to the number of PCs that explain 80% of the variance (here, five or six PCs). Thereafter a classical Hotelling's $t^2$ test ($\alpha$ = 0.05) was applied. The correlation between PCs and a variable's importance (estimated factor loading in a factor-analytical model) was used to examine the impact of single substrates (13).

**DNA extraction.** Suspended bacterial cells were disrupted by freeze-thawing with three cycles at −20°C and 30°C. An aliquot from the suspension's clear supernatant of the last thawing step was used for extracting DNA by phenol-chloroform extraction (32). The DNA pellet was dissolved in 50 μl of TE buffer (10 mM Tris-Cl [pH 8.0]–1 mM EDTA), yielding PCR-amplifiable DNA without additional cleaning steps.

**PCR.** PCR primers F-968-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-AA CGC GAA GAA CCT TAC-3′) and R-1401 (5′-CGG TGT GTA CAA GGC CC-3′) were used to amplify the segment of bacterial 16S rDNA from nucleotide 968 to 1401 (*Escherichia coli* numbering [7]). The dash in the F-968-GC sequence indicates the addition of the GC-clamp, used for the improvement of fragment separations in the TGGE analysis. The specificity of the primers is demonstrated by their similarity to conserved 16S sequence regions (>95% at 13 and 14 positions, respectively). For reamplification of eluted TGGE bands, a forward primer without a GC clamp (F-968) and a slightly different reverse primer, R-1401/1378 (5′-CGG TGT GTA CAA GGC CCG GGA ACG-3′), were used. For PCR, Ampli*Taq* DNA polymerase, Stoffel fragment, and a buffer kit from Perkin-Elmer Cetus were used. Reaction mixtures (50 μl) contained 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 3 mM $MgCl_2$, 5% dimethyl sulfoxide (vol/vol), 100 μM (each) dATP, dCTP, dGTP, and dTTP, 20 pmol (each) of primers R-1401 and F-968-GC, and 5 U of Ampli*Taq* DNA polymerase. Samples, overlaid with 50 μl of mineral oil, were incubated in a Hybaid OmniGene Temperature Cycler (Hybaid, Teddington, United Kingdom) after addition of 2 μl of target DNA. Programming was 5 min at 94°C; amplification for 35 cycles, each consisting of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C; and a final extension for 5 min at 72°C. Aliquots were analyzed by electrophoresis on a 1.5% (wt/vol) agarose gel stained with ethidium bromide. Before separation in TGGE, products were purified by the QIAquick PCR Purification Kit (Qiagen GmbH, Düsseldorf, Germany) and concentrated (5 times).

**TGGE.** For sequence-specific separation of PCR products, the Diagen TGGE system was used. Gels (6% [wt/vol] acrylamide, 0.1% [wt/vol] bisacrylamide, 8 M urea, 20% [vol/vol] formamide, 2% [vol/vol] glycerol) were polymerized overnight on the hydrophilic side of a gel support film (FMC, Vallensbaek Strand, Denmark). Runs were performed at 360 V for 4.5 h in 1× MOPS electrophoresis buffer (20 mM morpholinopropanesulfonic acid–1 mM EDTA [pH 8.0]) by using a temperature gradient of 37 to 50°C. After the run, the gels were silver stained (33).

**Reamplification of TGGE bands.** The preparative TGGE gel was polymerized on the hydrophobic side of the gel support film to allow detachment before staining with SYBR-Green I (Molecular Probes, Eugene, Oreg.) according to the manufacturer's instructions. Small slices of selected TGGE bands were extracted by puncturing the gel with a sterile glass pipette. The cut-outs were eluted (33) and reamplified (with primers R-1401/1378 and F-968; annealing temperature, 60°C). Bands of the silver-stained gels consistently were not reamplifiable.

**Cloning and sequencing.** Reamplified PCR products (≈430 bp) were ligated into a pGEM-T plasmid vector (Invitrogen, San Diego, Calif.). Transformation of *E. coli* DH5α and small-scale preparation of plasmid DNA were performed as described by Sambrook et al. (32), and purification was carried out with the Qiagen Plasmid Kit (Diagen GmbH). Plasmid DNA was used as a template in cycle sequencing reactions with the Thermosequenase Kit RPN 2436 (Amersham). Sequences of both strands of plasmid inserts were determined by using the multiple cloning site flanking primers M13 (−24)r and M13 (−20)f, both labeled with the fluorophore IRD-41 (MWG Biotech, Ebersberg, Germany). Products of sequencing reactions were analyzed by using a LI-COR 4000 automatic DNA sequencer (MWG Biotech).

