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Effects of fumigants on microbial diversity and persistence of *E. coli* O15:H7 in contrasting soil microcosms

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ABSTRACT

Persistence of *E. coli* O157 in the environment is a serious public health concern. However, little is known about the persistence of this pathogen after exposure to chemical compounds like fumigants in the environment. In this study, the persistence behavior of pathogenic *E. coli* O157:H7 was investigated after fumigation with methyl bromide (MeBr; CH_3Br) and methyl iodide (MeI, iodomethane; CH_3I) in soil microcosms under laboratory conditions. Our goal was to assess changes in soil microbial community structure and persistence of *E. coli* O157:H7 in microcosm soils after fumigation. PCR was used to amplify 16S rRNA genes from total bacterial community composition, and the products were subjected to denaturing gradient gel electrophoresis (DGGE). Microbial diversity as determined by DGGE was significantly higher in clay soil than sandy soil. Real-time PCR and plate counts were used to quantify the survival of *E. coli* O157:H7 in the two soils after fumigation with MeBr and MeI. The survival of the pathogen was higher in the non fumigated controls than the fumigated treatments when determined using plate counts. These results were confirmed by real time PCR analysis targeting the *stx*1, *stx*2, and the *eae* genes. *E. coli* O157:H7 survived for about 35 days when determined using the plate count method but continued to be detected at about the detection limit of 10^2 by real time PCR for more than 86 days. Our results showed that there was a fast inactivation of the pathogen during the first 35 days. After this period, a small proportion of the pathogen continued to survive in the soil microcosms. Subsequent enrichment of soil samples and immunomagnetic separation revealed the continuous presence of viable cells after 86 days of incubation. The data presented contribute to a better understanding of the behavior of *E. coli* O157:H7 in soil, and showed the need for more investigation of the role of dormant cells in soil that may be a source for recontamination of the environment.

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1. Introduction

Methyl bromide (MeBr; CH_3Br) is a broad spectrum, highly effective and relatively cheap fumigant used for pre-planting fumigation. It has been used extensively to control plant pathogens such as nematodes, soil-borne diseases, and weeds in economically important crops such as strawberries and nursery stock (Ferguson and Padula, 1994) in California and other parts of the world. Methyl bromide was scheduled for elimination in the United States and other developed countries by the year 2005 (USEPA., 2000) and in developing countries by 2015 because of its stratospheric ozone depletion potential. However, MeBr is still in use due to critical use exemptions for the cultivation of strawberries, tomatoes, and peppers in California and Florida. The proposed phase-out has resulted in an intensive search for alternative fumigants and the development of other integrated pest management strategies to replace MeBr. Methyl iodide (MeI, iodomethane, CH_3I) was reported as a potential alternative to the stratospheric ozone-depleting fumigant methyl bromide (MeBr) in the mid-1990s (Ohr et al., 1996; Sims et al., 1995). Methyl iodide is often referred to as the "drop-in replacement" because its fate, transport characteristics and effectiveness as a biocide are similar to those properties of MeBr (Ohr et al., 1996). MeI has a distinct advantage over MeBr in that its atmospheric lifetime is only <10 days, compared to 1.5 to 2 years for MeBr (Ruzo, 2006). Therefore, it is unlikely that MeI will reach the stratosphere and contribute to ozone depletion (Rassmussen et al., 1982; Solomon et al., 1994), although the volatilization of MeI may be similar to that of MeBr. As a preplant soil fumigant, MeI can be used alone, or in combination with chloropicrin (CP) to control plant pathogens, nematodes, insects and weeds on crops such as strawberries, tomatoes, peppers, ornamentals, turf, trees and vines (USEPA, 2010).

Methyl iodide use in US agriculture is receiving significant focus due to its recent registration (USEPA, 2010). California recently announced its decision to become the forty-eighth US state to register MeI (California Department of Pesticide Regulation, 2010). Concern over the use of MeI as an agricultural fumigant is based on its potential to cause serious health effects to humans after emission and inhalation. Recently, our laboratory has tested different methods of reducing emissions of methyl iodide from agricultural soils (Ashworth et al.,

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2011; Luo et al., 2010), but little or no studies have been done on the long term impact on soil bacterial composition. Changes in the soil microbial population can be observed following fumigation with MeBr and other fumigants (Dungan et al., 2003; Ibekwe et al., 2001; Martin, 2003). Ibekwe et al. (2010) observed decline in *E. coli* O157:H7 population in soils cultivated with lettuce and fumigated with MeBr and MeI in a growth chamber. This study was conducted for 60 days in two walk-in growth chambers. However, *E. coli* O157:H7 survived longer in non-fumigated soil than in fumigated soil. A subsequent study showed that the effect of these fumigants on rhizosphere and phyllosphere microbial composition was insignificant (Ibekwe et al., 2009). Due to the increased focus on food safety related to fresh produce, there are several other studies of *E. coli* O157:H7 survival in different environments such as soil, manure and water (Jiang et al., 2002; Kudva et al., 1998; Vital et al., 2008). These studies showed that the availability of nutritional resources and key abiotic conditions are critical to *E. coli* O157:H7 population survival and even growth in such environments. However, under fluctuating environmental conditions, such as those present in many soil environments, growth may be differential and gross bacterial death may ensue if the death rate exceeds the growth rate.

Our goal was to assess changes in soil microbial community structure and persistence of *E. coli* O157:H7 in soil microcosms after fumigation with MeBr and MeI. PCR was used to amplify 16S rRNA from total bacterial community composition, and the products were subjected to denaturing gradient gel electrophoresis (DGGE). The Shannon-Weaver index of diversity (*H*) was used to determine the effects of both fumigants on soil microbial community structure. For our main objectives, plate count and real-time PCR approaches were used to determine the survival of *E. coli* O157:H7 in the two soil microcosms. The survival data were fitted to a biphasic model as proposed by Coroller et al. (2006) with the Geeraerd and Van Impe inactivation model-fitting tool (GInaFiT) (Geeraerd et al., 2005) as described by Franz et al. (2008).

