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ORIGINAL ARTICLE

Structural divergence of bacterial communities from functionally similar laboratory-scale vermicomposts assessed by PCR-CE-SSCP

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Abstract

Aims: To evaluate bacterial community structure and dynamics in triplicate vermicomposts made from the same start-up material, along with certain physico-chemical changes.
Methods and Results: The physico-chemical parameters (pH, temperature, carbon, nitrogen, soluble substances and cellulose) evolved similarly in the triplicate vermicomposts, indicating a steady function. The 16S bacterial gene abundance remained constant over time. To monitor changes in the bacterial community structure, fingerprinting based on capillary electrophoresis single-strand conformation polymorphism was employed. A rise in bacterial diversity occurred after precomposting and it remained stable during the maturation phase. However, a rapid shift in the structure of the bacterial community in the vermicompost replicates was noted at the beginning that stabilized with the process maturation. Multivariate analyses showed different patterns of bacterial community evolution in each vermicompost that did not correlate with the physico-chemical changes.
Conclusions: The broad-scale functions remained similar in the triplicates, with stable bacterial abundance and diversity despite fluctuation in the community structure.
Significance and Impact of the Study: This study has demonstrated that microbial fingerprinting with multivariate analysis can provide significant understanding of community structure and also clearly suggests that an ecosystem's efficacy could be the outcome of functional redundancy whereby a number of species carry out the same function.

Introduction

Studies related to ecosystem functions and diversity has been carried out extensively in the past at both the producer and consumer levels (Schläpfer and Schmid 1999). The introduction of molecular techniques to microbial ecology has facilitated the analysis of the bacterial community structures and their contribution as ecosystem decomposers. Yet we know little about either the effects of abiotic resources on an ecosystem's stability or the impact of decomposer diversity. A number of studies have shown that the broad-scale functional ability of a microbial community is determined by its diversity. Redundancy of function may also be important for understanding the stability of microbial communities and how the ecosystem functions (Franklin and Mills 2006). Fernandez *et al.* (1999) found that an extremely dynamic community sustains a functionally stable ecosystem and also indicated that functional parameters like pH and chemical oxygen demand (COD) are insufficient indicators for understanding variations in community structure in a methanogenic reactor. Several studies on community

structure and functions have been performed on methanogenic and activated sludge reactors (Godon *et al.* 1997; Zumstein *et al.* 2000; Kaewpipat and Grady 2002; Saikaly *et al.* 2005; Nadarajah *et al.* 2007). All such recent observations in various laboratory-scale ecosystems have led to the conclusion that bacterial communities may evolve independently in identically operated bioreactors performing with similar efficiency. These findings have thus raised questions about the inter-relationship of a functionally stable ecosystem and a stable community.

A recent study has shown that sugar industry waste can be composted with the help of earthworm *Eudrilus eugeniae*: the C/N fell from 21 to 10 within 40 days, along with a reduction in the lignocellulosic fraction (lignin, cellulose and hemicellulose) and an increase in soluble substances (Sen and Chandra 2007). This study has also suggested that the vermicomposting process may be a model microbial ecosystem wherein a rapid and similar transformation of organic matter is replicated. Vermicomposting is an accelerated process of biooxidation and stabilization of organic material that, in contrast to traditional composting, involves the joint action of earthworms and micro-organisms and does not involve a thermophilic stage (Domínguez and Edwards 2004). Earthworms make the substrate more favourable to microbial activity and enhance decomposition by acting as mechanical blenders (Domínguez 2004). However, the resident bacteria play a major role in the decomposition of organic matter during the vermicomposting process.

Past studies on the microbiology of traditional composting achieved the isolation and description of various microbes present in compost using culture-dependent methods and classified the bacterial communities according to temperature profiles during composting (Ryckeboer *et al.* 2003). Up to now, there have been few reports on community dynamics during the composting process, using sequence analysis combined with single-strand conformation polymorphism (SSCP; Peters *et al.* 2000) and denaturing gradient gel electrophoresis (DGGE; Ishii *et al.* 2000). However, little attempt has been made in understanding the structure of bacterial communities and community assemblages during a functionally steady vermicomposting process at a constant temperature and pH.