**Comparative sequence analysis.** The phylogenetic positions of the clone sequences were determined as suggested by Stackebrandt and Rainey (36). Alignment of the unknown sequences to the database (about 4,000 sequences) of the ARB software package identified the most-similar organisms. Sequences of related organisms were chosen for calculation of partial trees by cluster analysis using the nearest-neighbor method. The classification of sequences was also cross-checked by similarity ranking with respect to the database of the Ribosomal Database Project (19).

**Nucleotide sequence accession numbers.** Nucleotide sequences for clones 3, 4, 5, 17, and 24 have been deposited in the EMBL database under accession no. AJ005063 through AJ005067, respectively.

## RESULTS

**Traditional testing protocols.** The effects of the pesticides on SIR, dehydrogenase activity, long-term respiration, and nitrogen mineralization are shown in Fig. 1. Experimental data represent the means of three replicates. The magnitudes of effects differ between applications and sometimes vary during the observation period. Consistently, corresponding to the findings of a variety of earlier investigations (6), the herbicide Herbogil (dinoterb) caused the greatest effects, despite its low (1×) concentration in soil (Table 1). Significant reductions in the two biomass-related activities, SIR (22%) and dehydrogenase activity (44%), indicated a decrease in microbial biomass. Long-term respiration was less affected, but its reduction became significant at the two later observation times. Significant stimulation of nitrogen mineralization after 1 and 2 weeks indicated mineralization of dead cells.