2. Materials and methods

2.1. Soils and chemicals

Clay soil (Willows silty clay, saline–alkaline) and sandy soil (Dello sand) were collected from Mystic Lake dry bed and the Santa Ana River bed, respectively, in Riverside County, California. The clay soil has a bulk density of 1.51 Mg m^{-3} with 3.7% sand, 49.1% silt, and 47.2% clay. The sandy soil has a bulk density of 1.67 Mg m^{-3} with 99.1% sand, 0.20% silt, and 0.70% clay. The soils were sieved through a 4 mm sieve before planting, and the high salt content (electrical conductivity = 15 ds m^{-1}) in the clay soil was reclaimed as described by Ibekwe and Grieve (2004). These soils were chosen because these are the two main soil types supporting cattle production in the area and cattle are the main source of *E. coli* O157:H7 in the environment.

Methyl iodide (>99% purity) was purchased from Chem Service (West Chester, PA) and methyl bromide (>99% purity) was obtained from Great Lakes Chemical Company (West Lafayette, IN).

2.2. Bacterial strain and growth conditions

E. coli O157:H7 strain 72 pGFP was kindly provided by Dr. Pina Fratamico (Fratamico et al., 1997). This strain contains Shiga-like toxin genes 1 and 11 (*stx*1, *stx*2) and the pGFP expressing the green fluorescent protein (GFP) containing an ampicillin resistance gene and was used for pathogen enumeration from soil. *E. coli* O157:H7 was cultured at 37 °C overnight in modified Tryptic Soy broth (mTSB) (Difco Laboratories Inc., Cockeysville, MD) supplemented with 100 μg of ampicillin ml^{-1} (Sigma, St Louis, MO). Cells were harvested by centrifugation at 5000 xg for 10 min and resuspended in phosphate buffered saline (PBS) (Fisher Scientific, Pittsburgh, PA) to a concentration of ~10^8 CFU ml^{-1}.

2.3. Microcosm experimental design

The microcosm experiment was set up in 1.0 l Mason Kerr self sealing wide mouth glass containers (Lima, OH). The soil (1.5 kg) was adjusted to a moisture content of about 12% (for equal distribution of fumigant in soil) by adding *E. coli* O157:H7 inoculants or water and mixing in a larger container before transfer to each microcosm. This was to maintain the same level of pathogen concentrations and moisture content. After the inoculation, fumigants were added. The experimental design consisted of two fumigants at three different concentrations in duplicates (0.5x, 1x, and 5x, where 1x is 48 kg ha^{-1} for MeBr and 40 kg h^{-1} for MeI). These resulted in spiking approximately 1.73 g ml^{-1} of MeBr and 2.28 g ml^{-1} of MeI per gram of soil into each microcosm for the 1x treatments. Microcosms were sealed for 24 h after fumigant application, and vented continuously through a small opening in the cover for the remainder of the experiment as previously described (Ibekwe et al., 2001). Soil samples were taken from individual microcosm (with a sterile spatula) for heterotrophic bacteria, *E. coli* O157:H7 concentration, and total bacterial DNA before fumigation and at days 1, 3, 7, 14, 21, 28, 35, 49, 56, and 86 after fumigant treatment. Bacterial concentrations were determined by plating soil on Tryptic soy agar (TSA; Becton Dickinson) plates containing 100 μg of ampicillin ml^{-1} (TSA-A). The GFP-labeled *E. coli* O157:H7 colonies were counted under an UV light. Total bacterial DNA was extracted from samples, and heterotrophic bacteria were counted on TSA.

2.4. DNA extraction, PCR amplification, and DGGE analysis

Total bacterial community DNA was extracted from soil samples (0.5 g) with the Power Soil DNA Kit (MoBio Laboratories, Solana Beach, CA) and stored at −20 °C. A 236-bp DNA fragment in the V3 region of the small subunit ribosomal RNA genes of eubacteria was amplified by using primer set PRBA338f and PRUN518r (Øverås et al., 1997). Ready-To-Go PCR beads (GC Healthcare Biotech, Piscataway, NJ) and 5 pmol of primers in a total volume of 25 μl were used in the PCR reaction. PCR amplifications were done under the following conditions: 92 °C for 2 min; 30 cycles of 92 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min followed by a final extension at 72 °C for 6 min.

DGGE was performed with 8% (wt/vol) acrylamide gels containing a linear chemical gradient ranging from 30 to 70% denaturant with 100% defined as 7 M urea and 40% formamide. Gels were run for 3.5 h at 200 V with the DCode™ Universal Mutation System (Bio-Rad Laboratories, Hercules, CA). DNA was visualized after ethidium bromide staining by UV transillumination and photographed with a Polaroid camera. Major bands were excised for identification of bacterial species. Bands were placed into sterilized vials with 20 μl of sterilized, distilled water and stored overnight at 4 °C to allow the DNA to diffuse out of the gel strips. Ten microliter of eluted DNA was used as the DNA template with the bacteria primers above but without the GC-clamp. DNA was cloned into the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Isolation of plasmids from *E. coli* was performed using the Qiagen plasmid mini kit (Valencia, CA). Four plasmids from each band were sequenced to check for purity of clones. The purified plasmids were sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase, FS (Applied Biosystems, Foster City, CA) with forward and reversed primer M13. Sequence identification was performed by using the BLAST database (National Center for Biotechnology Information: www.ncbi.nlm.nih.gov) to identify the major bands excised from DGGE.

2.5. Primer and probe design for real-time PCR

Genomic DNA was isolated from pure culture of *E. coli* O157:H7, grown for 12 h at 37° C and extracted with the Qiagen tissue kit (QIAamp DNA Mini Kit; Valencia, CA). DNA extracted from *E. coli* O157:H7 was used for the construction of a standard curve and for the determination of detection limits of the pathogen by real-time PCR. Primers and probes used for the detection and quantification of the *stx*1, *stx*2, and the *eae* gene in *E. coli* O157:H7 were as described (Ibekwe et al., 2002; Sharma, 2002). Real-time, quantitative PCR was performed with the iCycler iQ (Bio-Rad, Hercules, CA) as described by Ibekwe et al. (2002). Briefly, PCR was performed in a total volume of 50 μl volume containing 200 μM of dNTPs, 2 μl of genomic DNA from each concentration, 2.5 U of AmpliTaq Gold polymerase, 5 μl of 10x TaqMan buffer (PE Applied Biosystems, Foster City, CA), 0.3 μM of each primer, 0.1 μM of probe, and 3.5 mM of $MgCl_2$. Genomic DNA purified from *E. coli* O157:H7 was used as a template for the positive control and no template for negative control. PCR was performed using the following cycle conditions: denaturation at 95 °C for 10 min, 50 cycles of 94 °C for 20 s, 55 °C for 30 s, 72 °C for 40 s, followed by a 5 min extension at 72 °C and a hold at 4 °C. Standard curves generated from plotting the threshold cycle (C_T) versus $\log_{10}$ of starting DNA quantities (pg) were used for determining the detection limit of the assay. The standard curves were constructed by using known quantities of genomic DNA (2.5×10^{0} to 2.5×10^{-9} pg ml^{-1}) extracted from samples containing 1.6×10^{-2} to 1.6×10^{8} CFU ml^{-1} of *E. coli* O157: H7. Optimization of the multiplex assay was done as previously discussed (Ibekwe et al., 2002; Sharma, 2002). For a comparison of PCR amplification efficiency and detection sensitivity among different experiments, slopes of the standard curves were calculated by performing a linear regression analysis with the iCycle iQ software. The 10-fold serial dilutions of genomic DNA were used to quantify the concentration of the *stx*1, *stx*2, and the *eae* genes. Amplification efficiency (E) was estimated by using the slope of the standard curve and the formula: $E=(10^{-1/\text{slope}})-1$. Reaction with 100% efficiency generated a slope of −3.32.

