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ORIGINAL ARTICLE

Survival of genetically marked *Escherichia coli* O157:H7 in soil as affected by soil microbial community shifts

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A loamy sand soil sampled from a species-rich permanent grassland at a long-term experimental site (Wildekamp, Bennekom, The Netherlands) was used to construct soil microcosms in which the microbial community compositions had been modified by fumigation at different intensities (depths). As expected, increasing depth of fumigation was shown to result in progressively increasing effects on the microbiological soil parameters, as determined by cultivation-based as well as cultivation-independent (PCR-DGGE, PLFA) methods. Both at 7 and at 60 days after fumigation, shifts in the bacterial, fungal and protozoan communities were noted, indicating that altered community compositions had emerged following a transition phase. At the level of bacteria culturable on plates, an increase of the prevalence of bacterial r-strategists was noted at 7 days followed by a decline at 60 days, which also hinted at the effectiveness of the fumigation treatments. The survival of a non-toxigenic *Escherichia coli* O157:H7 derivative, strain T, was then assessed over 60 days in these microcosms, using detection via colony forming units counts as well as via PCR-DGGE. Both data sets were consistent with each other. Thus, a clear effect of fumigation depth on the survival of the invading strain T was noted, as a progressive increase of depth coincided with a progressively enhanced inoculant survival rate. As fumigation depth was presumably inversely related to community complexity, this was consistent with the hypothesis that soil systems with reduced biological complexity offer enhanced opportunities for invading microbial species to establish and persist. The significance of this finding is discussed in the light of the ongoing discussion about the complexity–invasiveness relationship within microbial communities, in particular regarding the opportunities of pathogens to persist.

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Introduction

Escherichia coli O157:H7, a severe pathogen of humans and animals, has often been found involved in cases of very severe food poisening. The pathogen may infect humans from environmental sources such as food sources grown in manured soils or in soils treated with contaminated waters. Following invasion, the organism can cause death in elderly and/or immunocompromised people (Ritchie *et al.*, 2003). In 2002, *E. coli* O157:H7 was placed on the list of potential bioterrorist (BT) agents by the US government, followed by a range of nations and international bodies, and it hence deserves extra attention with respect to its risk and hazard in the environment. One major issue, when considering the risks associated with this organism in the environment, is its actual survival in open environments, for example, soils and sediments (Jiang *et al.*, 2002). Several experiments on *E. coli* survival have been performed before (Kudva *et al.*, 1998; Jiang *et al.*, 2002; Ritchie *et al.*, 2003; Topp *et al.*, 2003), and, in general terms, the population size of the organism in soil and soil-related (manure) habitats was shown to progressively decline in most of the cases. Also, evidence was found for strain- as well as habitat-specific variability in the decline rates

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(Kudva *et al.*, 1998; Ritchie *et al.*, 2003; Topp *et al.*, 2003). However, we still lack a sound understanding of the biotic factors in the recipient environment that govern the persistence of this organism, including the effects of the diversity and community composition of the microbial communities in these ecosystems.

In macroecology, it has long been surmised that ecosystems that contain a higher level of biodiversity are less amenable ('vulnerable') to disturbances, such as those brought about by biological invasions, than ecosystems that have a lower diversity (Tilman, 1997). Theories about the invasibility of ecosystems by microorganisms have been dominated by the now classical concept of microbiostasis, which, in simple terms, dictates that most natural ecosystems commonly have 'filled' ecological niches and are therefore difficult to penetrate. How the degree of microbiostasis relates to microbial community structure and complexity is still a largely unanswered question, although recent data in microbiology may provide support for the above contention from macroecology. Thus, a recent study tested the possible relationship between microbial complexity and invasibility with respect to *Pseudomonas aeruginosa* invading the rhizosphere of wheat; indeed, an inverse relationship was found between the survival rate of the inoculant and the extent of microbial diversity (Matos *et al.*, 2005). In these experiments, the microbial diversities and community structures were modified by using a dilution–reinoculation approach. In another recent study in artificially established microcosms (Irikiin *et al.*, 2006), the chances of *Ralstonia solanacearum* biovar 2 to cause disease in tomato, and thus to presumably establish, were also found to be inversely related to the complexity of the bacterial communities present. However, both studies involved the rhizosphere, which represents a very particular environment in soil. We have no clues whatsoever, which would indicate that a similar phenomenon might be applicable for microbial communities in the bulk soil.

In this paper, we describe experiments on the survival of an *E. coli* O157:H7 derivative strain, denoted T, in soil with different community compositions and diversities, established on the basis of fumigations of different intensities (depths). Our working hypothesis was indeed that the microbial community compositions in the experimental systems would be modified by fumigation at varying intensity, resulting in changed (reduced) microbial diversities, and that the survival of the inoculant organism would show an inverse relationship with the level of modification/presumed diversity.

Materials and methods

Soil

The soil used (denoted W) was a loamy sand soil obtained from permanent species-rich grassland (pH 5.2–5.5, 2.5% organic matter) at Wildekamp, Bennekom, The Netherlands. This soil has been extensively described by Garbeva *et al.* (2003, 2005). We selected this soil as it contained a high microbial diversity as well as a high level of phytopathogen suppressiveness, which relates to microbiostasis (Garbeva *et al.*, 2004, 2005). Shortly before the onset of the experiments, soil was freshly sampled from the permanent grassland plot (treatment G; Garbeva *et al.*, 2004) by inserting an approximately 5-cm diameter cork borer vertically into the soil and removing the top 10–15 cm just below the grass. Three subplots of roughly 2×2 m were sampled and at least 20 randomly chosen replicate cores per plot were used to yield one composite sample per plot. Following sampling, the soil was used within 2 weeks to set up soil microcosms with modified microbial communities. Soil was manually cured from root parts and stones before experimentation.