In order to investigate community dynamics during a functionally steady process and discover whether such dynamics reoccur in vermicomposts, we monitored the temporal changes in the three vermicompost bacterial communities by using capillary electrophoresis (CE)-SSCP fingerprinting of the 16S rRNA gene. Although rRNA-based approaches may be affected by a number of factors such as biased sample collection, differential cell lysis during nucleic acid extraction or differential polymerase chain reaction (PCR) amplification (von Wintzingerode *et al.* 1997), such techniques appear to offer broader scope than culture-based analyses (DeLong and Pace 2001). Fingerprinting analyses provides a faster and less labour-intensive approach for comparing community composition in many different samples than the sequencing of clone libraries (Delbès *et al.* 2000; Burr *et al.* 2006; Peu *et al.* 2006). However, all fingerprinting methods only generate banding patterns and do not provide any sequence information (Schmalenberger and Tebbe 2003). Moreover, band identification is time-consuming and only provides limited information due in part to the shortness of DNA fragments used. Capillary analysis, in contrast to gel banding patterns, has notable advantages such as better resolution and the presence of an internal standard. In addition, the complex data sets obtained from CE-SSCP profiles can be subjected to multivariate statistical analysis that can quantitatively interpret the genetic similarity of samples in a two-dimensional plot in which the most dissimilar samples appear at the greatest distance apart. Such similarity is not readily interpretable from raw CE-SSCP profiles (Fromin *et al.* 2002). Multivariate techniques have the advantage that datasets can comprise a variety of variables and need not be limited to species lists (Rees *et al.* 2004).

The aim of this work was to evaluate the bacterial community dynamics and diversity of a triplicate laboratory-scale vermicomposting process and to assess any relationship between physico-chemical changes and microbial community structure. The application of the CE-SSCP technique and multivariate statistical approaches to microbial community analysis of complex ecosystems was also evaluated. We believe this study provides a cogent report on the divergent dynamics of microbial communities in replicate vermicomposts.

Materials and Methods

Vermicomposting setup

A laboratory-scale vermicomposting of sugar industry waste, made up of pressmud, trash, bagasse and dried cattle manure in 7 : 1 : 1 : 1 w/w ratio, as start-up material (SM), was carried out in cement bins with an internal top dimension of 35·5 × 35·5 cm, a bottom dimension of 26·5 × 26·5 cm and a depth of 33·5 cm. To get a homogenous SM mixture, pressmud and dried cattle manure were powdered while trash and bagasse were finely cut into sizes of <0·5 inches. Three separate bins were set up for the experiment to replicate the same process. Each bin contained 2·5 kg of SM mixture that was precomposted for 4 days to lower the initial rise in temperature (35 ± 1°C), the equivalent in this experiment to the usual

thermophilic phase. As earthworms are sensitive to high temperature, an identical biomass of earthworms (*E. eugeniae*) was inoculated (15 g kg^{-1} of SM) after the end of the precomposting (thermophilic) stage. Day 0 indicates the day when earthworms were added after the four days of precomposting. After complete mixing of the composted SM, 200 g were sampled at regular 10-day intervals, representing *c.* 1/12th of the original SM mixture. The earthworms were removed from the collected samples and put back into the respective bins. Each sample (*c.* 200 g) from each bin, consisting of both decomposed and undecomposed material as well as earthworm castings, was thoroughly mixed in a blender for homogenization. The moisture content was kept at a level of 60–70% by sprinkling an equal volume of water at regular intervals.

Analysis of physico-chemical parameters

Samples were oven-dried at 105°C, then ground in a blender and sieved. Total organic carbon (carbon content) was determined by wet oxidation with potassium dichromate. Total Kjeldahl nitrogen (nitrogen content) was determined (Jackson 1973). pH was determined in the water extract (sample : water, 1 : 2·5) at 30°C. The temperatures were taken with a thermometer inserted deep within the vermicompost bins. Soluble substances (water-soluble or ethanol : benzene-soluble) and cellulose content were analysed after lignocelluosic biofractionation (Thronber and Northcote 1961).

Genomic DNA extraction

Nucleic acid extraction was performed using the Ultra Clean Soil DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA, USA) according to the manufacturer's instructions. Samples weighting about 0·25 g were processed in each case for DNA isolation. DNA was isolated at a regular interval of 10 days. Isolated genomic DNA was checked for its quality on 0·8 % agarose gel and stored at −20°C prior to analysis.