At the end of the study (86 days) soil samples were enriched in a 10× volume of buffered peptone water (Lab M, Bury, United Kingdom) supplemented with vancomycin (8 mg l^{-1}) for 6 h at 42 °C. This was followed by immunomagnetic separation (IMS) by plating onto Harlequin cefixime–tellurite sorbitol MacConkey (CT-SMAC) agar with BCIG (5-bromo-4-chloro-3-indoxyl-ß-D-glucuronide) containing 0.05 mg of cefixime l^{-1} and 2.5 mg of tellurite l^{-1} (LAB M; IDG). The main aim was to determine if viable cells were still present in the soil after 86 days of incubation.

2.6. Statistical analysis

The comparison of bacterial diversity was done using one-way analysis of variance, and Tukey's studentized range test for post hoc analysis (SAS Institute, 2009). Diversity was calculated by using the Shannon index of diversity (H') to compare changes in diversity of microbial communities within all treatments at each time (Shannon and Weaver, 1963) by using the following function:

$$H' = -\sum P_i \log P_i$$

when $P_i = n_i/N$, n_i is the height of peak, and N is the sum of all peak heights in the curve.

E. coli O157:H7 concentrations were converted to log CFU g^{-1} for regression analysis. The population data were log transformed to obtain a normal distribution of the data. Comparisons between pairs of treatment means at any date were accomplished with the Tukey's test. Plate counts and real time PCR data were transformed to Log_{10} values and survival curves were obtained by plotting the logarithm of survivors against the treatment time. The survival data were fitted to a biphasic model as proposed by Coroller et al. (2006) with the Geeraerd and Van Impe inactivation model-fitting tool (GInaFiT) as shown in Eqs. 1 and 2 and as described by Franz et al. (2008):

$$N(t) = \frac{N_0}{1+10^{\alpha}}\left[10^{-\left(\frac{t}{\delta_1}\right)^{p}+a} + 10^{-\left(\frac{t}{\delta_2}\right)^{p}}\right] \quad (1)$$

$$\alpha = \log_{10}\left(\frac{f}{1-f}\right) \quad (2)$$

where N is the number of survivors, N_0 is the inoculums size; t is the time; p is the shape parameter, when $p>1$ a convex curve is observed; when $p<1$ a concave curve is observed, and when $p=1$a linear curve is observed. The scale parameter, δ, represents the time needed for first decimal reduction. The f, varying from 0 to 1, is the fraction of subpopulation 1 in the population. Another parameter, α, varying from negative infinity to positive infinity, is obtained by logit transformation of f as shown in Eq. 2. The strong correlation between the scale (δ) and the shape (p) parameters makes it possible for the double Weibull model to fit most of the shapes of deactivation curves. Additionally, when $\delta_1=\delta_2$, the double Weibull model can be simplified into a single Weibull model, and the survival curve can be described by only three parameters. A very important and useful parameter, time to detection limit (Td) can also be calculated when using GInaFiT to fit the experimental survival data.

3. Results

3.1. Changes in soil microbial community diversity following fumigation

In the microcosm study, PCR-DGGE was used to examine the effects on soil microbial communities after fumigation with MeBr and MeI. Fig. 1a and b shows the DGGE patterns of the 16S rRNA fragments amplified from the sandy soil as an example, 1 and 12 weeks after fumigation. DGGE patterns in sandy soil are shown because the bacterial community did not completely recover after 12 weeks, as was the case in clay soil (Fig. 1c and d). Diversity indices were significantly higher ($P=0.05$) in clay soil than sandy soil (Fig. 1c and d). Analysis of variance was performed on the total data set to determine the effects of soils, time in weeks, fumigants, and fumigant treatment rates on total microbial community diversity in the two soils. Soil, time, treatment ($P<0.0001$), and fumigant ($P=0.05$) were significant factors affecting diversity indices (data not shown). Within each soil, the Shannon-Weaver index of diversity (Fig. 1c and d) showed that during the first week of the experiment microbial diversity was higher in clay soil than sandy soil ($P<0.0001$). MeBr treated sandy soil with five times the normal application rate showed the most significant decrease in diversity (Fig. 1d). The same treatment during week 2 produced the lowest microbial diversity for sandy soil. During week 5, instability was still observed as differences were observed between treatments. This observation continued during week 12 in sandy soil, but stability in diversity was restored in clay soil (week 5; $P=0.001$; week 12; $P=0.005$ for sandy soil). Major bands were excised, cloned, and sequenced. Most of the bacteria recovered were dominated by *Proteobacteria* (M2, M5, M6, M8, M9), *Firmicutes* (M1 and M4) phyla (Table 1), with some bands from week one disappearing while new bands appeared during week twelve, suggesting the formation of new communities.