Soil fumigation

The W soil, at 60% of its water holding capacity (50 g), was placed in replicate 250-ml presterilized cotton-stoppered Erlenmeyer flasks to establish a suite of soil microcosms with modified microbial communities. Then, different depths of fumigation were applied, notably 0 h (no fumigation), 0.5 h (low-depth), 2 h (intermediate-depth) and 24 h (high-depth) fumigation, in accordance with Griffiths *et al.* (2000). Following fumigation, the microcosms thus established were kept at 20°C (85% air humidity) for a total of 60 days. Evaporation of water was checked weekly and water was replenished when needed. The microcosms were sampled after 7 and 60 days to assess the changes in the microbial communities.

Bacterial strain and cultivation

Escherichia coli O157:H7 derivative strain Tn*5 luxCDABE* (hereafter denoted strain T), described by Ritchie *et al.* (2003), was kindly furnished by Professor K Killham, University of Aberdeen, Scotland, UK. This strain is a rifampicin-resistant non-toxigenic derivate of strain O157:H7, which carries a transposon Tn*5*:*luxCDABE* construct, allowing its easy detection from soil by plating on selective media, notably rifampicin (chromosomal mutation) and kanamycin (resistance conferred by the *nptII* gene carried by the Tn*5* construct). The strain has been shown to retain its growth rate as well as the key O157, H7 and intimin phenotypes. It also survived in soil in a manner indistinguishable from its parent (Ritchie *et al.*, 2003), indicating its relevance as a non-toxigenic model to predict *E. coli* O157:H7 behaviour. Strain T was cultured in Luria-Bertani (LB; Sambrook *et al.*, 1989) medium supplemented with rifampicin (10 μg/ml) and kanamycin (50 μg/ml) at 37°C (with shaking, 100 r.p.m.), until the end of the exponential growth phase, after

which it was used as an inoculant for the survival studies in soil microcosms.

Survival experiment

The soil microcosms with modified microbial community structures at 60 days were used as the recipient habitats for strain T. To establish a set of replicates that would warrant (destructive) analyses at set times over a full 60-day period, a total of 60 microcosms were thus used. A freshly grown culture (50 ml, end of exponential growth phase) was washed twice by centrifugation (5000 *g*, 10 min) after which the cell pellet was resuspended in sterile ultrapure water. The final colony forming units (CFU) density was estimated to be about 10^9 cells/ml. Aliquots of 2.5 ml were then used in order to inoculate each 50-g soil microcosm by gentle pipetting and mixing, establishing a cell density of approximately 5×10^7 cells/g dry soil.

The microcosms (three replicates for each treatment, plus uninoculated control) were then incubated at 20°C (85% air humidity) for a total of 60 days. Three replicates per treatment were destructively sampled (inlcuding homogenization) after 3 h (time 0), 3, 7, 15, 30 and 60 days, and analyses were performed as detailed below. The soil moisture content was regularly measured by weighing and the lost water was replenished by adding sterile ultrapure water.

Soil analyses

Soil pH. Soil pH was measured according to standard techniques (ISO/DIS 10390, ISO, 1992). Soil pH varied marginally, from 5.2 (pH-H_2O) in the unfumigated soil to 5.3 in all fumigated soils.

Soil enzymes. The soil total dehydrogenases, nitrate reductases and phosphatases were measured in accordance with Zvyagintzev *et al.* (1980). Sixty days after fumigation, these values either were stable along the different fumigation depths (total dehydrogenases) or showed an increasing (nitrate reductase – from 0.25 to 0.8 mmol NO_3^-/min/mg protein/g dry soil) or decreasing (phosphatase – from 2.3 to 1.9 mmol *p*-nitrophenyl phosphate (pNP) transformed/min/mg protein/g dry soil) trend.

Microbial biomass carbon (C). Microbial biomass carbon was determined by the fumigation/extraction method of Vance *et al.* (1987).

Soil respiration. To assess soil respiration, 10 g of soil were placed in closed 150-ml vials (dry weight was determined in a parallel sample) and vials were incubated at 20°C for 4 days. The evolved CO_2 was trapped in 3 ml of 1 N NaOH contained in a subvial and then assayed by titration with HCl using $BaCl_2$ as an indicator (Alef and Nannipieri, 1995). Seven days following fumigation, soil respiration was significantly different between the differently treated samples, respiration rate being highest at the highest fumigation depths (Table 1). At 60 days, the respiration rates were consistent among all treatments and comparable to those at 7 days.

Total microscopic bacterial counts. Total bacterial counts were obtained using microscopy of 4,6-diamidino-2-phenylindole (DAPI)-stained cells in accordance with Bloem (1995).