Amplification of the V3 region of the 16S rRNA gene

One microlitre of genomic DNA samples was analysed by PCR amplification of the 16S rRNA gene V3 region using the primers w49 (5′-ACGGTCCAGACTCCTACGGG-3′, *Escherichia coli* position F330) and 5′-6FAM labelled w104 (5′-TTACCGCGGCTGCTGCTGGCAC-3′, *E. coli* position R533) (Delbès *et al.* 2000). Each reaction tube contained 1·3 μl of each primer (100 ng μl^{-1}), 1 μl of template DNA, 5 μl of buffer (10× Pfu Turbo), 4 μl of dNTP (2·5 mmol l^{-1}), 0·5 μl of Pfu Turbo (2·5 U μl^{-1}; Invitrogen) and was adjusted to a final volume of 50 μl. The reaction tubes were placed in a thermocycler (Mastercycler epgradientS; Eppendorf) for 2 min at 94°C. Twenty-five cycles were then performed for 30 s at 94°C, 30 s at 61°C, 30 s at 72°C, with a final extension step of 10 min at 72°C. The PCR product sizes were checked on 2% agarose gel and the bands were detected with ethidium bromide. The expected size of the band was around 200 bp in length, and was checked using a 100 bp DNA ladder (Invitrogen).

CE-SSCP procedure

The PCR products were diluted between 3 and 10 times depending on the band intensity on the gel. One microlitre of each PCR product was mixed with 18·8 μl of formamide and 0·2 μl of internal standard GeneScan ROX (Applied Biosystems). Samples were heat-denatured at 95°C for 3 min and immediately placed on ice. They were then subjected to capillary electrophoresis for 35 min in an ABI Prism 3130 genetic analyser (Applied Biosystems) with four 50-cm capillary tubes. The capillary tubes were filled with 5·6% of conformation analysis polymer (Applied Biosystems) in the corresponding buffer and 10% glycerol. Raw binary data were converted into csv text files using Chromagna (M.J. Miller, personal communication) for statistical interpretation.

Real-time PCR assay conditions

The amplification reactions were carried out in a total volume of 25 μl. All reaction mixtures contained 5 μl of template DNA and 1× TaqMan universal PCR master mix (Applied Biosystems). A mix of 756 nmol l^{-1} of primer w102 (5′-CGGTGAATACGTTCYCGG-3′ *E. coli* position F1369), 500 nmol l^{-1} of primer w105 (5′-GGWTACCTTGTTACGACTT-3′, *E. coli* position R1492) and 250 nmol l^{-1} of TaqMan probe w101 (5′-(6FAM) CTTGTACACACCGCCCGTC Tamra-3′, *E. coli* position 1389) was added to amplify 16S rDNA (Suzuki *et al.* 2000). Amplification reactions were run on an ABI Prism 7000 SDS (Applied Biosystems). Reactions took place after a sequence of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles: 15 s at 94°C and 1 min at 56°C. All tests were performed in triplicate. Results were then compared with a standard curve to obtain the number of target copies in the sample. The standard DNA curves were generated by amplification of the serial 10-fold dilutions of a reference clone (Rousselon *et al.* 2004). The cycle threshold (C_T) corresponded to the cycle number at which the reaction became exponential. The sample's C_T was compared with a linear standard curve plotted with C_T *vs* gene copies number. The results were expressed as the number of target copies in the sample. The regression coefficient value for the standard DNA curve was always above 0·99.

Statistical analysis

Both principal component analysis (PCA) and redundancy analysis (RDA) were performed on CE-SSCP profiles. Raw CE-SSCP data were log-transformed to minimize the contribution of large peaks in the analysis. PCA is useful for discerning patterns within the CE-SSCP data itself while RDA can be used to test hypotheses regarding the importance of physico-chemical parameters in explaining variation in CE-SSCP data. PCA describes the axes of maximum variability in the multivariate data set. RDA is a constrained ordination technique in which ordination axes are constrained to be linear combinations of environmental variables (Legendre and Legendre 1998). This later method allows direct assessment of the relationship between known environmental factors and variation in the multivariate data. The significance of the relationship was tested using Monte Carlo tests with 100 permutations. The R package (version 2·1·0) was used (R Development Core Team 2005). CE-SSCP profiles were aligned with the internal standard (GeneScan ROX) in the CE-SSCP procedure and the entire area of CE-SSCP patterns was normalized prior to statistical analysis using MATLAB ver. 7·0·4 (The MathWorks, Inc., Natick, MA). The relative sub-peak background areas were computed using SAFUM (Zemb *et al.* 2007). As parameters were measured in different units, physico-chemical data were standardized to zero mean and unit variance prior to redundancy analysis.