3.2. Impact of fumigants on survival E. coli O157:H7 in laboratory microcosm

The survival of the pathogens was studied for 86 day in the microcosm. This provided us with the opportunity to study the relationship between time of survival (days) and population over a longer period of time that may mimic a contamination event in an open

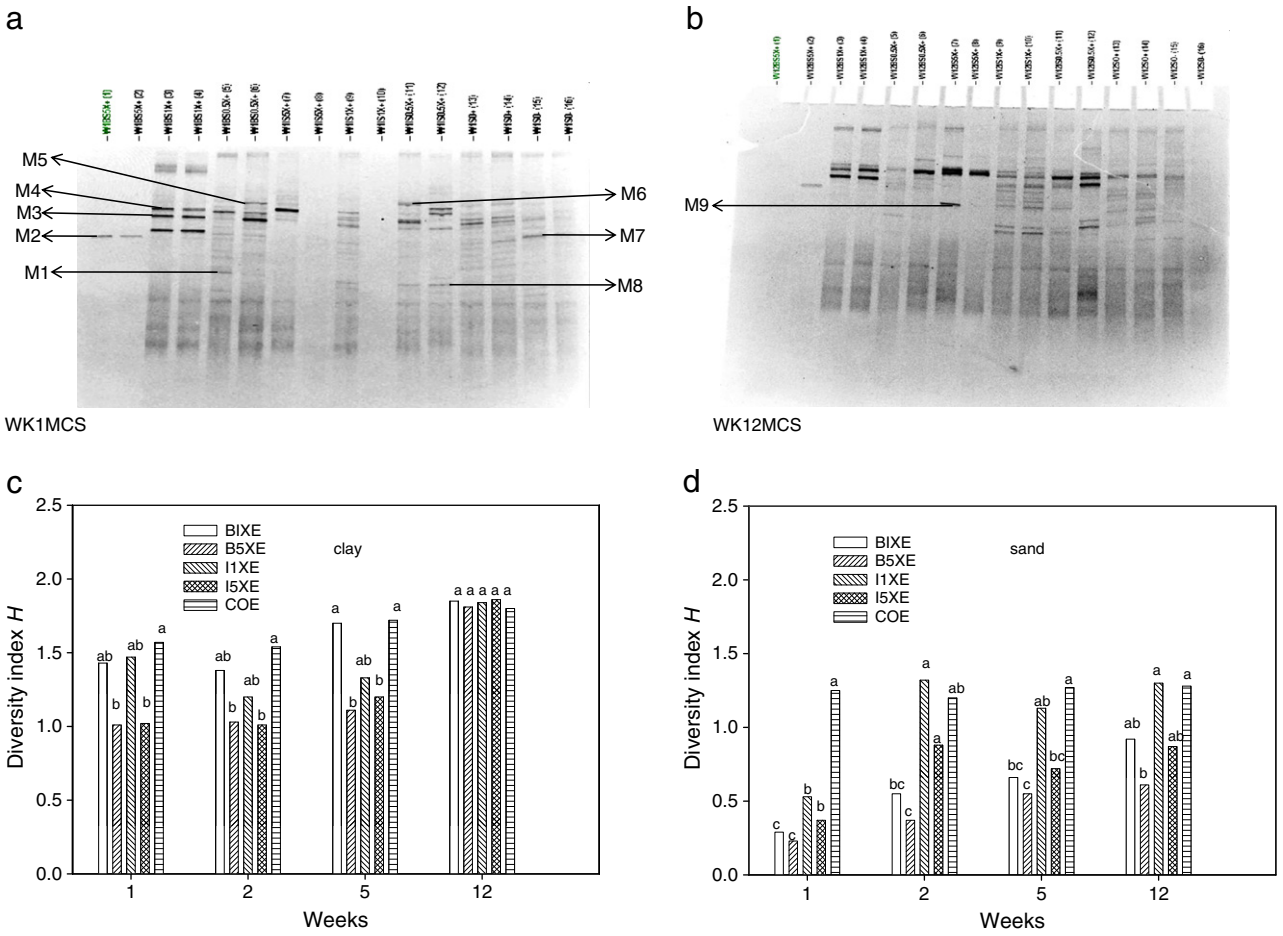

Fig. 1. DGGE analysis of 16S rRNA fragments of total bacterial population extracted from microcosm soil during the study (a) sandy soil one week after fumigation and (b) sandy soil 12 weeks after fumigation. Symbols in (a) indicate W1 (week 1), (B) methyl bromide or (I) methyl iodide treatments, (S) sandy soil, 5x, 1x, 0.5x, and 0 means five times, normal, 50% below normal, and no addition of fumigants, respectively. The + indicates soils were inoculated with *E. coli* O157:H7 and – indicates no *E. coli* O157:H7 were added. Symbols in (b) are similar for week 12 (W12). The numbers in parenthesis represent gel lanes. M1–M9 indicates bands cut from DGGE for bacterial sequence analysis (see Table 1). Numerical analysis of DGGE bands by Shannon index of diversity (*H*) from microcosm soils: clay soil; 1c: sandy soil; 1 day. Means with the same letter are not significantly different at $P \leq 0.05$ using Tukey's Studentized Range Test. B1XE and B5XE indicate treatment with methyl bromide at 5 times and normal field application rate with the inoculation of *E. coli* O157:H7 to the soil. I1XE, I5XE indicate treatment with methyl iodide at five times and normal field application rate, and COE indicates treatment inoculated with the pathogen but without the fumigants.

environment or soil in the presence of other bacterial populations. To this end, background concentrations of heterotrophic bacteria were determined. The initial heterotrophic plate count in soil was 2.1×10^8 CFU g^{-1}. After storage at room temperature in the microcosm, the total aerobic plate counts decreased steadily from ca. 10^8 to ca. 10^6 CFU $g^{-1} \pm 10^2$ during the experimental period in both soils. There were no differences in the levels of heterotrophic plate count in the two soils during the study period (data not shown). Regression analysis with non-linear fitting was used to determine survival of the pathogen in soil after 86 days incubation. Overall, the numbers of *E. coli* O157 showed a significant non-linear decline over time using plate count method ($P = 0.029$ to 0.0018 and $r^2 = 0.47$ to 0.68; Table 2 and Fig. 2). When soils were kept without fumigation, *E. coli* O157 showed a significant decline in population for both sandy (except *stx*1 gene) and clay soils (Table 2). Based on regression analysis, soils fumigated with MeI showed a greater decline in pathogen population than soils fumigated with MeBr. Since there was a non-linear significant fitting in our data, enrichment of the soil samples with IMS showed that the pathogen was still present in our soils (data not shown) thereby confirming the accuracy of statistical analysis and RT-PCR.

