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The effects of pesticides on the diversity of culturable soil bacteria

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P.S. NICHOLSON AND P.R. HIRSCH. 1998. The numbers of culturable soil bacteria in plots that had received either no pesticides or the full combination (aldicarb, chlorfenvinphos, benomyl, glyphosate, plus chlorotoluron or triadimefon) over a 20 year period were compared. Differences were very small although there were consistently higher numbers on the treated plot, possibly reflecting the greater crop yields which had been reported previously. There was no significant difference in numbers of bacterial colonies with homology to a *nif* gene probe in soils from the two plots. Genetic fingerprinting of *Pseudomonas fluorescens* isolates from the plots, using ERIC-PCR, showed that the dominant strains in the two populations were not the same although there was no obvious difference in the degree of diversity. Substrate utilization by microbial populations from the two plots was compared using Biolog plates. The population from the pesticide-treated plot showed a higher rate of substrate utilization which could reflect a slightly higher inoculum of heterotrophic bacteria, but could also indicate greater metabolic diversity in the population.

INTRODUCTION

The ability of soil microbes to degrade complex substrates is well known. In addition to decomposition of plant residues, degradation of more unusual compounds arising from both industrial pollution and the deliberate application of agrochemicals, particularly pesticides, has been well documented (Chakrabarty *et al.* 1982). There has been concern that build-up of pesticide residues in soil could be deleterious to certain soil microbes and consequently damage soil fertility. However, it has been observed that some soil-applied pesticides become less efficacious after several years continued application, due to accelerated breakdown mediated by soil microbes which appear to become adapted to the novel substrate (Munnecke *et al.* 1982). It is not clear which groups of microbes and whether single strains or consortia are involved. Soil microbial populations are large and diverse; fertile agricultural soils at Rothamsted contain more than 10^9 bacterial cells g^{-1} estimated by direct counts on soil, although only 1–10% of these are culturable (Skinner *et al.* 1952). The relative numbers of the most prevalent isolates in culturable populations are subject to change, but the relevance of such shifts is unclear.

In this paper, the effects on the diversity of the culturable soil bacterial populations of long-term pesticide applications to field plots at Rothamsted were investigated. These 'Chemical Reference Plots' have received combinations of up to five pesticides annually between 1974 and 1993, with spring barley grown each year. The pesticides were: aldicarb (insecticide/nematicide); chlorfenvinphos (insecticide); benomyl (fungicide); glyphosate (herbicide, applied 1980 onwards) and chlorotoluron (herbicide, applied 1974 and 1976 only) or triadimefon (applied 1982 onwards). Details of these experiments (a fully randomized, single replicate 2^5 factorial design), their effects on crop yields and soil fertility have been reported recently (Bromilow *et al.* 1996). There were no deleterious effects of the pesticides on crop yield or soil processes although aldicarb improved yields significantly until the last 4 years of the experiment. No residues of any of the pesticides could be detected in soil samples taken 17–22 months after the last application, indicating that no accumulation had occurred. Previously, both aldicarb (Smelt *et al.* 1987) and chlorfenvinphos (Rouchaud *et al.* 1989) have been reported to be subject to accelerated breakdown by soil microbes. However, in the Chemical Reference Plots experiment, only aldicarb appeared to be degraded at a sig-

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nificantly greater rate in treated soils compared with those with no history of aldicarb treatment. This was observed in samples taken 17 months after the last application of aldicarb (Bromilow *et al.* 1996).

Pesticide effects on the size and activity of the total soil microbial population were investigated in 1992. Overall, aldicarb-treated plots had slightly higher microbial biomass carbon contents but showed no significant difference in the rate of mineralization of soil organic carbon or nitrogen (Hart and Brookes 1997).

The aim of this study was to investigate the effect of long-term pesticide applications on soil bacterial populations. Numbers of culturable bacteria on different media were compared, functional diversity was investigated using Biolog substrate utilization assays (Garland and Mills 1991) and the genetic diversity of pseudomonad populations was compared by rep-polymerase chain reaction (PCR) genetic fingerprinting (de Bruin 1992).