Results

Physico-chemical changes during vermicomposting of sugar industry waste

Figure 1 shows the variations in carbon, the C/N ratio, soluble substances and cellulose content during the 60-day process of vermicomposting sugar industry waste combined with dried cattle manure. Temperature and pH changes were not significant during the 60-day process, with temperature maintained around 30 ± 2°C and a neutral pH of 7·3 ± 0·2. A constant reduction in carbon from 38% to 27% was observed with an increase in nitrogen from 1·8% to 2·8%. A typical steady decrease in the value of C/N ratio from 22 to 10 was noted throughout the process. Soluble substances (sugars, amino-acids, organic acids, fats, oils, waxes, resins, pigments, proteins and minerals) were found to increase from 142 to 360 mg g^{-1} dry weight, whereas a decreasing trend in cellulose content from 320 to 136 mg g^{-1} dry weight was noted.

Bacterial community structure during the period of vermicomposting process

The bacterial abundance at three different stages, i.e. early (day 0), mid (day 30) and end (day 60) was evaluated through the quantitative PCR technique. The mean 16S rRNA gene copies per gram of sample was stable

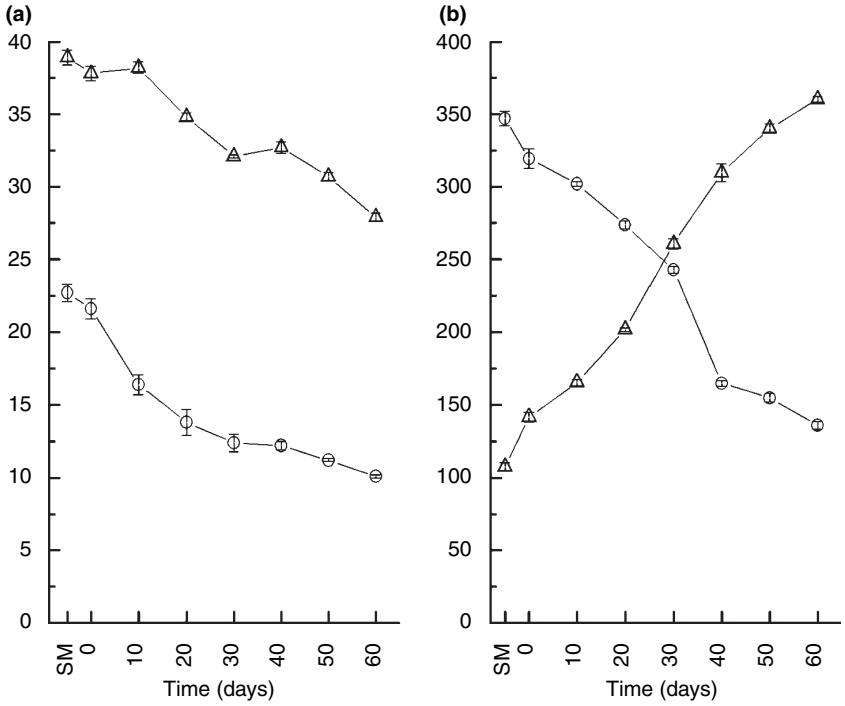

Figure 1 Variation over time in the physico-chemical parameters during the vermicomposting process. Values of symbols indicate mean ± SD of triplicates. (a) (—△—), carbon (%) and (—○—), C/N (ratio); (b) (—△—), soluble substances (mg g^{-1}), (—○—) cellulose (mg g^{-1}).

($P < 0{\cdot}05$), varying within the range of $1{\cdot}7 \times 10^8$–$6{\cdot}8 \times 10^8$. Figure 2 illustrates the CE-SSCP fingerprints representing progressive changes in the bacterial community over time based on a seven-point time frame (0–60 days) for triplicate samples along with that of the starting material. The CE-SSCP fingerprint of the starting material appears as very different from the time-series profiles. The background area in the CE-SSCP fingerprints as shown in Fig. 3a varied within 80–90% of the total area in the time series, indicating large overlaps of CE-SSCP peaks. On the contrary, SM sample had much lower diversity, with a background area of 70%, possibly indicating an initial rise in bacterial diversity during the precomposting phase.

The mean change of genetic profiles between two consecutive samplings at a 10-day interval decreased over time (Fig. 3b). Furthermore, the replicate variation also lessened with compost maturation (Fig. 3c). Finally at the end of maturation, the mean genetic distance of CE-SSCP profiles of replicates were similar to the mean distance of 10-day consecutive samples taken after day 30. This clearly indicates that the CE-SSCP fingerprints do in fact represent the actual variations in the entire community structure of the vermicomposting process and, thus, profiling of sub-samples from each replicate was not required.