Effect of soil type on the survival of *E. coli* O157:H7 in clay and sandy soils after fumigation was modeled by fitting the experimental data to the double Weibull equation shown in Eqs. 1 and 2. Mean comparison was used to determine the impact of fumigants on the survival of *E. coli* O157:H7 in the two soils after fumigation (data not shown). Direct comparison of the two fumigated treatments and the control was done using plate count and real-time PCR to quantify the concentrations of *E. coli* O157:H7. The majority of the survival curves (Fig. 2) showed a concave shape, with a relatively fast initial decline followed by a slower decline phase. Survival reached the detection limit faster in sandy soil (21 days) than in clay soil (28 days) without fumigation using plate counts (Fig. 2a and b). When the pathogen was exposed to MeBr, at the normal application rate, inactivation was faster than in the control,

Table 1
Sequence analysis of bands excised from DGGE gels derived from bacterial 16S rRNA extracted from microcosm soil.

Bands	Related bacterial sequences	Sequence similarity	Accession no.
MI	*Bacillus sp.* 12	100	AY269875
M2	*Gamma Proteobacteria* MS-1	100	AF005656
M3	Unidentified eubacterium (clone LRE12)	99	AJ232875
M4	*Painibacillus lactis* strain MB 1871	99	AY257868
M5	*Photorhabdus luminescens*	100	AY444555
M6	Uncultured *Gamma Proteobacteria*	100	AY911444
M7	Uncultured Bacterium	100	AY853674
M8	*Pseudomonas sp.* 4	98	AY269867
M9	*Pseudomonas aeruginosa*	97	X06684

Table 2
Concentration of *E. coli* O157:H7 recovered from microcosm soils after 86 days of incubation.

Treatments/concentration	Analysis techniques	Regression equation	Pr>F	R^2
S-MB-1x	Plate count	$5.59-0.32\,x+0.003x^2$	0.0072	0.59
	Stx1	$8.01-0.07x+0.0008x^2$	0.2738	0.25
	Stx2	$6.46-0.15x+0.0014x^2$	0.4030	0.18
	eae	$9.01-0.04\,x+0.0004x^2$	0.0001	0.99
S-MB-5x	Plate count	$6.30-0.34\,x+0.0032x^2$	0.0097	0.64
	Stx1	$7.72-0.015x+0.0013x^2$	0.0679	0.45
	Stx2	$7.48-0.15x+0.0014x^2$	0.2746	0.25
	eae	$7.15-0.16x+0.0017x^2$	0.3517	0.21
S-MI-1x	Plate count	$4.93-0.29\,x+0.0027x^2$	0.0292	0.47
	Stx1	$8.37-0.11\,x+0.0008x^2$	0.0009	0.79
	Stx2	$7.59-0.21x+0.0021x^2$	0.0742	0.44
	eae	$9.19-0.06x+0.0004x^2$	0.0028	0.73
S-MI-5x	Plate count	$5.12-0.30\,x+0.0028x^2$	0.0195	0.51
	Stx1	$8.37-0.11x+0.0008x^2$	0.0009	0.79
	Stx2	$8.20-0.22x+0.0019x^2$	0.0560	0.47
	eae	$8.43-0.19\,x+0.0023x^2$	0.0039	0.71
S-0x	Plate count	$8.76-0.28\,x+0.0021x^2$	0.0001	0.90
	Stx1	$8.37-0.09\,x+0.0009x^2$	0.2754	0.25
	Stx2	$9.21-0.21\,x+0.0018x^2$	0.0391	0.51
	eae	$9.19-0.11\,x+0.0012x^2$	0.0025	0.73
C-MB-1x	Plate count	$6.69-0.36\,x+0.0033x^2$	0.0020	0.68
	Stx1	$7.69-0.10\,x+0.0007x^2$	0.0980	0.40
	Stx2	$7.31-0.21\,x+0.0018x^2$	0.0172	0.60
	eae	$7.89-0.11\,x+0.0012x^2$	0.5530	0.12
C-MB-5x	Plate count	$6.40-0.35\,x+0.0032x^2$	0.0021	0.68
	Stx1	$7.64-0.11x+0.0010x^2$	0.1245	0.37
	Stx2	$7.03-0.25\,x+0.0023x^2$	0.0097	0.64
	Eae	$8.04-0.07x+0.0002x^2$	0.0026	0.73
C-MI-1x	Plate count	$6.47-0.35\,x+0.0032x^2$	0.0019	0.68
	Stx1	$9.03-0.24\,x+0.0025x^2$	0.0073	0.67
	Stx2	$8.65-0.29x+0.0031x^2$	0.0001	0.94
	eae	$9.67-0.26x+0.0028x^2$	0.0193	0.58
C-MI-5x	Plate count	$6.67-0.35x+0.0033x^2$	0.0018	0.68
	Stx1	$8.10-0.13x+0.0013x^2$	0.2763	0.25
	Stx2	$8.85-0.29\,x+0.0029x^2$	0.0001	0.94
	eae	$9.35-0.27x+0.0029x^2$	0.0305	0.54
C-0x	Plate count	$6.71-0.36\,x+0.0033x^2$	0.0018	0.68
	Stx1	$9.65-0.25x+0.0026x^2$	0.0060	0.68
	Stx2	$8.90-0.23x+0.0024x^2$	0.0130	0.62
	eae	$9.49-0.24x+0.0022x^2$	0.0462	0.50

S or C represents sandy or clay soil; MB or MI represent methyl bromide or methyl iodide. The numbers 5x, 1x, and 0x represent five times the normal application rate, normal fumigant application rate, and no fumigant application, respectively. *E. coli* O157:H7 were enumerated from microcosm soils by plate count and by real-time PCR (RT-PCR) using *stx*1, *stx*2, and the *eae* genes. Regression equations are $\log_{10}$ data showing all the treatments presented in Fig. 2.

especially using plate counts (Fig. 2c and d). The same pattern was observed with MeI (Fig. 2e and f). However, for both control treatments the population size did not reach the detection limit (ttd) of 10^2 CFU g^{-1} during the experiment due to an earlier onset of tailing at about 35 days using real-time PCR. Furthermore, both soils showed that it took less than a day to inactivate the first $\log_{10}$ of microbial population in most of the fumigated samples. At a higher concentration of fumigants (5x), it took less than 10 days for the inactivation of the pathogen to fall below the detection limit of 10^2 CFU g^{-1} (Fig. 2g and h) using plate counts.