Cultivation-based analyses

CFU counts. Soil samples (5 g) were transferred to Erlenmeyer flasks containing 45 ml of sterile 0.1% sodium pyrophosphate solution, and placed in an ultrasonication bath (50 kHz, 4 min, room temperature). Serial tenfold dilutions in sterile tap water were then made, and 200 μl of each dilution were spread-plated onto selective and unselective agar media. To assess total bacterial communities growing on unselective medium, R2A agar (Difco, pH 7.2)

Table 1 Effect of different fumigation depths on soil microbial parameters relative to unfumigated soil samples[a]

Parameter	*Percentage of value in unfumigated soil*					
Fumigation time (h)	*0.5*		*2*		*24*	
Days after fumigation of soil (days)	*7*	*60*	*7*	*60*	*7*	*60*
Total biomass C	66.8±7.4	96.0±20.8	64.5±7.2	108.4±13.3	74.5±13.1	107.7±9.3
Viable biomass	100.0±20.3	61.4±4.8	84.8±18.4	52.0±6.4	59.3±9.7	55.6±4.8
Respiration	130.2±17	143.3±5.8	144.8±11.4	132.7±28.3	161.0±14.2	164.3±5.2
Dehydrogenase activity	82.1	ND	89.8	41.3	89.8	155
CFU counts	29.3±5.5	79.9±36.6	18.7±2.3	187.2±36.9	10.6±3.3	202.0±26.1
Total bacterial counts (DAPI)	66.3±1.6	74.3±2.9	45.5±2.8	81.8±18.5	38.3±3.7	57.6±36.3
C/T ratio[b]	46.0±2.8	111.1±12.5	40.5±8.8	233.4±15.5	27.0±6.8	355.6±22.4
EP index[b]	81.9±2.7	113.0±9.1	73.2±6.1	93.4±25.4	47.9±14.3	91.6±9.1

Abbreviations: CFU, colony forming units; DAPI, 4,6-diamidino-2-phenylindole; ND, not determined.
[a]Values in unfumigated soil (day 7/day 60): total biomass C (mg C_{mic}/g), 301 (42)/660 (67); viable biomass (nmol $PLFA_{tot}$/g), 27.5 (2.3)/42.4 (0.7); respiration (μg C-CO_2/g/h), 0.92 (0.39)/1.29 (0.28); dehydrogenase activity (mKMoH$^+$/min/mg/g protein $\times 10^{-3}$), 0.0004/0.18; log 10 CFU counts, 7.26 (0.06)/7.48 (0.05); log total bacterial counts, 9.72 (0.07)/9.51 (0.07); C/T ratio, 0.0037/0.009; EP index, 0.67/0.51.
[b]C/T ratio, ratio of CFU to total bacterial counts; EP index, ecophysiological index as defined by de Leij *et al.* (1993).

was used, and such plates were incubated at 20°C. Colonies on plates were enumerated in accordance with de Leij *et al.* (1993); see below under 'ecophysiological (EP) indices'. To enumerate strain T CFU in soil, selective plating was performed using tryptic soy agar (TSA, Becton-Dickinson Co., Franklin Lakes, NJ, USA) supplemented with 10 μg/ml of rifampicin and 50 μg/ml of kanamycin. These plates were incubated at 37°C, and counting was after 24 h. From the uninoculated microcosms, no colonies were observed in dilution plating on the selective plates used to enumerate strain T. Moreover, colonies growing on the selective plates from the inoculated systems were all confirmed to be strain T by phospholipid fatty acid (PLFA) analysis on the MIDI system.

EP indices. The EP indices of the bacterial communities were determined from the colony formation curves obtained by enumerating colonies appearing on R2A in the different classes (in accordance with time of appearance; de Leij *et al.*, 1993). The formula of de Leij and co-workers was used to calculate the EP index. We defined r-strategists as the colonies appearing in the first 3 days of incubation, and K-strategists as those appearing thereafter.

Culturable-to-total (C/T) cell ratio. The C/T ratio was calculated from the total bacterial counts on R2A and those obtained by microscopical enumeration of the DAPI-stained cells in accordance with Sigler *et al.* (2002).

PLFA analyses
PLFA were extracted from prehomogenized 2-g subsamples of soil obtained from each replicate microcosm, according to Frouz *et al.* (2006). The MIDI system (MIDI Inc., Newark, DE, USA) was used to assess the total soil community PLFA. Analyses were performed in accordance with the instructions from the manufacturer, and in line with Frouz *et al.* (2006). Following the measurements, the complete PLFA profiles were used to assess the microbial community compositions as well as to estimate the presumably active microbial biomass. Selected specific biomarkers were used to estimate the total bacterial (i15:0, a15:0, 15:0, 16:1ω7c, i17:0, cy17:0, cy19:0; Frostegård and Bååth, 1996), actinomycetal (10Me18:0, 10Me17:0, 10Me16:0; Kroppenstedt, 1985), fungal (18:2ω6; Frostegård and Bååth, 1996) and protozoal (20:4ω6; Erwin, 1973) community shifts.

Soil DNA extraction
Total community DNA was extracted from soil using the Ultra Clean soil DNA isolation kit (MoBio Laboratories, Biozym TC, Landgraaf, The Netherlands). To achieve maximal cell lysis, a bead beating step was included as follows. A 50 mg volume of glass beads (0.10–0.11 mm diameter) and 0.5 g of soil in 0.1% sodium pyrophosphate were added to the tubes provided in the kit, which were then placed in a mini-bead beater (BioSpec Products Inc., Bartlesville, OK, USA) and bead beaten two times for 30 s. After bead beating, the DNA was further extracted according to the protocol furnished by the supplier. The soil DNA obtained was checked for amount and molecular size by agarose gel electrophoresis (Sambrook *et al.*, 1989). DNA sizes ranged from approximately 10 to 25 kb, and 2–5 μg DNA per gram soil was commonly obtained. DNA was sufficiently pure to be subjected to successful amplification by PCR.