To visualize and distinguish between the bacterial communities present in the replicates and in the starting material, a PCA was done on the CE-SSCP profiles (Fig. 4). The entire CE-SSCP signal was taken for analysis, as opposed to the detectable peak areas/heights (e.g. in the Applied Biosystem GeneMapper software), because it takes into account the complete picture of the fingerprint. PCA on raw data lowers any bias associated with the accuracy of peak detection and the common assumption that each peak denotes a single species, which is not the case in complex molecular fingerprints with overlapping peaks (Loisel *et al.* 2006). PC 1 and 2 displayed 69% and 12% of the variance, respectively (Fig. 4). Consecutive CE-SSCP fingerprints in the time series from each replicate grouped separately, indicating that the microbial communities developed differently.

Relationship between bacterial community dynamics and physico-chemical evolution

A redundancy analysis was carried out to relate the bacterial community dynamics to physico-chemical parameters (Fig. 5). Variance explained by the first and second RDA axes constrained by the physico-chemical parameters were 12·6% and 2·1%, respectively. Both axes together accounted for only 14·7% of the variance in the CE-SSCP data. In addition, the Monte Carlo test for physico-chemical parameters (carbon, C/N ratio, soluble substances and cellulose) were not significant enough (P-value of 0·51) to explain the differences observed in CE-SSCP data. This indicated that the physico-chemical evolution was not correlated to bacterial community dynamics.

Discussion

We monitored the overall bacterial community structure, dynamics and abundance in three replicates of vermicompost using molecular fingerprinting and quantitative PCR techniques.

Using multivariate statistical analysis, we found significant differences in the bacterial composition and dynamics within the three replicates that did not correlate significantly with the physico-chemical changes.

Methodological considerations concerning CE-SSCP fingerprinting

Molecular fingerprinting (CE-SSCP) analysis is a PCR-based method, which can be biased (Suzuki and Giovannoni 1996; von Wintzingerode *et al.* 1997). However, it has been shown that both gel and capillary-based PCR-SSCP may adequately reflect the relative composition of 16S rRNA genes (Schwieger and Tebbe 1998; Fraccia *et al.* 2006). Regarding the composition of agri-industrial residues as substrates and the use of the same earthworm species, the samples evaluated in this study were of a similar nature, and they were all investigated using the same DNA extraction method and PCR conditions. The current CE-SSCP approach does not prevent hetero-dimer formation (Schwieger and Tebbe 1998). However, as all samples were probably affected equally by a potential bias, comparison of the different CE-SSCP fingerprints remains accurate. In addition, CE-SSCP fingerprinting patterns contain extractable data on structure and diversity. Here the background area under the CE-SSCP profiles was taken as a parameter of bacterial diversity as peak (p) saturation is seen when diversity is very high, i.e. $P \geq 50$ (Loisel *et al.* 2006).

Steady function and bacterial dynamics of triplicate vermicompost communities

During the vermicomposting process, the rates of physico-chemical changes (Fig. 1) were very similar in the triplicates with constant temperature and pH. In our study, the similarity in the broad-scale ecosystem function, i.e. the reduction of C/N, the most commonly used index of decomposition for organic matter, indicated a steady