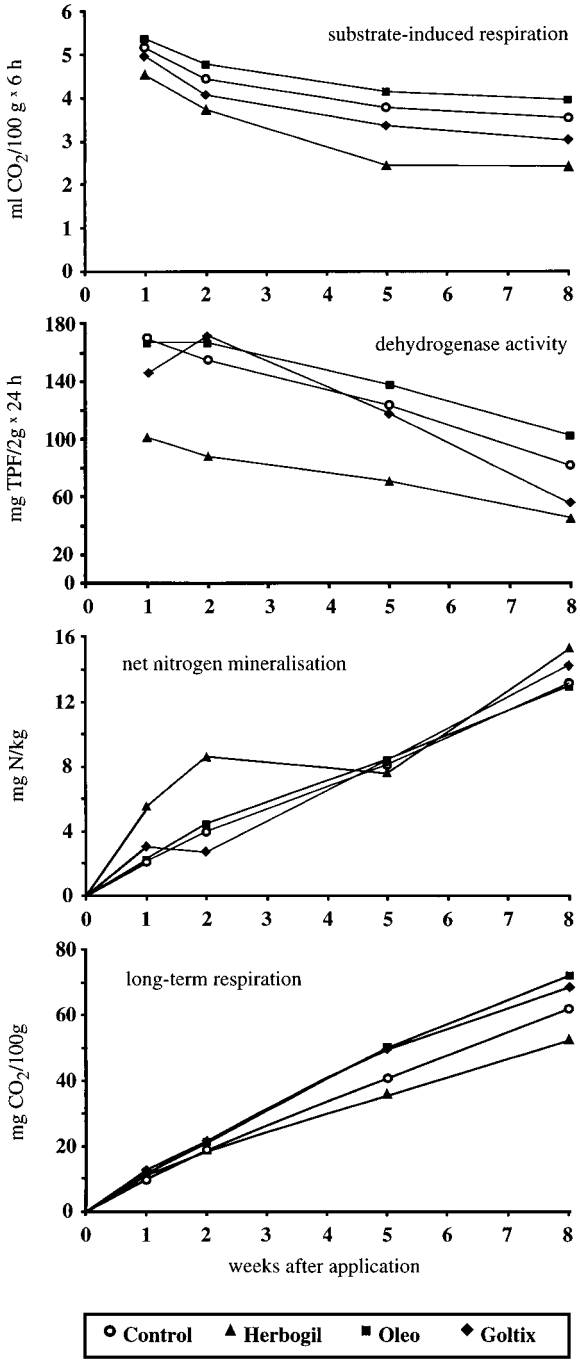


FIG. 1. Results of the traditional methods. SIR data represent the integration of $CO_2$ production of soil samples taken at the given sampling times for 6 h after the addition of glucose. For dehydrogenase activity, amounts of (reduced) triphenyl formazan (TPF), as determined with a spectrophotometer at the different sampling times, are shown. Changes in nitrogen mineralization were analyzed by a colorimetric assay at each sampling time. The concentrations observed at the beginning (day 0) were subtracted from actual experimental data. For long-term respiration, the cumulative carbon mineralization, determined by titration in a KOH solution, is shown.

Even though it was applied at a high dose (10×), the additive Oleo was the least-active compound. It seemed to stimulate SIR to some extent (not significant in pairwise comparisons to the control). Dehydrogenase activity and long-term respiration were significantly enhanced at later times. These effects indicate the good degradability of small amounts of oil (11) and its use as a carbon source.

Consistently, the magnitudes of the effects of the herbicide Goltix (metamitron), which also was used at a high dose (10×), are minor in comparison to those of Herbogil. Statistically significant inhibition of biomass-related activities (SIR at week 5 and dehydrogenase activity at weeks 1 and 8) and stimulation of long-term respiration (week 5) by this herbicide could be demonstrated only at some sampling times.

**Metabolic fingerprinting: BIOLOG.** Six replicates of pooled bacterial suspensions of each experimental variant were inoculated into the wells of BIOLOG GN microtiter plates as described in Materials and Methods. Figure 2 shows the means of the replicate BIOLOG data 5 weeks after inoculation in a pixel pattern representation of the color development after 32 h. Differences from the control are easily identified for the Herbogil treatment. A reduction in color development is predominant (Fig. 2, rows A through C in particular), and an increase may be noted in very few wells (G6 and H5). In contrast, there are fewer deviations from the control in the Oleo and Goltix results. In the Oleo wells, some stimulation of color development and metabolic activity can be observed (Fig. 2, wells A4, B2, B12, G1, G6, and G9), and color development is reduced in two wells only (A12 and C1). The Goltix results, also very similar to the control data, demonstrate minor suppressions of metabolic activities (Fig. 2, wells B4, B10, C1, E9, G2, G7, and G8) and few stimulations (wells C6 and F9).

Analysis of the carbon source utilization patterns by multivariate statistics followed an approach described recently (see Materials and Methods) (13). In pairwise comparisons, significant group differences separate the Herbogil results from the control data at all times after inoculation. The difference from the control is significant for the Goltix treatment only after 5 weeks ($P = 0.03$). Analysis of the Oleo data in general does not show a significance of grouping with the predetermined α-level ($\alpha = 0.05$). However, some tendency for differences to increase with time after application was noted ($P = 0.07$, 5 weeks after inoculation). Visualization of group differences by plotting of two PCs clearly separates the Herbogil data from the control. The data point distribution of treatment and control overlaps for the other treatments in such a diagram. This is also true for the Goltix data, where mathematical analysis by use of five PCs demonstrated significant group differences (data not shown).

The contributions of individual carbon sources to the factors describing group differences and the kinds of impacts can be quantified through factor weights (13). This analysis may also be compared with univariate tests and the pixel pattern comparison in Fig. 2. Table 2 represents the wells with high-ranking factor weights in a two-group comparison (Herbogil treatment versus control) at least once during the 8 weeks of observation. It is evident that significance in the univariate *t* tests is not a prerequisite for a high ranking of the factor weight. The first 10 ranks of factor weights 5 weeks after application are represented in the table. Except for well A8 (rank 7), all substrates contribute to differences which can also be observed in the pixel pattern representation (Fig. 2). The varying factor weights of individual carbon sources over the observation period should be noted as well. These variations include high ranking at all observation times (e.g., Table 2, well A12), and decreasing (well C6) or increasing (wells C2 and A8) weights, and significant maxima or minima at one observation time (wells H12 and A4 at 2 weeks).