PCR-DGGE
Amplification of 16S ribosomal RNA gene fragments from soil DNA was performed by using the bacterial primers F986-GC (GC denotes the presence of a GC-clamp at the 5′-end) and R1378, in accordance with Garbeva (2005). PCR mixes were set up and run as described by Garbeva (2005), using 50-μl reaction volumes. One to five nanograms of soil DNA was used as targets, and 5 U of AmpliTaq DNA polymerase, Stoffel Fragment (Roche, Basel, Switzerland) as the extending enzyme. An MJ Research thermal cycler was used. The PCR products obtained were checked as to quantity and molecular size by common agarose gel electrophoresis (Sambrook *et al.*, 1989). PCR products of high quality were purified and analyzed by denaturing gradient gel electrophoresis (DGGE).

DGGE analysis was performed in an Ingeny PhorU2 apparatus (Ingeny International, Goes, The Netherlands) using 40–70% gradients of denaturants in accordance with Garbeva (2005) and Garbeva *et al.* (2003, 2004). Gels were stained by SybrGold or silver staining and analyzed using GelCompar v 4.1 software (Applied Biomaths, Kortrijk, Belgium) after digitizing the bands. The DGGE profiles were compared within gels and clustered using the unweighted pair group method with mathematical averages (UPGMA)-based clustering algorithm of GelCompar.

Statistical treatment of data
The experiment encompassed triplicate experimental units and all data obtained were analyzed in respect of the statistical significance of the differences observed within treatments and between treatments. Response variables such as microscopic counts, CFU numbers, DGGE bands and PLFA signals were thus analyzed using two-way analysis of variance (ANOVA) and principal components analysis (PCA). For ANOVA, the data were considered to be significantly different using the 5% *P*-value ($P < 0.05$) as the criterion.

Results

Establishment of soils with different microbial communities by fumigation

Both 7 and 60 days after the fumigations, the progressively increasing fumigation depths had induced significantly changed microbial community structures (Table 1). Considering all the data, the situation at day 7 indicated that the system was in transition in microbiological terms, whereas the data at day 60 indicated that a more mature microbial community had established (Table 1). In the following sections, we describe what the specific microbiological indicators tell us.

Microbial biomass carbon (C). Seven days after fumigation, microbial biomass C had decreased in all fumigated soils as compared with the unfumigated control soil. Specifically, the losses ranged from 26% (high fumigation depth) to 35% (low as well as intermediate depths). However, 60 days after fumigation, the differences between the various treatments had disappeared, as all showed statistically similar microbial biomass C estimates, at levels roughly similar to those of the unfumigated samples.

Total bacterial counts obtained by DAPI staining. Seven days after fumigation, all fumigated soils had lost bacterial cells detectable via microscopic counting, at 34% (low fumigation depth), 54% (intermediate depth) and 62% (high depth). However, after 60 days, these losses had strongly diminished, revealing regrowth of organisms over this period of time, resulting in the putative replacement of original populations by novel ones.

Total bacterial CFU counts. Seven days after fumigation, the CFU counts from all fumigated soils were strongly reduced as compared with those in the unfumigated samples, and progressively so with increasing fumigation depth (Table 1). However, 60 days after fumigation, the total bacterial CFU counts had strongly increased in all fumigated samples, and a trend was noted towards increasing densities of culturable forms with increasing fumigation depth. The average values (standard deviations in parentheses) for unfumigated and low-, intermediate- and high-depth fumigated soils were, respectively, log 7.48 (0.05), log 7.49 (0.2), log 7.71 (0.19) and log 7.78 (0.15) CFU per gram of dry soil.

C/T ratios. C/T ratios were used as indicators of the community culturability levels. Unfumigated soil had a C/T ratio of 0.0037. Fumigation initially lowered this ratio for all three fumigation depths, by 54, 59 and 73%, respectively, for low-, intermediate- and high-depth fumigation, as evidenced 7 days after fumigation (Table 1). Sixty days following fumigation, the C/T ratios showed increases to values roughly equal to (low-depth fumigation), or even overshooting (intermediate- and high-depth fumigation), those in the unfumigated soil, in all fumigated samples. Hence, 60 days following fumigation, the bacterial communities apparently recovered culturability, resulting in an outgrowth of culturable forms, this effect being progressively stronger with increasing depth of fumigation (Table 1).

EP indices. The EP index of unfumigated soil was 0.68. Fumigation of soil lowered this value progressively with fumigation depth, in particular after 7 days (Table 1). This was notably due to an increase in the relative dominance of r-strategists after 7 days. However, the system appeared to veer back to normal, as, following the depression, the values measured after 60 days in all unfumigated soils were again akin to those in the unfumigated soil (Table 1), and the relative dominance of r-strategists had diminished.