Modeling parameters (alpha (α), delta (δ), and the shape parameter-p) were calculated from Eqs. 1 and 2 and used to explain the inactivation kinetics. More variations were observed in δ values from different soils (Fig. 3). When the strain was characterized in sandy and clay soils, distinct δ_1 and δ_2 were observed (as indicated by the differences in bar and error bar sizes) indicating the existence of two subpopulations and these behaved differently in both soils, thus the survival data in both soils might not be simplified into the single Weibull model that can be described by only three parameters, α, δ and p, in the majority of the cases. The initial sharp decrease in cell numbers in sandy soil (concave shape; Fig. 2) is attributed to the faster decline of the initial subpopulation as shown with smaller δ_1 (Figs. 2 and 3). However, with longer incubation time, the subpopulation with greater δ_2 (i.e. the more resistant population to fumigants) dominated the cell population, leading to a slower and steady decline of the cell concentration as the curves showed little or no decline, especially with the real time PCR method. Similar modeling parameters (α, δ, and p) were calculated when they were inoculated into the same soil (Fig. 3a–h). When the pathogen was characterized in sandy and clay soils, distinct δ_1 and δ_2 were observed indicating that the two subpopulations behave differently in both soils.

3.3. Influence of microbial diversity on E. coli O157:H7 survival

Bacterial species richness and microbial diversity as determined by Shannon Weaver index of diversity, *H*, from DGGE was significantly lower in sandy soil than in clay soil (data not shown). Microbial diversity was negatively correlated with survival of *E. coli* O157:H7 in the clay soil ($r^2=0.39$; $P=0.019$), but positively correlated with survival of *E. coli* O157:H7 in sandy soil ($r^2=0.49$, $P=0.043$) using the plate count method (Fig. 4a and b). However, survival of *E. coli* O157:H7 using data from real-time PCR analysis were positively correlated with microbial diversity in both clay and sandy soils (data not shown).

4. Discussion

We have shown from this study that *E. coli* O157:H7 can persist for more than 86 days in soils under different rates of MeBr and MeI application and different soil types. Our study showed that MeI

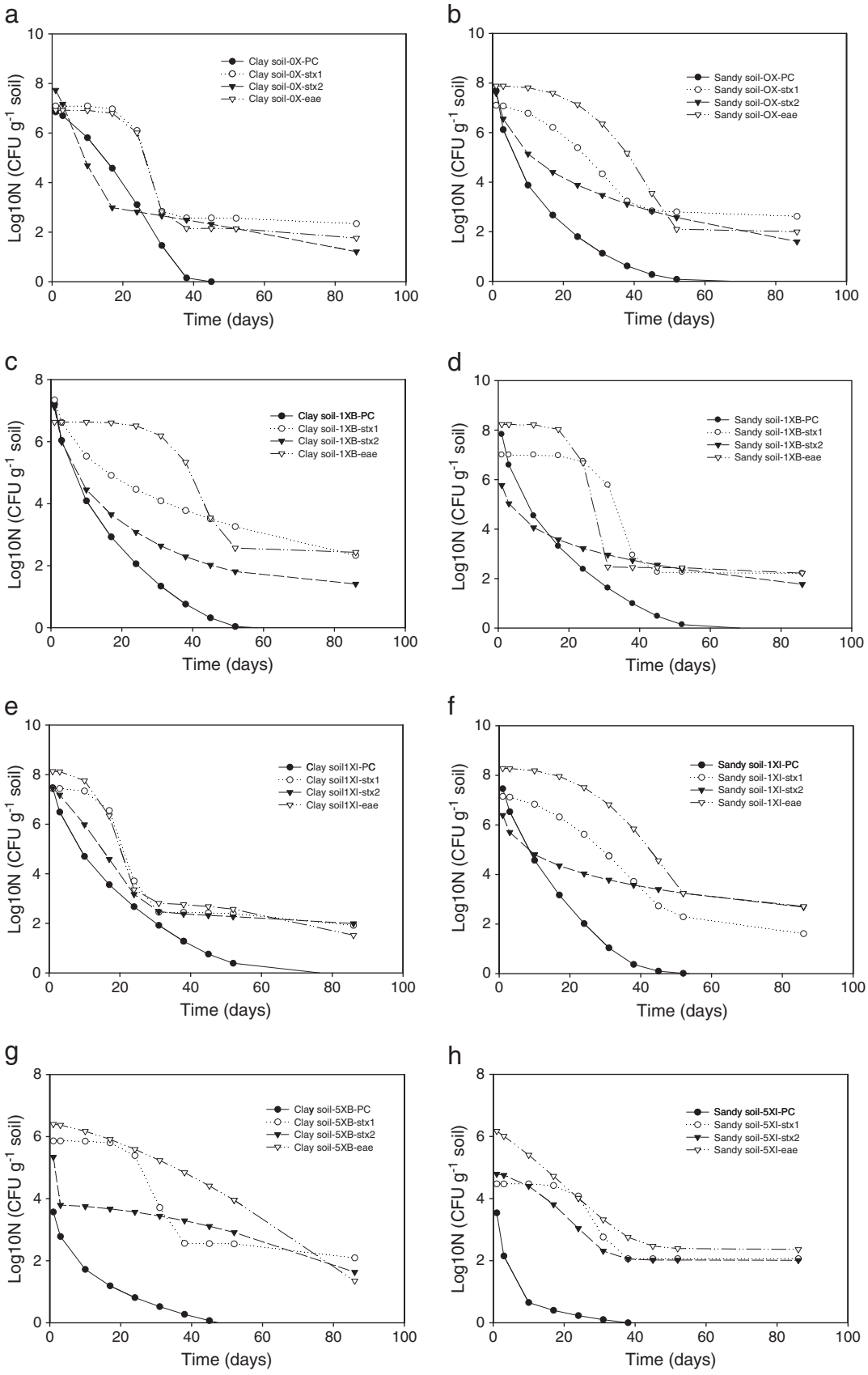

Fig. 2. Quantification of *E. coli* O157:H7 persistence in soil microcosms after 86 days in non-fumigated and fumigated soils inoculated with *E. coli* O157:H7. None fumigated control: a (clay soil), b (sandy soil) without fumigant treatments; c (clay soil), d (sandy soil) with normal application rate of MeBr; e (clay soil), f (sandy soil) with normal application rate of MeI; g (clay soil with five times the normal application rate of MeBr), h (sandy soil) with 5 times normal application rate of MeI; *E. coli* O157:H7 were enumerated from microcosm soils by plate count (PC) and by real-time PCR (RT-PCR) using *stx*1, *stx*2, and the *eae* genes.