**Genetic fingerprinting by TGGE.** A 16S rDNA region was selectively amplified from the molecule population extracted from the bacterial fractions as described in Materials and Methods. PCR products were separated by migration behavior

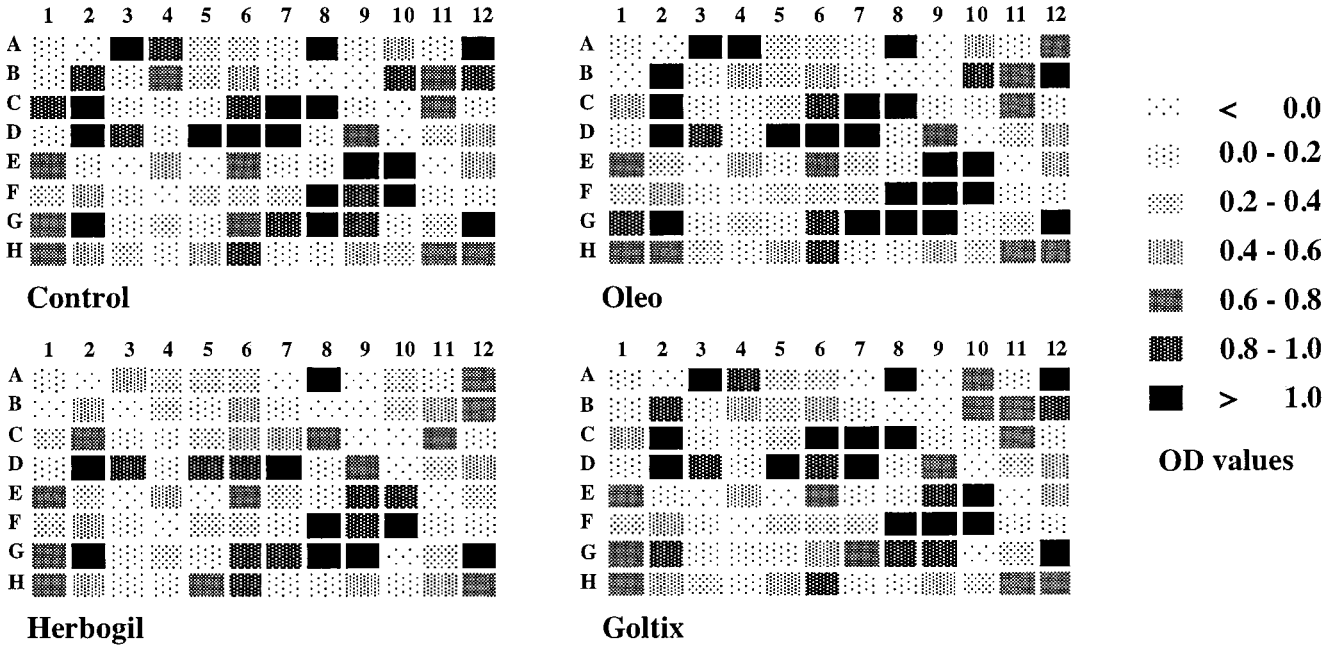


FIG. 2. Pixel pattern representation of BIOLOG community patterns. Readings of BIOLOG GN microplates, 5 weeks after inoculation with bacterial fractions of the experimental variants, are shown. The different pixel patterns represent the means of six replicates each after incubation for 32 h at 28°C, corrected by blank subtraction ($OD_0$).

in TGGE. Band patterns obtained by this sequence-dependent separation are highly reproducible, as shown by the patterns of replicates resulting from individual preparations in Fig. 3. This figure compares the molecule populations 8 weeks after applications, and apparently, the patterns are also characteristic for the experimental variants. Different patterns of the analyzed communities indicate a shift in their structural composition; in particular, such shifts are caused by the herbicide Herbogil (Fig. 3, lanes 4 to 6, in comparison with lanes 1 to 3) and the mineral oil Oleo (lanes 7 to 9). The pattern obtained from the Goltix application (Fig. 3, lanes 10 to 12) looks more similar to that of the control. Bands characterizing pattern variations by their increased abundance (clones 3 through 5 and 17 [Fig. 3 and 4]) and clone 24 [Fig. 3 and 5]) were analyzed by isolation and sequencing.