PLFA. Total PLFA analysis after 7 and 60 days revealed that depth of fumigation affected the extractable PLFA. Specifically, after 7 days, soils subjected to low- and intermediate-depth fumigation had incurred rather low losses ($<15\%$), whereas high-depth fumigated soil had lost 41% of extractable PLFA. At 60 days, the fumigated samples had lost between 37% (low depth) and 43–45% (intermediate and high depth) of total PLFA. Principal components analysis (PCA-71% of the variation explained by two PCA axes) of the PLFA analyses performed 7 and 60 days after the fumigation clearly showed a separation of the microbial communities in the differently treated soils into four groups, separated by fumigation depth (Figure 1). Specifically, after 7 days, a clear separation was seen between unfumigated/low-depth (together)–intermediate/high-depth fumigation. At 60 days, the separation according to fumigation depth was strong and all treatments were ordinated at quite different positions in the biplot.

PLFA biomarkers. The analysis of PLFA at 60 days after fumigation revealed a statistically similar relative abundance of the specific bacterial biomarkers i15:0, a15:0, 15:0, 16:1ω7c, i17:0, cy17:0 and cy19 in all fumigated samples, whereas an approximately 38% decrease of the abundance of these markers (significant at $P<0.05$) was noted in the fumigated soils as compared with the unfumigated one. This decrease was also observed for the actinobacterial/actinomycete-specific markers 10Me18:0, 10Me17:0 and 10Me16:0. Concerning the fungal biomarker 18:2w6, low-depth fumigation did not affect its abundance, whereas intermediate and deep fumigation did so (reduction by 50%). The microeukaryote marker 20:4w6 decreased by about 50% at low- and intermediate-depth fumigation, and to below detection by deep fumigation.

PCR-DGGE. The microbial diversity in the differently treated soils was determined by soil

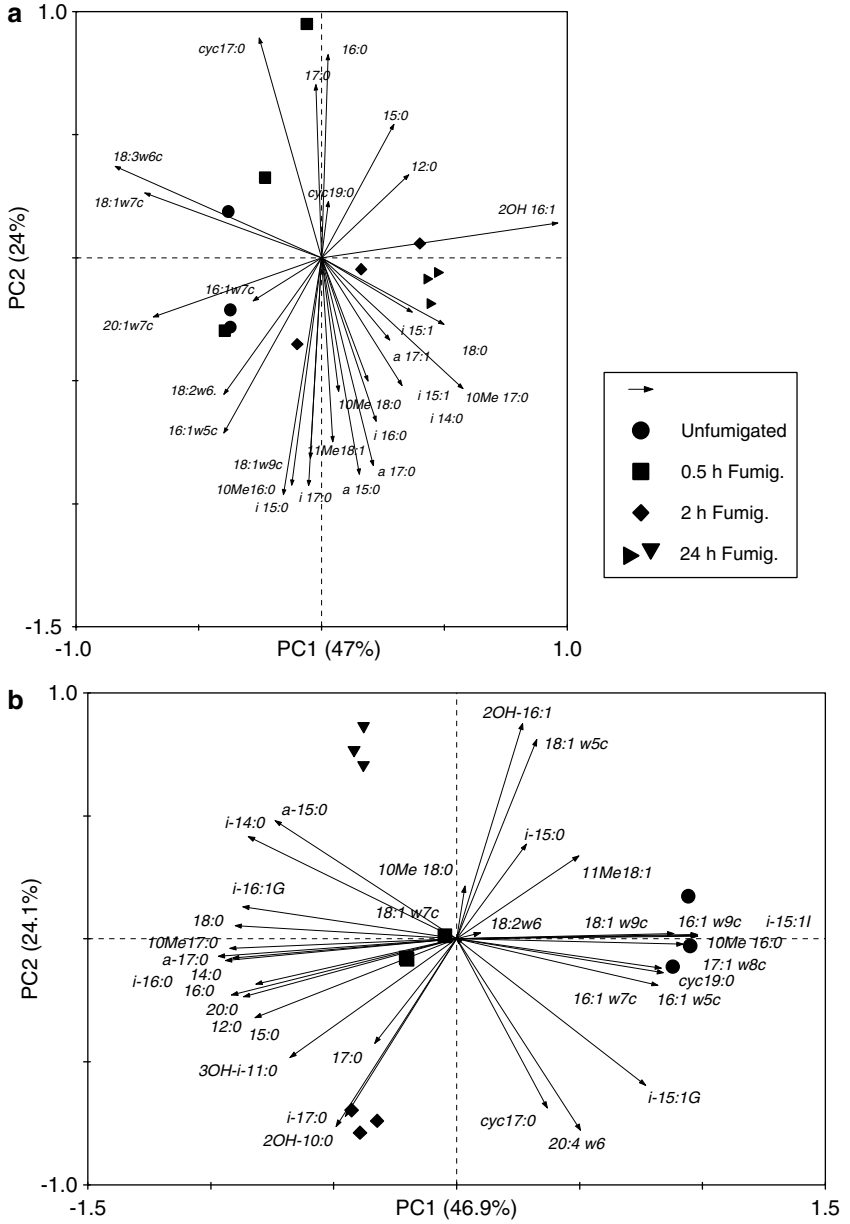

Figure 1 PCA of PLFA patterns obtained from soils with modified microbial communities. (**a**) Seven days after fumigation. (**b**) Sixty days after fumigation. PCA, principal components analysis; PLFA, phospholipid fatty acid.