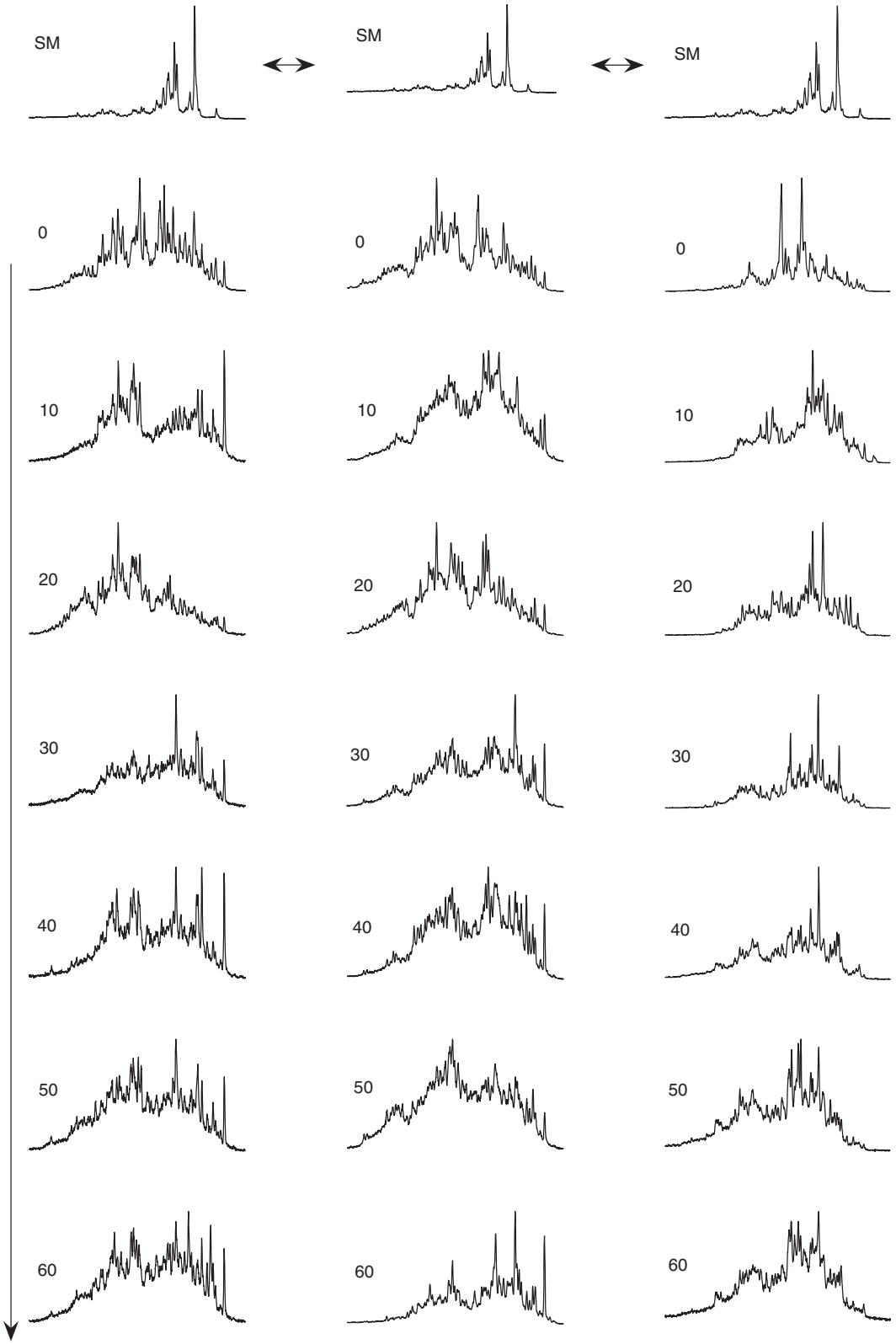

Figure 2 Capillary electrophoresis single-strand conformation polymorphism profiles of the vermicompost samples collected at 10-day intervals (days 0 to 60) from the triplicates, along with the start-up material sample.