Pattern differences were analyzed and quantified by image analysis. Figure 4 shows the average OD distribution of the control pattern in comparison to the Herbogil treatment pattern. The densities of the upper gel section (Fig. 3) comprising the well-recognizable part of the patterns are represented as percentages of the total OD of the respective lanes for normalization. The calculated averages of the experimental replicates are shown. In the Herbogil pattern, increases in some bands and a novel appearance of some bands, as well as missing bands and reduced abundances, are noticeable.

The scale of differences in OD distribution can be used to characterize the different impacts of the herbicide applications. The results of the Oleo and Goltix treatments are represented as a pattern of their differences from the control in Fig. 5. Normalized averages of the treatment replicates from the same

TABLE 2. Most important carbon sources for the differentiation of the Herbogil-affected community from the control[a]

| Well | Carbon source | Factor wt (rank) at the following time after application: | | | |
|---|---|---|---|---|---|
| | | 1 wk | 2 wk | 5 wk | 8 wk |
| A3 | Dextrin | 1.8 (13)* | 5.8 (4)* | 3.7 (10)* | 3.9 (9)* |
| A4 | Glycogen | 11.4 (1)* | 2.2 (15) | 5.2 (6)* | 6.1 (4) |
| A8 | *N*-Acetyl-D-glucosamine | 0.9 (29) | 0.3 (41) | 5.0 (7) | 5.3 (5) |
| A12 | Cellobiose | 8.2 (4) | 11.6 (1) | 10.8 (1) | 6.9 (2) |
| B10 | Maltose | 3.5 (7)* | 6.4 (3)* | 3.8 (8)* | 3.6 (11)* |
| B12 | D-Mannose | 1.1 (24) | 0.6 (33) | 1.1 (25) | 6.7 (3) |
| C1 | D-Melibiose | 1.3 (20) | 2.9 (13) | 6.4 (4) | 1.9 (16) |
| C2 | β-Methyl D-glycoside | 0.5 (39) | 3.0 (12) | 8.2 (2) | 11.7 (1) |
| C6 | D-Sorbitol | 9.3 (2) | 3.8 (6) | 3.7 (9) | 4.1 (8)* |
| C7 | Sucrose | 7.0 (5)* | 3.3 (10)* | 6.0 (5)* | 5.2 (6) |
| C8 | D-Trehalose | 9.2 (3) | 9.4 (2)* | 7.3 (3)* | 4.6 (7)* |
| H12 | Glucose-6-phosphate | 0.4 (42) | 5.1 (5) | 0.8 (28) | 3.3 (13) |

[a] Selected variables (wells and carbon sources) attain at least rank 5 at one or more observation times. A factor weight is the percentage representing a carbon source's contribution to group differentiation, determined by PCA using five PCs. Each asterisk indicates a significant result of the univariate *t* test for the given variable at the given observation time ($\alpha = 0.05$).

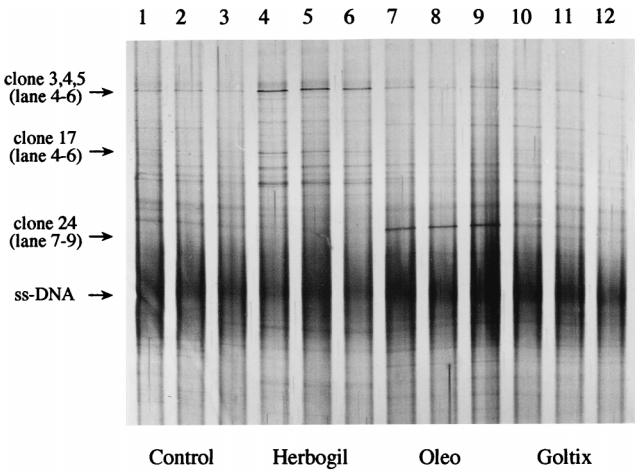


FIG. 3. TGGE community patterns 8 weeks after pesticide application. PCR-amplified 16S rDNA fragments (positions 968 to 1401) of control and challenged bacterial communities separated on a TGGE gel. Bands extracted, reamplified, and cloned for sequencing are indicated (clones 3 to 5, 17, and 24). A broad region with single-stranded DNA (ss-DNA) can be identified by its reddish color in the silver-stained gel. Lanes: 1 through 3, control; 4 through 6, Herbogil; 7 through 9, Oleo; 10 through 12, Goltix.