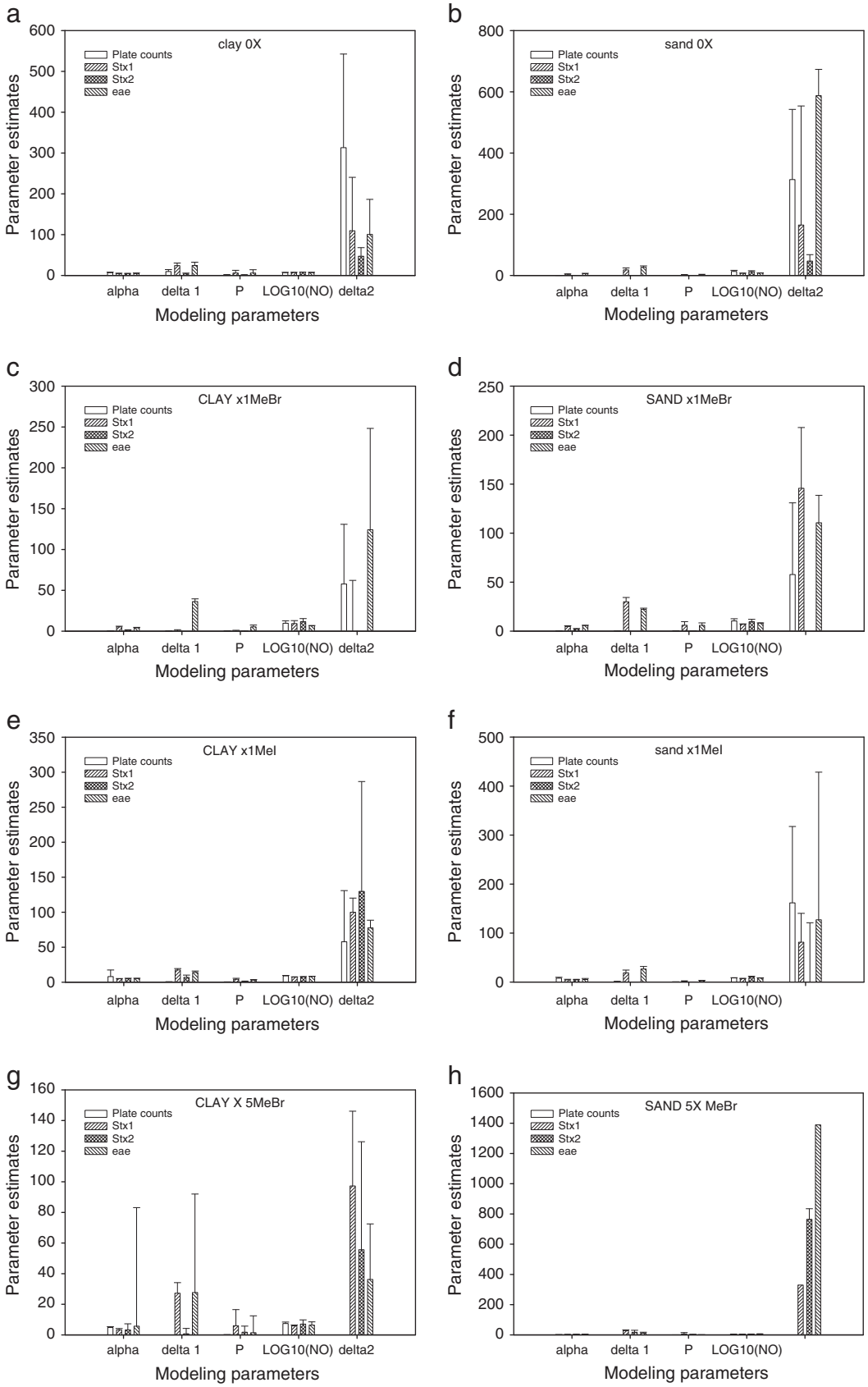

Fig. 3. Double Weibull Model parameters of *E. coli* O157:H7 in nonfumigated clay and sandy soils (3a and b); fumigated with normal rate of MeBr application in clay and sandy soils (3c and d); fumigated with normal rate of MeI application in clay and sandy soils (3e and f) and fumigated with five times the normal application rate of MeBr in clay and sandy soils (3g and h). There is very little variability in delta 1 because this is a fast and rapid die off with very small error bars. However, in delta 2 there are much more variabilities resulting in higher error bars.

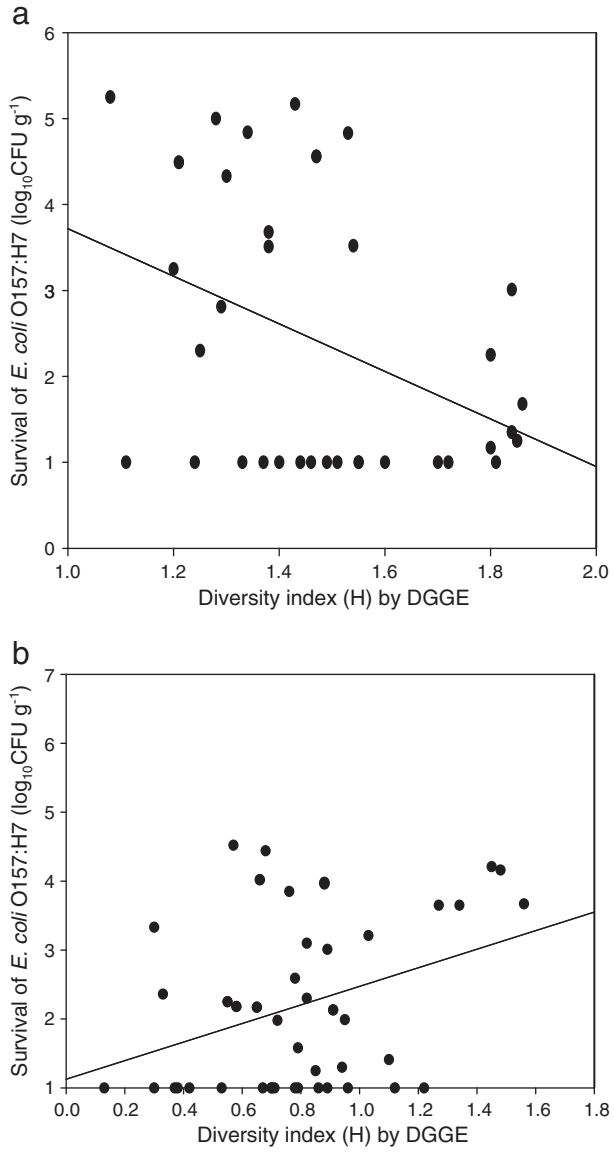

Fig. 4. Influence of microbial diversity on the survival of *E. coli* O157:H7. Observed points (●) and relations (solid lines between survival and microbial diversity in soil). (a) Clay soil and (b) sandy soil. Microbial diversity was negatively correlated with survival of *E. coli* O157:H7 in the clay soil ($r^2 = 0.39$; $P = 0.019$), but positively correlated with survival of *E. coli* O157:H7 in sandy soil ($r^2 = 0.49$, $P = 0.043$) using the plate count method in clay and sandy soils, respectively.