DNA-based polymerase chain reaction (PCR)-DGGE using the 16S ribosomal RNA gene as the marker. Sixty days after fumigation, the bacterial DGGE profiles in all fumigated soils were quite different from those in the unfumigated soil (Figure 2). Moreover, the low-depth fumigation soils (two samples) were clearly separate, at about 68% of similarity, from the intermediate- and high-depth treatments. With one exception, the latter treatments were quite similar, as they separated only at 79% of similarity. We also found that each treatment clustered separately from the others (except one 2 h replicate), indica-

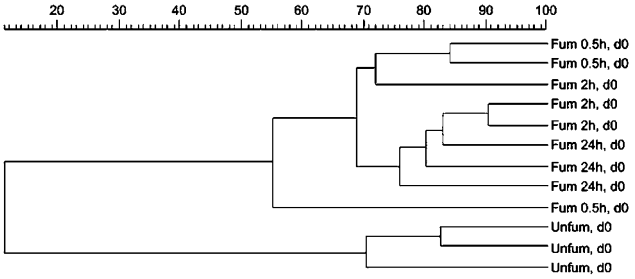

Figure 2 Clustering of bacterial DGGE patterns in soils with modified microbial communities 60 days after fumigation – time 0 of strain T survival experiment. Fum, fumigated; Unfum, unfumigated; 0.5, 2 and 24 h, fumigation times; d0, day 0.

ting that different depth of fumigation affected microbial community development each time in a specific way.

Survival of E. coli *O157:H7 derivative strain T in soil with different microbial communities*

The invading *E. coli* strain T showed differential behaviour in the differently treated soils. In unfumigated soil, the invader showed a progressive decline of the CFU numbers per gram of dry soil, from the initial log 7.2 to the detection limit over the 60-day incubation period, that is, a decline of at least six orders of magnitude. In comparison with this behaviour, the survival of the invader in all fumigated soils was strongly and significantly ($P<0.05$) enhanced (Figure 3). The highest survival rate was recorded in the soil that had been subjected to the highest fumigation level (24 h), and, in this case, the CFU decrease recorded amounted to just about two orders of magnitude. Progressively increasing fumigation depth thus resulted in progressively lowered death rates.

The data on the abundance of strain T in the differentially treated soils were supported by direct molecular observation via PCR-DGGE at 0, 30 and 60 days after introduction (see also next section). Clear bands comigrating with (and thus indicative of) strain T, which were not present in DGGE patterns of control (no-inoculant) soils were visible in the zero-time samples. These bands progressively decreased in intensity over time, in most cases, to extinction. Specifically, in the unfumigated and in the 0.5- and 2-h fumigated samples, the bands indicative of strain T became undetectable after respectively 30 days (unfumigated control) and 60 days (0.5- and 2-h fumigation), whereas in the 24-h fumigated samples, weak bands were still visible in two out of three replicate soil samples at 60 days.

Microbial diversity assessment in soil with E. coli *strain T*

PLFA. MIDI analyses at days 30 and 60 of the survival experiment showed that the differences in extractable PLFA established at day 0 largely persisted over the 60-day period. Specifically, the differently treated soils maintained their extractable PLFA (intermediate- and high-depth fumigation treatments) or incurred additional losses of around 20% at day 60 (unfumigated and 0.5 h fumigation). No clear evidence for the abundant presence of *E. coli*-specific PLFAs was found. The above picture of stability versus around 20% loss was clearly duplicated in both the bacterial and the actinobacterial biomarkers. In contrast, a complex picture emerged for the fungal biomarkers, that is, stable to 1.6-fold enhanced values in intermediate- and high-depth fumigation treatments, and 25–60% reduced values in unfumigated and low-depth fumigated samples. The microeukaryote biomarker values remained stable in the unfumigated samples, increased to 90% of these in the low- and intermediate-depth fumigation samples, and remained below detection in the high-depth fumigation samples.

PCR-DGGE. The bacterial diversities as determined by PCR-DGGE consistently showed visual differences between treatment. Overall and throughout the experiment, there was a decreased 'apparent phylotype richness', as judged by the number of bands in the 2-h (21 ± 1) and 24-h (21 ± 4) fumigated samples as compared to the unfumigated samples (31 ± 7). The clustering of DGGE profiles obtained at the onset of the survival experiment, that is, a consistent clustering of the bacterial communities by treatment (Figure 2), was roughly maintained over the entire 60-day experimental period, that is, at each time point, a similar grouping of samples was observed (data not shown). When samples were compared across time and treatments (only two

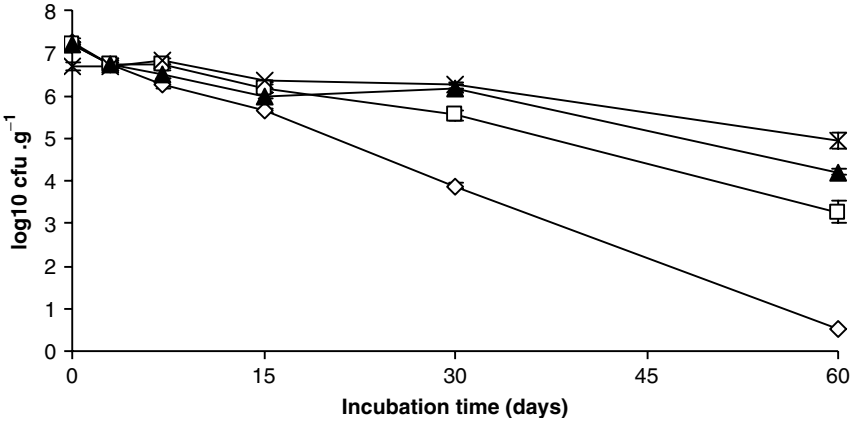

Figure 3 Survival of *E. coli* strain T in soil with modified microbial community structures. Explanation: diamonds, unfumigated soil; open squares, 0.5 h fumigation (low depth); triangles, 2 h (intermediate depth); crosses, 24 h (high depth); means $\pm$ s.d. of nine replicates. Inoculation of approximately 7×10^7 cells/g soil ($5–9\times10^7$ cells/g dw).