MATERIALS AND METHODS

Soil samples

Soil was collected, in August 1994 and February 1995, from two plots: Plot 4 which had received all pesticides (pesticide plot) and Plot 24 which had received none (control plot). Samples were taken randomly from the top 20 cm of soil in 10–20 positions across a plot, mixed and sieved (3 mm mesh) without drying and stored at 4 °C overnight before the first plating, and for several months for subsequent platings. This sampling strategy was devised to overcome the effects of spatial heterogeneity in soil while avoiding the need for simultaneous processing of many replicate samples. Soil moisture contents were assessed by drying 20 g samples for 24 h at 80 °C. Dry matter in both soils sampled in August was 88%; in February it was 83%. All results are expressed on an oven-dry soil basis.

Microbiological methods

Aseptic techniques were used for all subsequent procedures involving the soil samples. To decrease sampling errors, relatively large samples were used; 12 g moist soil (10 g dry weight) was resuspended in 100 ml sodium hexametaphosphate (2%), shaken for 30 min, diluted in sterile water and 100 μl aliquots spread. At least five replicate plates were inoculated at each dilution, and incubated at 21 °C for 4–5 d before counting, which precluded slower-growing microorganisms such as actinomycetes. Bacterial fluorescence was examined by illuminating colonies on PSA agar under u.v. light at 260 nm. Identification using API 20NE kits (bioMérieux, Marcy l'Etoile, France) was performed according to the manufacturer's instructions.

Media

Nutrient Agar (NA; Oxoid) and Tryptone Soy Agar (TSA; Difco) were used to enumerate fast-growing heterotrophic bacteria (TSA at the second sampling time only). Pseudomonas Agar Base (PSA) with CFC selective supplement (Oxoid) was used to select pseudomonads; SY, a non-selective minimal medium for rhizobia (Beringer 1974), was used for putative nitrogen fixers. It contains (l^{-1}): 0·1 g $MgSO_4.7H_2O$, 0·22 g $CaCl_2.6H_2O$, 0·22 g K_2HPO_4, 0·02 g $FeCl_3$, 1·5% agar; 1·1 g sodium glutamate, 135 mg sodium succinate, 0·75 mg biotin, 0·75 mg thiamine and 0·75 mg pantothenic acid were added after autoclaving. To inhibit fungal growth, 100 mg l^{-1} cycloheximide and 7·5 mg l^{-1} benomyl was added before pouring the agar.

Colony lifts

Transfer of colonies from plates to nylon membrane filters (Boehringer Mannheim) and subsequent DNA release from cells was performed as described previously (Hirsch 1995). These colony lifts were carried out from the 10^{-3} dilution of soil plated onto SY media. Filters were baked at 120 °C for 30 min to fix the DNA. Before hybridization, they were washed in 2 × SSC, 0·1% SDS at 68 °C for 1 h (2 × SSC is 0·3 mol l^{-1} NaCl, 0·03 mol l^{-1} sodium citrate). Hybridization was carried out according to the manufacturer's protocols for the DIG/CSPD chemiluminescent detection system (Boehringer Mannheim) using a 2·45 kb *nif* gene probe (Hirsch *et al.* 1993) labelled by random priming.

PCR fingerprinting

To extract DNA from colonies, growth from PSA plates was taken with a sterile tip and resuspended in 50 μl aliquots of lysis buffer (0·05 mol l^{-1} NaOH, 0·25% SDS) which were heated for 15 min at 95 °C, cooled on ice and centrifuged for 5 min at 12 000 *g* in a Microfuge (MSE). Aliquots of this supernatant fluid (20–30 μl) were removed, stored at −20 °C and then diluted 1/10 with water before use. Primers for ERIC PCR were as described by Verlasovic *et al.* (1991), but with ERIC1R replaced by a reversed sequence, R1CIRE, which had previously been found to improve results (Arora *et al.* 1996):

R1CIRE: 5′-CACTTAGGGGTCCTCGAATGTA-3′

ERIC2: 5′-AAGTAAGTGACTGGGGTGAGCG-3′

The PCR reaction mix contained: 17·3 μl sterile water, 2·5 μl 10 × buffer (Boehringer Mannheim), 0·5 μl (200 μmol) of each nucleotide, 0·2 μl (1 U) Taq polymerase (Boehringer Mannheim), 1 μl (50 pmol) of each primer and 1 μl DNA. The conditions for PCR were: initial denaturation for 3 min at 95 °C; 30 cycles for 1 min at 94 °C, 1 min at 52 °C, 8 min

at 72 °C, final extension for 16 min at 72 °C. Products were visualized when 10 μl reaction mix was run on a 1·5% agarose gel, stained in ethidium bromide and illuminated under u.v. light.