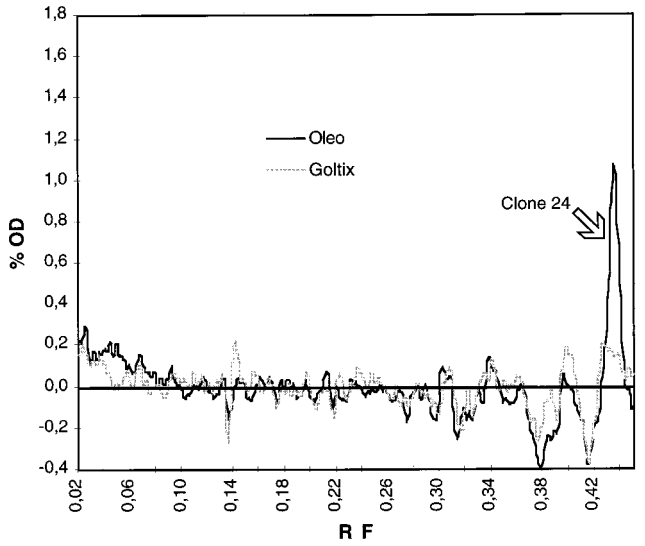


FIG. 5. TGGE pattern comparisons: impacts of Oleo and Goltix. The differences between the median OD readings of the Oleo and Goltix patterns and those of the control lanes were calculated by subtraction. The OD readings were adjusted and corrected as described for Fig. 4.

section of the gel are analyzed again. The differential spectrum indicates the general similarity of the patterns. Some noticeable differences at higher $R_f$ values, which are similar for the two treatments, might be due to imperfect correction for inhomogeneities in fragment migration. But the prominent peak in the Oleo pattern is a clearly distinct feature demonstrating the increased abundance of the fragment which was sequenced. The intensity of this peak increased from the beginning of the observation period (1 week after application) to its end (8 weeks).

By integrating the differences between control and application patterns of the analyzed gel section in absolute terms (irrespective of increase or reduction in OD values) a quantitative measure of the impact was obtained. If this measure is used for ranking, the Herbogil treatment (61% differences in total) again ranks first in such a measure of pattern modification. More than 20% of the pattern difference of the Oleo treatment (55% differences) results from the highly significant intensity of one band. The pattern modification of the Goltix treatment ranks lowest in this ordering (38%). The value is close to the variability of individual replicates (about 30%).

To investigate pattern differences in some detail, the marked bands were extracted, reamplified, cloned, and sequenced as described in Materials and Methods. The identities of cloned bands were cross-checked by TGGE separation. Homology relationships of the sequences are shown in Fig. 6. Clones 3 through 5 (435 to 379 bp) from one band of the Herbogil

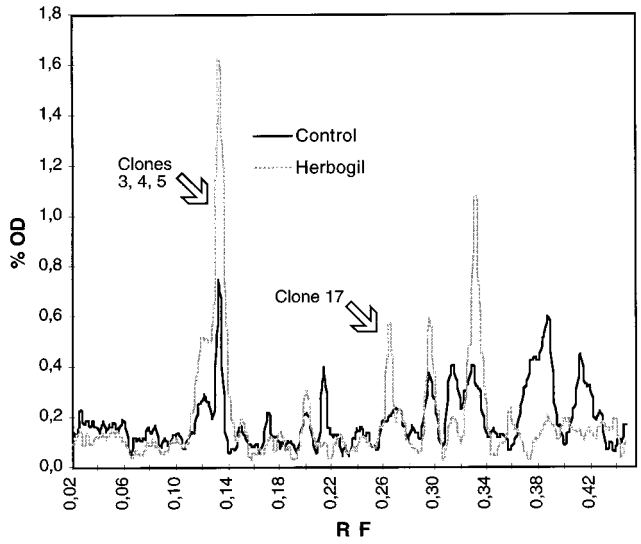


FIG. 4. TGGE pattern comparison: control versus Herbogil treatment. Data of Fig. 3 are represented as a scan of OD readings. The calculated median OD readings from the upper gel sections (approximately 40%) of replicate lanes were used. Data were adjusted by plotting the OD as a fraction of the integrated pattern intensity. The slightly different migration of corresponding bands was corrected according to visual inspection.