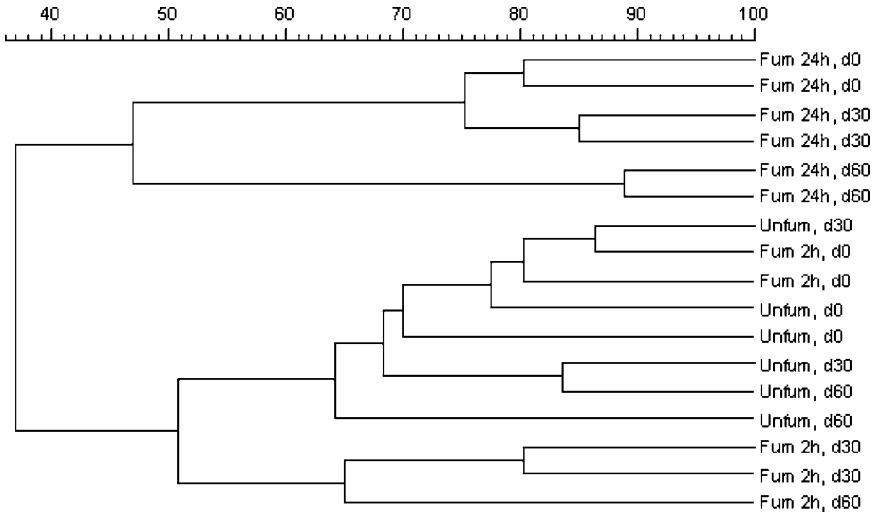

Figure 4 DGGE fingerprints of bacterial communities in soils with modified communities over 60 days in the presence of the bacterial invader *E. coli* O157:H7 strain T. Fum, fumigated; Unfum, unfumigated; 2, 24 h, fumigation times (0.5 h not shown due to space limitation on gel); d0, d30 and d60: days 0, 30 and 60.

replicates used because of space constraints, and low-depth fumigation not included), the following three main clusters emerged (Figure 4):

(1) unfumigated samples, separated into 0, 30 and 60 days (mixed with the zero-time intermediate-depth fumigation samples),
(2) intermediate-depth fumigation samples, 30 and 60 days and
(3) high-depth fumigation samples, 0, 30 and 60 days.

Clusters 1 and 2 could actually be seen as subclusters of an overall clustering at about 50% similarity, whereas cluster 3 (encompassing all high-depth fumigation treatments) was clearly separate. Within cluster 3, there was a neat subclustering by time, that is, 0, 30 and 60 days. With the exception of the zero-time intermediate-depth fumigated samples, it is apparent that the clustering by treatment was apparently maintained throughout the whole experimental period.

Discussion

In this study, we assessed the behaviour of a key bacterial pathogen, *E. coli* O157:H7 derivative strain T, in a loamy sand soil obtained from species-rich grassland, in which the microbial community composition had been modified by progressively enhanced fumigation depths. Changes in the key microbial populations of this soil, and concomitant effects on the suppressiveness against the fungal potato pathogen *Rhizoctonia solani* AG-3, both resulting from changed land use, have been shown before (Garbeva, 2005). The current assessment of the survival of the potential BT agent *E. coli* O157:H7, in relation to soil microbial diversity and community composition, is important, as this organism has been amply shown to be able to colonize edible plants such as radish (Itoh *et al.*, 1998) and lettuce (Solomon *et al.*, 2002) from manure, irrigation water (Solomon *et al.*, 2002) or contaminated seeds (Itoh *et al.*, 1998). The organism thus clearly poses a risk for the food chain. The study further aimed to provide data that would support measures against bacterial BT agents.

Fumigation of soil is known to result in the temporarily enhanced availability of (carbonaceous) resources from lysed cells, upon which the chloroform survivors are able to grow, resulting in an altered microbial community in which primarily the chloroform survivors abound. That the fumigation treatments imposed on our soil indeed resulted in changes in the microbial communities was convincingly shown from the assessment of a range of parameters, which addressed both the total microbial communities by cultivation-independent methods and the culturable fractions of these communities using cultivation-dependent ones. Concerning the former, both at seven and 60 days following fumigation, shifts in a range of overall bacterial, actinobacterial, fungal and microeukaryotic PLFA biomarkers were noted, and, at both time points, clustering of the samples along treatment was found. Zelles *et al.* (1997), who studied changes in soil microbial properties and PLFA fractions shortly (10 days) after chloroform fumigation, also showed clear effects of (deep) fumigation on soil microbial community structures. Specifically, they revealed that branched-chain fatty acids may resist chloroform treatment more than mono-unsaturated and cyclopropyl fatty acids, and that, hence, Gram-positive bacteria may become less injured by chloroform fumigation than Gram-negative ones. The formation of spores by the former bacteria may be