fumigation had a greater impact on *E. coli* O157:H7 population decline than MeBr in sandy soil but no differences were found in clay soil. The main reasons for this may be soil texture and microbial diversity. In soil, *E. coli* O157:H7 will interact with the local biota, including the microbial communities, and the cumulative effect of the total indigenous microflora on *E. coli* survival is often negative as a result of predation, substrate competition and antagonism (Jiang et al., 2002; Unc et al., 2006; Semenov et al., 2007). The diversity of the indigenous microbial communities has been brought up as an important factor that regulates the population dynamics of *E. coli* (van Elsas et al., 2007). According to these authors, ecosystems with a higher level of biodiversity are more resistant to perturbances than those with a lower diversity (Tilman, 1997; Trevors, 1998). Consequently, the former habitats would be less susceptible to invasion by *E. coli* than the latter (Girvan et al., 2005; Semenov et al., 2008).

For field plots located in the main strawberry production areas of California, Stromberger et al. (2005) reported that, MeI eliminated soilborne fungal pathogens and reduced culturable fungal populations up to 4 weeks after fumigation. Soil microbial respiration, enzyme activity, and potential nitrification rates were also decreased with fumigant application, indicating a significant impact of the fumigants on the microbial flora and fauna. Comparative studies of MeBr and MeI (Hutchinson et al., 2000) showed that MeI was, on average, 2.7 times more efficacious than MeBr at killing fungal species. These studies and others from our laboratory and review papers have confirmed the efficacious abilities of MeI compared to MeBr in preplant fumigation as a strong alternative replacement for MeBr (Ashworth and Yates, 2010; Luo et al., 2010). It has also been shown that MeBr and MeI behave differently in soils under the same environmental conditions because MeI degrades rapidly by photolysis and has an estimated atmospheric lifetime of <10 days, compared to 1.5–2 years for MeBr (Ruzo 2006). Ibekwe et al. (2007) reported that MeI and MeBr were effective in reducing both the concentration of *E. coli* 0157 in soil, and the survival of the pathogen on lettuce leaf surface (Ibekwe et al., 2009), suggesting that the fumigants may have played some role in reducing the transfer of *E. coli* 0157 from soil to leaf. This was a short term study with plants and did not show if the pathogen could persist in soil after fumigation for more than the five weeks used in the growth chamber study. However, the detection of the pathogen by IMS and by RT-PCR during the long term microcosm study suggests that very few cells were still viable in the soil after 86 days, but at numbers below the detection limit of 10^2 CFU g^{-1}. This result was confirmed by the non-linear fitting of our data in Table 2 and in Fig. 2. This was also confirmed by the presence of distinct δ_1 and δ_2 subpopulations (Fig. 3). Most of the subpopulations in δ_2 may be viable but non-culturable cells (dormant) as cells at this stage cannot be easily recovered on standard laboratory media, but are still present as viable cells. For instance, in an experiment with *E. coli* O157:H7 in manure, significantly higher numbers of the organism were found by direct microscopic counts than by plating on a selective medium (Semenov et al., 2007). The state can be triggered by stress conditions that are imposed on the pathogen, for instance, as in our study; application of fumigants to the soil may induce stress on *E. coli* O157:H7.

We have shown from this study that *E. coli* O157:H7 will persist for over 86 days due to the presence of dormant but viable cells that were resuscitated by enrichment. We did not study the genetic mechanisms of the subpopulations responsible for long term persistence in the fumigated soils. However, in a biofilm environment, bacterial tolerance to antimicrobials has been hypothesized to involve growth-stage dependent production of specialized survivor cells termed 'persisters' (Harrison et al., 2005; Spoering and Lewis, 2001; Keren et al., 2004). It has also been shown that genetically homogenous bacterial populations, grown in planktonic culture or surface adherent biofilm, produce subpopulations that survive exposure to high concentrations of bactericidal antibiotics (Stewart, 2003; Balaban et al., 2004). These authors have concluded that persisters are highly tolerant of antimicrobials because they do not die, and further, may represent a recalcitrant population that can seed a new culture with normal susceptibility. In *E. coli*, Balaban et al. (2004) associated persister populations with slow growth phenotypes. It has also been suggested that persisters represent the stationary phase bacterial population relative to the logarithmic-growing bacterial cultures (Keren et al., 2004; Mulcahy et al., 2010; Spoering and Lewis, 2001). Persistence was therefore linked to preexisting heterogeneity in bacterial populations because phenotypic switching occurred between normally growing cells and the persister cells having reduced growth rate. Data from our study has shown that persistence phenomenon observed in biofilm (Harrison et al., 2005) and microfluidic devices (Balaban et al., 2004) may be likely occurring in soil. It is also of great concern when dealing with pathogens such as *E. coli* O157:H7 where cattle are the main reservoir, because manure management to kill off this pathogen remains an unresolved issue both in developed and developing countries.

In conclusion, the non-linear fitting observed in the study suggested that *E. coli* O157:H7 persisters may have been selected during the long incubation period with fumigants, and this may have

increased the chances of *E. coli* O157:H7 population survival in the microcosm soils. Further studies are needed on treatment technologies that will kill 100% of the pathogen in manure waste on site. In this way, the potential for leaching to sub-surface soil from the manure pile, and for runoff to surface water, could be prevented. The observed persistence of *E. coli* O157:H7 in soil may be a significant factor in its contamination cycle that may result in recontamination of produce, surface, and ground water after the initial contamination event.

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