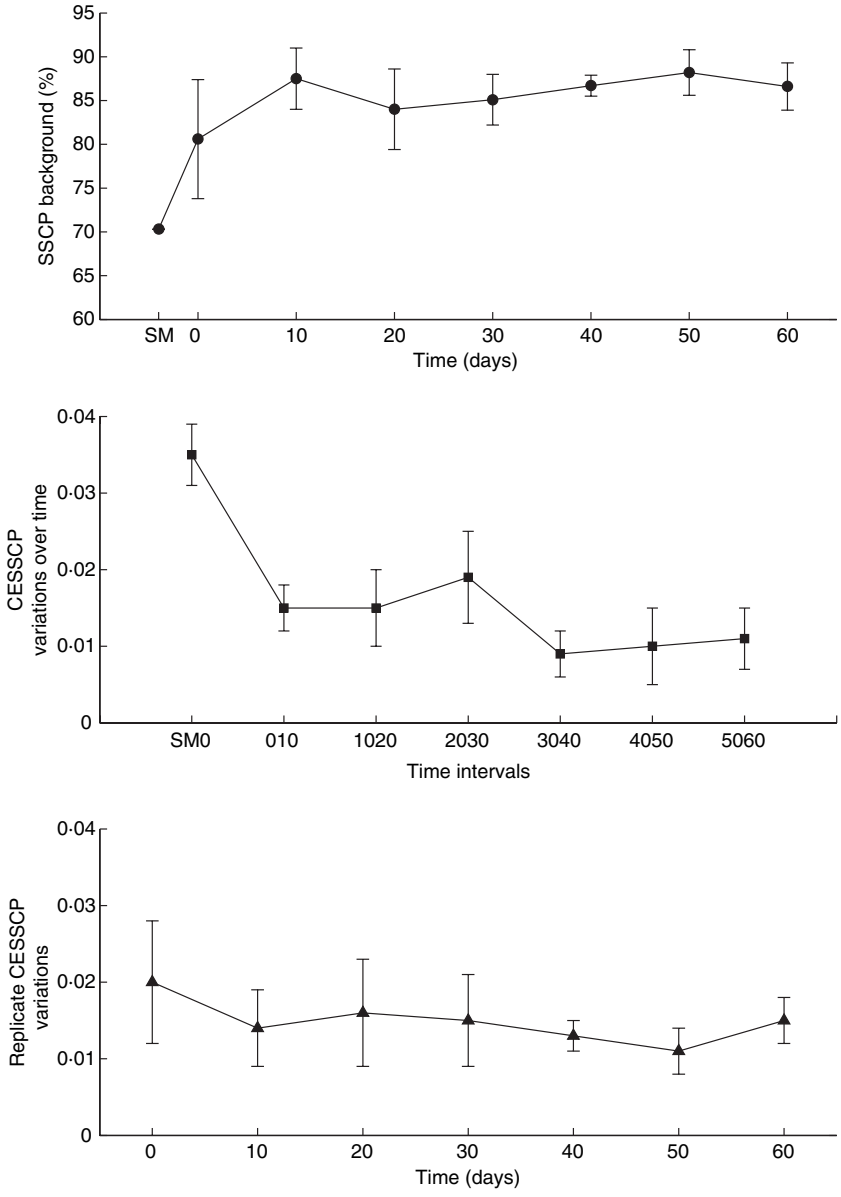

Figure 3 Plots of: (a) capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) background of samples collected at regular intervals during vermicomposting process in triplicates; (b) mean variations over time in bacterial community structure; (c) replicate CE-SSCP fingerprint variations. Values indicate mean ± SD of triplicates and the *y*-axis in (b) and (c) represents the Euclidean distance between two different SSCP profiles.

performance of the communities in the triplicates. We also observed a stable density of bacteria measured as 16S rRNA gene copies during the process, suggesting a constant bacterial abundance throughout the decomposition process. In addition, the sub-peak background area remained very high (80–90%) and stable during the vermicomposting process, which further indicates practically unchanging bacterial diversity. The increase in bacterial diversity (Fig. 3a) after the precomposting stage in all replicates indicates a rise of microbial diversity at the start of the decomposition process. Despite the high background in the CE-SSCP fingerprints that imposed limitations on evaluating diversity indices based on the relative proportion of peak areas/heights (Hill *et al.* 2003), we were still able to evidence subtle changes in bacterial diversity during the process in all replicates.

It has been reported that temperature in the compost environment plays a crucial role in the microbial community succession (Strom 1985). Yet during the vermicomposting process, despite a narrow range of temperature fluctuation (28–30°C) we observed shifts in the bacterial community in all the replicates after the biggest shift that occurred in the initial thermophilic phase between SM and day 0 (Fig. 3b). Interestingly, we also found that the dynamics in the community structure as well as the relative species abundance in the triplicates, based on the CE-SSCP peak profiles, were dissimilar (Figs 2 and 3c). These results indicate that microbial community succession during the

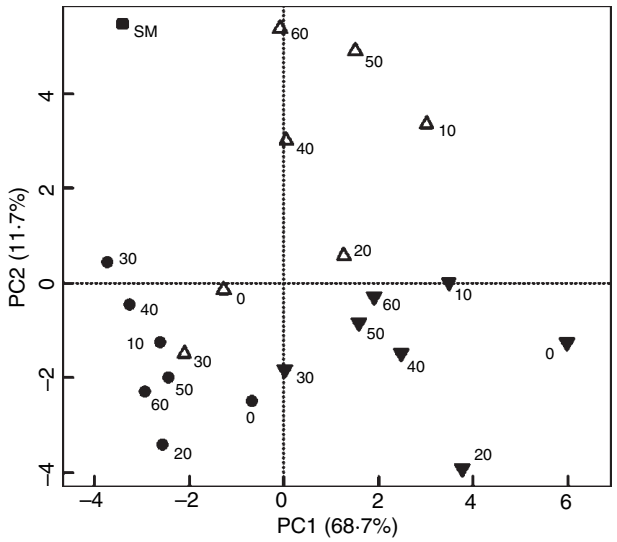

Figure 4 A principal component analysis plot of capillary electrophoresis single-strand conformation polymorphism profiles of start-up material (■, SM) and replicate vermicompost samples (●, ▼, Δ) collected at different time points. Percentages on axes are per cent of total variation in dataset explained. Labels (0–60) indicate the time (days) of sampling.