the reason for them to better survive unfavourable periods. Also, organisms that contain high amounts of branched-chain fatty acids might be to a larger extent situated in sites in soil that are less accessible to chloroform (Ross, 1987). On another notice, in our study, reductions in the amounts of extractable PLFAs were found, which were quite akin to those reported by Zelles *et al.* (1997) 10 days after fumigation and by Griffiths *et al.* (2000). These reductions indicate a population size reduction of the groups of organisms measured by these PLFA. The modified bacterial community structures could further be confirmed by PCR-DGGE, which also revealed a clustering largely by treatment. As a cautionary note, it should be stressed here that PCR-DGGE, as usual, only described members of the dominant populations in the system, and does not allow an appreciation of the full complexity of the system. In this context, we used the term 'apparent phylotype richness', indicating the DGGE-derived richness levels, in a comparative manner to allow an angle, albeit biased and incomplete (for obvious reasons), at the effects of the fumigation treatments.

At the level of the culturable bacterial communities, a striking effect was the shift in the relative dominance of r-strategists, which reached a maximum level after 7 days and diminished after 60 days. Such an increase in r-strategist dominance is indicative of the emergence of novel ecological opportunities for organisms in an otherwise largely microbiostatic system, in this case driven by the microbial changes induced by fumigation. In addition, the total bacterial counts on plates indicated a temporary depression of culturable forms after 7 days, followed by an outgrowth of specific populations after 60 days to levels akin to, or above, those in the unfumigated soils.

Together, the data indicated that increasing fumigation depth treatments had progressively modified the structures of the microbial communities that were subjected to these treatments. Whereas at day 7 the indicators pointed to the systems still being in transition, at day 60 it appeared the microbial communities had reached a more stable situation. Such so-called climax communities may resist the influx of immigrant/invading species to a larger extent, since, in order to persist, such invaders must either compete with the resident species for available resources or occupy as-yet-unoccupied niches. We surmised that progressively higher numbers of unoccupied niches could be found in the soils that had been subjected to higher fumigation depths.

The behaviour of strain T in the soils with modified community structures was clearly consistent with the hypothesis that within the single selected habitat (soil), which was relatively unaffected in respect of abiotic conditions like pH, moisture and soil chemical conditions, microbial community structure was the main determinant of the survival of the invader. We determined strain T survival at two levels, that is, (1) at the CFU level using dilution plate counting and (2) at the DNA level, by detection of the strain T-specific band in DGGE. The latter is possible for populations that make part of down to about 0.1% of a community, as evidenced by Gelsomino *et al.* (1999), and such a cut-off value was also found in this study. Over time, both analyses were largely consistent with each other, which indicated that the surviving strain T population largely occurred in a culturable form, and that thus viable-but-non-culturable forms presumably did not accumulate to a large extent in the system.

Specifically in respect of inoculant survival, the progressively changed microbial diversities and community compositions – which indicated an increasing temporary ecological disturbance leading to an altered microbial community and a presumed reduction of diversity – coincided with an enhancement of the survival rate of the invader. In establishing the changes in the soil microbiota, we obviously tinkered with, and most likely reduced, the interactive forces of the natural biotic system, notably those that, together, constitute soil microbiostasis, for example, microbial competition, predation and antagonism. Strikingly, predatory protozoa may have been one factor affected, as the microeukaryote PLFA indicators in all cases were reduced as a result of fumigation, and most strongly so in the fumigation treatment of highest depth. However, in the low- and intermediate-depth fumigation treatments, these values veered back to almost the levels in the unfumigated soils over the course of the survival experiment. Hence, in the light of the enhanced inoculant survival over the unfumigated control, predation by protozoa was not the only factor involved in determining survival. We surmised that the most likely factor involved in the enhanced survival was indeed the lowering of the complexity of the soil microbiota, resulting in a lower degree of overlapping functionalities, which putatively enhances the chances for invaders to find, conquer and establish in, a niche and subsequently persist as part of the community.

The fact that the susceptibility of an ecosystem with a lowered biological complexity to invasion by exogenous (micro)organisms might indeed be enhanced (as compared with that in a more complex community) may be mainly due to the legerdemain of interactive forces reigning in a mature system (Girvan *et al.*, 2005), which is presumably disrupted to varying degrees in systems with shifted communities. The nature of such interactive forces is likely to be complex and involve the varying types of microbial signalling, sensing and antibiosis systems that are currently known to play a role in microbial life in soil (Van Elsas *et al.*, 2006).

Recent developments in decontamination technology have emphasized the need to reduce the toxicity and environmental damage of decontaminants to be used in practice (Raber *et al.*, 2001). In this experiment, chloroform had 60 days in which to

evaporate before the incubation started, so residual toxicity was absent. This mimics the effect of an environmentally benign decontaminant such as hydrogen peroxide steam or ultraviolet radiation. Paradoxically, decontamination may lower the natural soil suppressiveness due to microbiostasis, thus making it easier for subsequent invading pathogens to survive in the soil. On the other hand, toxic decontaminants such as paraformaldehyde may prevent further pathogen colonization through their residual toxicity.

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