Substrate utilization assays

Soil (12 g; 10 g dry weight) was gently suspended in 88 ml quarter-strength Ringer solution (Oxoid) to 100 ml final volume using a Spiramix roller for 3 h at 21 °C, then diluted to 10^{-4} in quarter-strength Ringer solution. This 10^{-4} suspension was used to inoculate triplicate GN MicroPlate™ plates (Biolog Inc., Hayward, USA), which allow simultaneous testing of 95 separate carbon sources, the oxidation of which is determined by colorimetrically measuring tetrazolium dye reduction. The plates were wrapped in clingfilm to reduce evaporation and incubated at 21 °C. Plates were scanned immediately after inoculation and then at intervals over the next 7 d (21, 27, 45, 51, 69, 72, 94 and 189 h) using a Titertek Multiscan MCC/340 MKII at 540 nm.

RESULTS

Viable counts

Figure 1 shows that at each sampling time there were either slightly more culturable bacteria or no significant differences compared with the control plot, when soil from the pesticide plot was plated on TSA, PSA and NA. The numbers of colonies on SY agar with homology to the *nif* probe were not significantly different. The largest variation observed overall was a fourfold difference in PSA counts from the control plot soil sampled in August, compared with February. The greatest difference between the two treatments at any one time was the twofold difference in PSA counts observed at the August sampling. A similar range of variation between the lowest and highest plate counts in samples taken on seven different dates from Rothamsted soil has been recorded previously (Skinner *et al.* 1952).

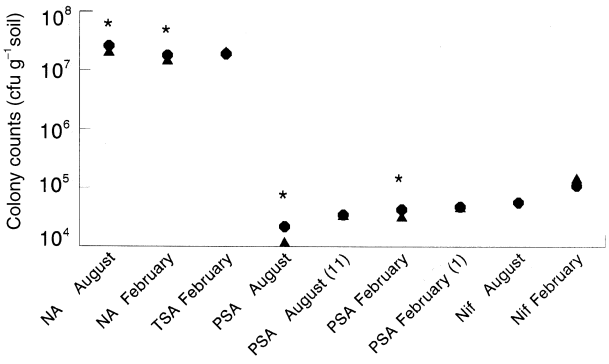

Fig. 1 Comparison of viable counts on different media. Pairwise comparisons of counts on the different media are shown, standard errors (not shown as error bars would be obscured by symbols) were calculated from at least four replicate plates. *Denotes pairs where difference between means was greater than either mean ± S.E. NA, nutrient agar; TSA, tryptone-soy agar; PSA, fluorescent pseudomonad-selective agar; Nif, colonies from SY agar with homology to a *nif* DNA probe. Initial sampling date of soil is given (number of weeks stored at 4 °C before plating in parenthesis). No TSA count was made from the August sampling

Since pseudomonads are particularly important rhizosphere colonizers in arable agriculture, they were the main focus of the studies on population diversity. The PSA plates from soil collected in August were viewed under u.v. light to identify fluorescent pseudomonads; in the pesticide plot, 8% of the colonies were fluorescent compared with 27% in the control plot. On TSA, 10% of colonies from the pesticide plot were pigmented compared with 19% from the control plot. On NA, there were no obvious differences in colony morphology. Colony morphologies were noted for the pseudomonads (on PSA), and isolates with distinctly different genetic profiles generated by ERIC-PCR (see below) were subcultured for API 20NE identification. Despite the variety of colony types, all of the isolates were identified as *Pseudomonas fluorescens*. The isolates from pesticide Plot 4 were generally flat matt colonies whereas those from the control plot were predominantly of a very wet, mucoid type. Single plates from each soil dilution series were selected for further study and every colony (approximately 30–40) from these plates was subcultured for PCR fingerprinting. When the soil collected in February was plated, fluorescent colonies represented 1% and 7% of isolates in the pesticide and control plots, respectively. All colonies from two plates in each soil dilution series were subcultured for DNA extraction, colony descriptions and subsequent storage at −20 °C. Colonies were taken from two plates of each soil on this occasion to assess plate-to-plate variation which would affect diversity studies. However, there were no obvious differences in the distribution of colonies between each pair of plates.