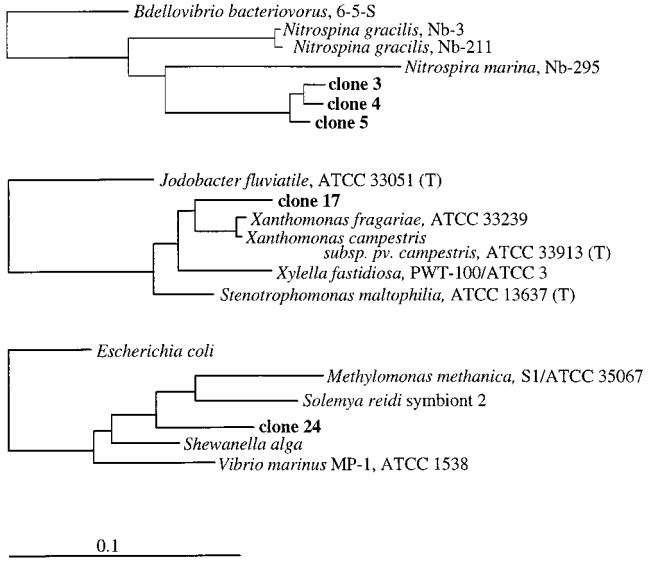


FIG. 6. Phylogenetic positions of the cut-out sequences. Clones correspond to the bands and peaks marked in Fig. 3. Bar, sequence difference of 10%.

pattern demonstrated 99% homology with each other. The sequence most similar to those of these clones in the database was that of *Nitrospina gracilis* (85 to 87%). A closer relationship to a database sequence was detected for the other clone from the Herbogil pattern. Clone 17 (350 bp) had a similarity of 97% to *Xanthomonas campestris* within the 16S region analyzed. Clone 24 (435 bp), isolated from the prominent band, characterizing the shift in the Oleo pattern, had a similarity of 92% with *Shewanella alga*.

## DISCUSSION

The results of the BIOLOG and TGGE approaches to monitoring the impact of pesticide applications on soil microbial communities demonstrated some correspondence with those of the traditional testing protocols. The latter summarize the metabolic activities of the total microbial biomass, including fungi, whereas BIOLOG and TGGE exclusively measure impacts on the bacterial communities. But the extent and significance of treatment effects were comparable. Also, the sensitivities of the novel methods proved to be similar to those of the traditional methods. However, the structure of the information about the effects of impacts is quite different. Both the BIOLOG approach and TGGE analysis provide details about changes in the diversity of functions and structural composition of the bacterial communities.

In contrast to glucose-induced respiration or dehydrogenase activity analysis, the BIOLOG system tests the stimulation or reduction of the catabolism of 95 substrates simultaneously. BIOLOG results may be checked for effects on individual substrate uses. Observations were categorized here with respect to the significance of the impact by multivariate statistics, which also indicates the relative contributions of single variables (substrates) to group differentiation by factor weights. Independent experimental replicates, as used in our analysis, are required to assess group variability.

Corresponding to the reduction of biomass-dependent parameters in the traditional tests, the Herbogil application was the only stressor that consistently demonstrated an inhibiting effect on catabolic potentials at all sampling times. The time-dependent dynamics of this stress reaction can only be inferred from the varying scaling of the effect in the traditional tests, e.g., as observed for nitrogen mineralization (significant at the first two samplings) or long-term respiration (significant after 5 and 8 weeks), which indicates the severe impact on microbial activity (Fig. 1). The varying factor weights in the BIOLOG test represent more dimensions of these dynamics (Table 2). A time course of responses may also be inferred from the divergent significances of sum parameters and BIOLOG test results after applications of Oleo and Goltix (see Results), although all methods are close to their detection limits with these applications.