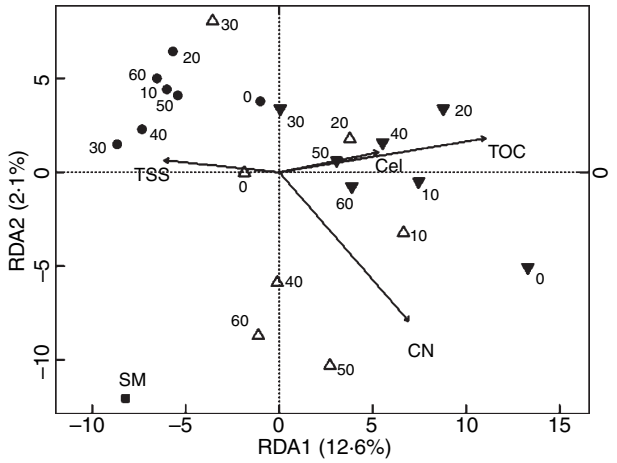

Figure 5 A biplot of redundancy analysis on capillary electrophoresis single-strand conformation polymorphism data and physico-chemical parameters of start-up material (■, SM) and replicate vermicompost samples (●, ▼, Δ) collected at different times. Percentages on axes are per cent of total variation in dataset explained; 14·7% of the variability that can be attributed to physico-chemical parameters is shown in the plot. Labels (0–60) indicate the time (days) of sampling. Lines represents physico-chemical parameter vectors showing their contribution to the ordination; TOC, total organic carbon; C/N, ratio of total organic carbon to Kjeldahl nitrogen; TSS, total soluble substances; Cel, cellulose.

decomposition process is neither a temperature-dependent phenomenon nor influenced by any of the other environmental factors tested. Several reasons may explain this discrepancy between bacterial community structure in the triplicates. First, it could be an effect of earthworms that are known to modify the diversity and the abundance of the microflora directly, by selective feeding or by the stimulation of particular taxa of micro-organisms (Brown 1995; Tiunov and Scheu 2000). Second, it may be the effect, individually or in combination, of several biotic and abiotic factors such as resource competition (Huisman and Weissing 1999), predation and bacteriophages (Weinbauer and Rassoulwadegan 2004). Indeed, predation and bacteriophage effects, which clearly remain in need of further study, may contribute substantially to bacterial community structure dynamics.

Schloss *et al.* (2003) reported the possible correlation of variables like pH and temperature with changes in community structure during the initial stages of composting. Conversely, in the present study when temperature and pH remained nearly constant, the physico-chemical variables (carbon, C/N ratio, soluble substances and cellulose) were not significant in explaining the variations in the bacterial community structure dynamics (Fig. 5). However, we do agree that a more complete analysis of the function of microbial communities would include measures of other compounds and processes and would compare the assemblages based on both general (e.g. overall decomposition rates) and specific functions (Franklin and Mills 2006).

Consequence of divergent bacterial community structure on ecosystem functions

Despite the discrepancy in bacterial community structure in the triplicates as revealed by CE-SSCP fingerprinting, we still observed a steady broad-scale ecosystem function, i.e. a reduction in the C/N ratio, with constant bacterial population and diversity. This raises a question as to whether functional parameters are really important in revealing variations in the microbial community structure. The present study supports previous reports (Fernandez *et al.* 1999; Zumstein *et al.* 2000; Kaewpapit and Grady 2002) in which an extremely dynamic community sustained a functionally stable ecosystem. In fact, fluctuations in the abundance of species having different adaptive modes may be a mechanism that stabilizes community function in a varying environment. We consider that at least some minimum number of species is indispensable for an ecosystem to function under steady conditions and that a large number of species is perhaps necessary for maintaining stable processes in a varying environment. The three replicates functioned in the same way (Fig. 1) displaying the same overall diversity (Fig. 3a) but with fluctuating dominant populations (Fig. 2) that differed between the replicates (Fig. 3c). Our results

certainly agree with the insurance hypothesis (Yachi and Loreau 1999), which proposed that biodiversity provides insurance, or a buffer, against environmental fluctuations in so far as different species respond differently to such variations, leading to a more predictable aggregate community or ecosystem properties.

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