ERIC-PCR fingerprinting

From the August sampling, 32 colonies from the pesticide plot and 27 colonies from the control plot were picked. The colonies were plated out again on the same medium, DNA was extracted and full descriptions made for single colonies, i.e. pigmentation, size and appearance. In both soils, mucoid colonies accounted for 50% of isolates. The DNA from the isolates was used in PCR with ERIC primers. The PCR products were grouped together on the gel according to colony types of the original isolates. The PCR produced a large number of profiles (Fig. 2). Isolates which appeared identical on the selective media were often quite different in ERIC-

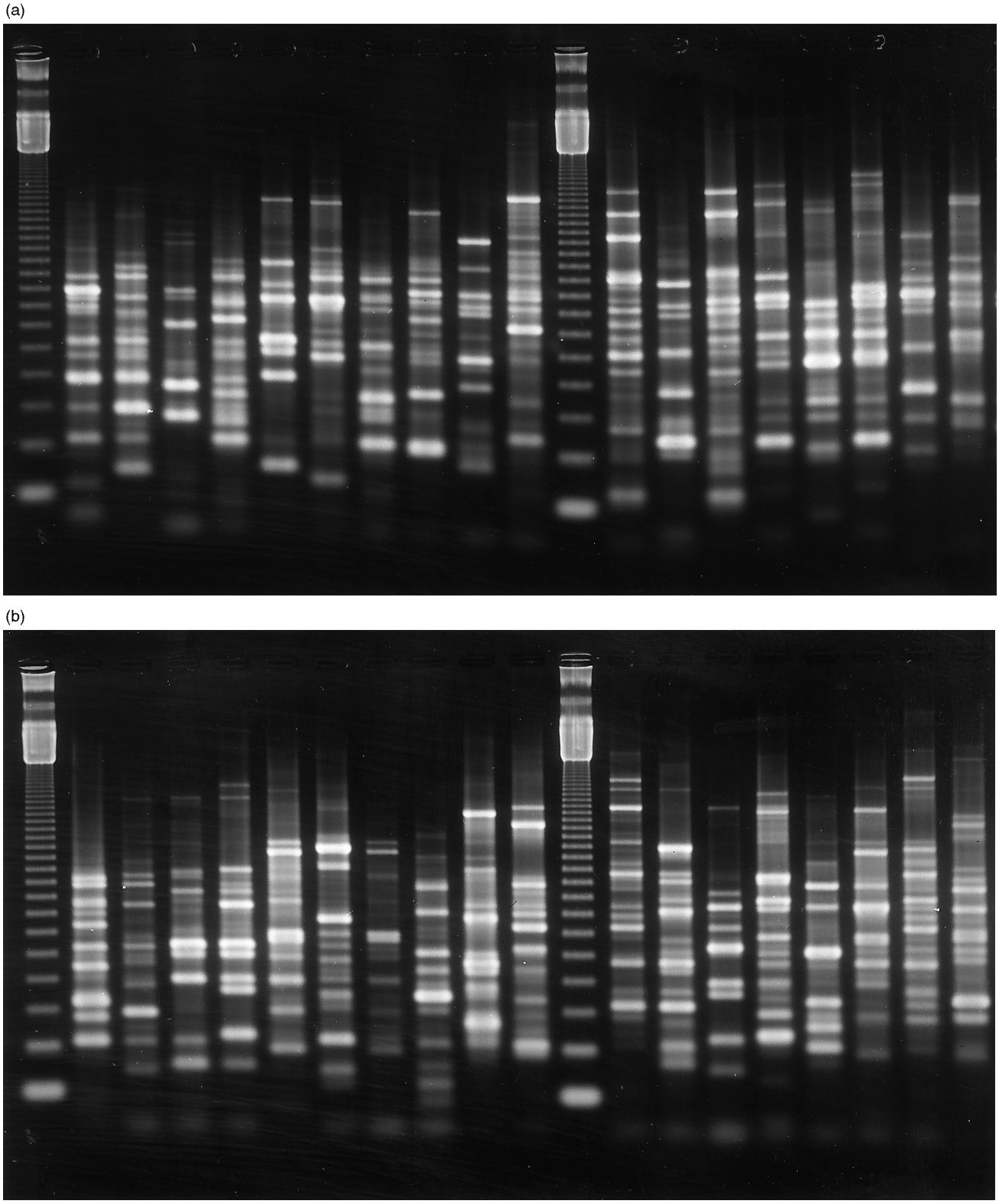

Fig. 2 Representative ERIC profiles. (a) Pesticide-treated soil; (b) control soil. First and 12th tracks from the left on each gel contain size markers which increase in multiples of 123 bp

PCR profile, particularly the mucoid isolates. The pesticide plot soil contained 21 different profiles compared with 17 in the control plot, with only seven profiles common to both soils. Also, of the three dominant profiles in the control plot, one was absent in isolates from the pesticide plot and the other two profiles were represented by single isolates. To confirm similarity, it was necessary to compare isolates on the same gel, which prevented comparison of large numbers of profiles and made comparison between profiles from the first and second sampling impractical.

At the February sampling time, mucoid colonies represented 60% of the pesticide plot isolates and 65% from the control plot. As before, colonies which appeared morphologically similar yielded very different DNA profiles. The pesticide-treated and control soils contained 16 and 19 different profiles, respectively; only six profiles were common to both. The dominant profile in the pesticide plot, represented by eight isolates, was not present in the control. Of the dominant profiles in the control, one was not present and the other was represented by a single isolate in the pesticide plot. The results are summarized in Table 1.

Substrate utilization