By monitoring the oxidative catabolism of readily degradable substrates, the signal generation of the BIOLOG system provides a limited observation window. It is biased in that it favors gram-negative over gram-positive bacteria (16). Signal generation depends on the physiological states of inoculated cells as well as on their—selective—growth. It affords a detailed and sensitive view of the functional spectrum of the community and of induced changes. The indirect and summarizing nature of BIOLOG data, however, might generally preclude an interpretation with respect to details of cause-and-effect mechanisms in community functionality, e.g., by relating the BIOLOG data to the degradation of the herbicide in our case. With respect to an evaluation of ecological significance, any reduction in the spectrum of catabolic functions indicated by the test system might be considered an unfavorable effect. Depending on the knowledge about functions in a particular habitat, the approach of the system may be modified to include more specific endpoints by the use of alternative or additional carbon sources and signal generation conditions. Also, the sensitivity of data analysis might be improved by alleviating the restriction of the statistical data analysis to color development at a single reading time (13).

Taking the TGGE pattern as a representation of species abundance, the Herbogil application results in a pronounced repression of a variety of species (bands), which is compensated for by an increased abundance in, or novel appearance of, others. We have no straightforward interpretation concerning the correspondence between this modified diversity, as analyzed by the TGGE pattern, and the results of the BIOLOG analysis. The BIOLOG data demonstrate a predominant repression of catabolic potentials only. The impact of Oleo is most clearly characterized by TGGE analysis, demonstrating the time course-dependent significant increase in the intensity of one band (Fig. 3). This is an unequivocal response in comparison with the results of the other tests. The observations indicate a significant change in species frequency distribution with a particular impact on its evenness, in both cases. The reproduction of independent replicates from the Goltix application did not allow for the clear identification of pattern differences in the TGGE analysis, indicating the limits of its present sensitivity, which might be improved, e.g., by choosing rRNA as target molecule for amplification. The ranking of quantified pattern changes resulted in a fair correspondence to the other test results, again identifying the application of Herbogil as having the most significant impact.

The observation window provided by TGGE analysis is also distorted and biased. The assignment of one band to one species is a simplified approximation. Heterogeneities of rRNA genes may result in divergent bands for a particular bacterial species (26). Also, nonidentical sequences may be found at an identical position by chance. Other biases are introduced by the different numbers of 16S rRNA genes per microorganism genome (8, 9) and by the details of the PCR, including primer specificities and elongation. Only the most abundant species of very complex communities will contribute to a pattern. The isolation method for target nucleic acid sequences is also important. For example, our technique of freeze-thawing bacterial cells is somehow selective because of difficulties in disrupting spores and microcells. However, different biases are shared by other more labor-intensive methods—and the biases of TGGE analysis are partly compensated for by the advantage of an approach relying on pattern comparisons. The TGGE approach can be modified for more-targeted investigations, too. Primer selection for a representation of single bacterial groups (17) or a modification of target sequence preparations are only two possibilities here.

Pattern variations were analyzed in more detail by investigating the phylogenetic relationships of responsive bands (Fig. 3 through 6). The weak similarity (85 to 87%) of clones 3 through 5 to *N. gracilis* somehow may correspond to the nitrogen mineralization data, since several organisms in this phylogenetic branch are described as nitrifying bacteria from marine and terrestrial habitats. Clone 17 may originate from a plant-pathogenic organism, as suggested by its high level of similarity (97%) to *X. campestris* ATCC 33913 (Fig. 5). The phylogenetic classification of the sequence from the characteristic band of the Oleo treatment (clone 24) shows a similarity of 92% to the sequence of *S. alga*. The fact that members of the genus *Shewanella* previously were isolated from soluble oil emulsions (30) somehow corresponds to this result. But in general, an interpretation of the results in terms of correlation to causal mechanisms of the impacts of the herbicides on community composition remains speculative, stressing the notion that a knowledge of increasing details of community varia-

tions may not automatically provide the potential for any but superficial interpretations. Clearly, the findings suggest further investigations into more details. For example, the observation of an increased abundance of sequences related to plant-pathogenic organisms might trigger more-specific testing for soil-borne pathogens.

The novel approaches represent a valuable complement to traditional test systems intended to obtain information of ecotoxicological significance from particular exposure to chemicals. They may contribute to a better understanding of induced changes in microbial communities, which normally will react to any change in "environmental" conditions. The intensity, character, and dynamics of changes are important factors for evaluation. Quantification, as demonstrated here, may be further studied by establishing dose-response relationships. To describe the "healthy" state of microbial communities in soil, including their natural variability, in order to define unfavorable changes, however, remains a scientific challenge.

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