The functional diversity of the microbial communities in the two soils sampled in February was examined using Biolog plates, which allow the rate of utilization of 95 separate carbon sources to be compared. Colony counts indicated only very small differences (less than twofold) in the numbers of culturable bacteria. No substrate usage was recorded in any plate until the fourth sampling time, 45 h after inoculation. Not all 95 possible substrates had been used by the end of the experiment at 189 h; nine wells with pesticide plot soil had absorbance readings of less than 0·3, and with control soil the number was 17 (these included the same nine as the pesticide plot). The plates inoculated with pesticide-treated soil also used substrates at a greater rate than the control. Average absorbance readings for each plate were examined by analysis of variance. This showed highly significant differences between soil samples at all times with no significant differences between replicates overall (Table 2). Initial examination of the results, comparing utilization of all 95 substrates, indicated that the difference between the two soils was greatest 94 h after inoculation. However, there was increased variation between replicates of each substrate by this time. The 10 most important substrates with the greatest significant difference at 45 h are illustrated in Fig. 3a; by 94 h, these differences were either reduced or no longer apparent (Fig. 3b).

DISCUSSION

The microbial population in the pesticide plot soil appeared to have more rapid and more varied metabolic activity than

Table 1 Microbial population diversity on pesticide-treated and control plots

	August 1994 sampling										February 1995 sampling									
			Isolates per profile shown (% total profiles)										Isolates per profile shown (% total profiles)							
Treatment	Isolates tested	No. ERIC profiles	8	7	6	5	4	3	2	1	Isolates tested	No. ERIC profiles	8	7	6	5	4	3	2	1
Pesticides*	35	21	0	1 (5)	0	0	0	0	5 (24)	15 (71)	42	16	2 (12)	0	0	0	1 (6)	3 (19)	3 (19)	7 (44)
Control	34	17	0	0	0	1 (6)	2 (12)	0	0	14 (82)	43	19	1 (5)	0	0	0	1 (5)	4 (21)	6 (32)	7 (37)

The number of isolates sharing the same profiles are shown for soil samples taken in August and February, on the pesticide-treated and control plots (i.e. 15 profiles are represented by single isolates, five profiles are each represented by two isolates and one profile is represented by seven isolates in the August-sampled pesticide plot soil).
*Aldicarb, benomyl, chlorfenvinphos, glyphosate, chlorotoluron (two applications only) or triadimefon (from 1982 onwards).

Table 2 Analysis of variance of substrate utilization assay absorbance readings

	Time (h)					
	45	51	69	72	94	189
Pesticide plot mean	0·5281	0·6613	1·0160	1·0554	1·3390	1·7860
Control plot mean	0·4468	0·5898	0·9190	0·9587	1·2070	1·6476
S.E.D.	0·00730	0·01416	0·02010	0·01720	0·02460	0·01372
P	<0·001	0·007	0·008	0·005	0·005	<0·001

Absorbance readings at 540 nm from all 95 wells in each of three replicates inoculated with soil from the pesticide and control plots, from 45 h onwards, were included.

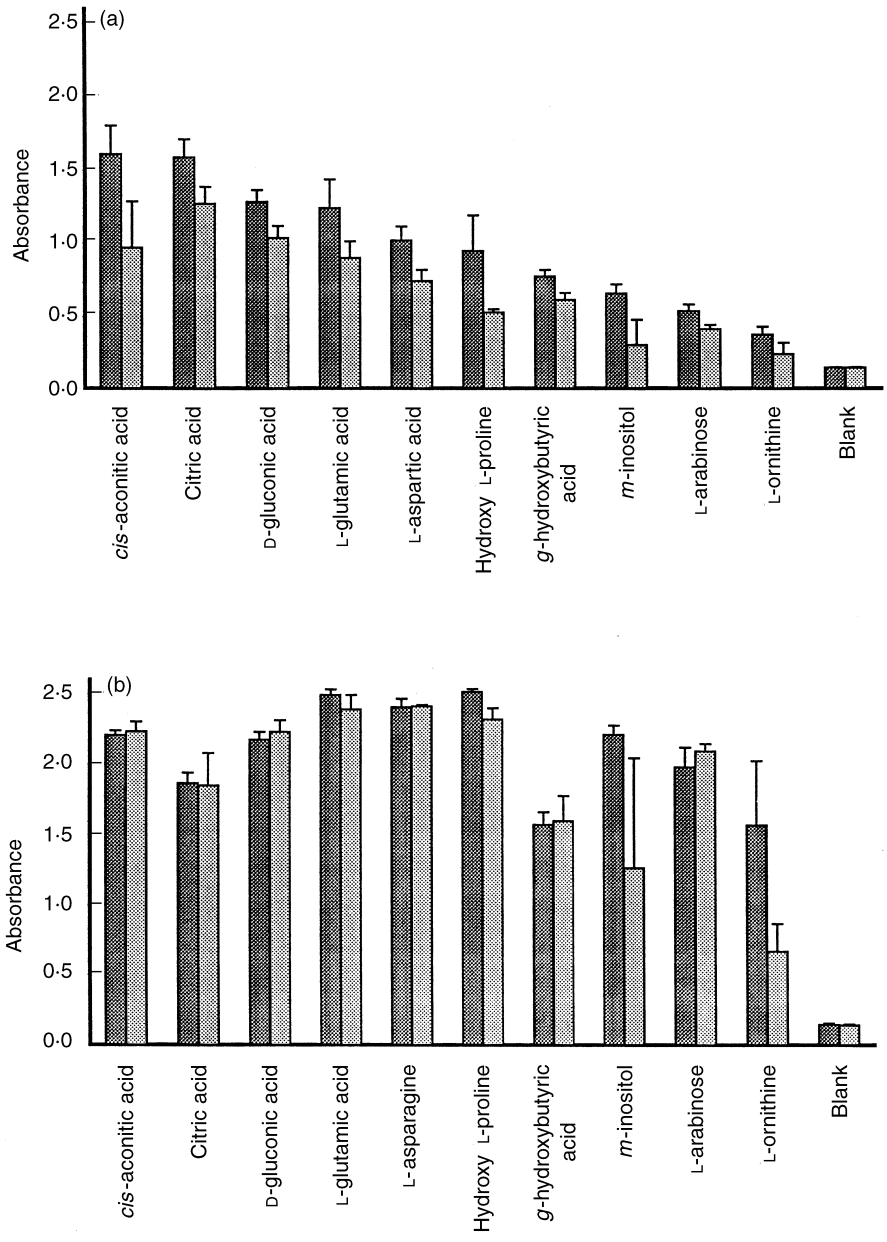

Fig. 3 Biolog substrate utilization. (a) The 10 substrates showing the largest significant differences between pesticide-treated and control soils; (b) the same substrates at 94 h, where use of hydroxy L-proline, m-inositol and L-ornithine are still significantly different. ■, Pesticide plot; □, control plot

the control, which could reflect the slightly higher inoculum size. Since Biolog substrate utilization in plates inoculated with soil dilutions almost certainly involves growth of fast-growing heterotrophic bacteria, particularly *Pseudomonas* spp., in the wells (K. Smalla, personal communication), a sample with a larger initial population of these bacteria would be expected to show a positive result more quickly and appear to use more substrates especially at the earlier time points. One difference between the two soils is the presence in the pesticide-treated soil of a population able to degrade aldicarb (Bromilow *et al.* 1996). However, a relatively small population of aldicarb degraders would be sufficient for a soil to exhibit accelerated degradation, and it is unlikely that any enzymes involved in catabolism of the Biolog substrates used more rapidly by the pesticide-treated soil would be involved; microbial degradation of aldicarb appears to involve hydrolysis of the carbamate linkage, mediated by an *N*-methylcarbamate hydrolase (Mulbry and Eaton 1991).

Although differences in numbers of culturable heterotrophic bacteria in soil from the two plots were very small, there were consistently more in the pesticide plot soil. This could reflect the higher crop yields (especially in the early years of the experiment) in the pesticide plot, attributed to the insecticidal and nematicidal activity of aldicarb protecting the barley from attack by pests (Bromilow *et al.* 1996). The mean annual yield of spring barley from 1974 to 1993 in the pesticide plot was 5·3 t ha^{-1} (S.E. 0·148) compared with 4·92 t ha^{-1} (S.E. 0·148) in the control plot (R. Bromilow and R. Todd, personal communication). For a definitive result, considerably more samples taken at many time points from sufficient numbers of replicated treatments would be needed to overcome the natural heterogeneity of soil; unfortunately this is not practicable.

Populations of root-colonizing soil bacteria are influenced by plants; both physiological activity and genetic fingerprints of fluorescent pseudomonads isolated from rhizospheres of tomato and flax grown in the same soil were shown to differ (Lemanceau *et al.* 1995). Similarly, the rhizosphere populations of wheat plants grown with different levels of N fertilizer, which affects plant growth and consequently the amount and possibly the composition of root exudate, were found to differ (G. Ross and P. Hirsch, unpublished observations). Over time, with continued experimental treatment, differences in the rhizosphere might be expected to be reflected in bacterial populations in bulk soil.

It is difficult to compare the diversity of the pseudomonads isolated from the two soils. There were obviously both phenotypic and genotypic differences, the former reflected by colony appearance (more mucoid, pigmented and fluorescent colonies in the control soil) and the latter by ERIC profiles. A significant finding was that colonies with similar phenotypes often had completely different genotypes. Difficulties in accurate comparisons of large numbers of profiles, and in comparing results within one plot on two different sampling occasions, compound this problem. Better methods for comparing PCR fingerprints of strains are essential for future screening of large numbers of isolates.

In conclusion, our results indicate that long-term treatment of soils with pesticides may have affected the size of the soil microbial population but it is uncertain whether such a small difference would have any effect on soil fertility. The composition of the microbial populations, based on genotype (ERIC-PCR), phenotype (colony morphology) and activity (substrate utilization) is different in the pesticide-treated and control soils. However, further work is needed to clarify whether this is due to the effects of pesticides, rather than the heterogeneity of soil itself.

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