ELSEVIER

Soil Biology & Biochemistry 36 (2004) 859–868

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Distribution of microbial communities in a forest soil profile investigated by microbial biomass, soil respiration and DGGE of total and extracellular DNA

Alberto Agnelli[a,*], Judith Ascher[b], Giuseppe Corti[a], Maria Teresa Ceccherini[b], Paolo Nannipieri[b], Giacomo Pietramellara[b]

[a]*Dipartimento di Scienze Ambientali e Produzioni Vegetali, Università Politecnica della Marche, Via Brecce Bianche, 60131 Ancona, Italy*
[b]*Dipartimento di Scienza del Suolo e Nutrizione della Pianta, Università degli Studi di Firenze, Piazzale delle Cascine 28, 50144 Firenze, Italy*

Received 10 July 2003; received in revised form 18 November 2003; accepted 3 December 2004

Abstract

We studied the distribution of the indigenous bacterial and fungal communities in a forest soil profile. The composition of bacterial and fungal communities was assessed by denaturing gradient gel electrophoresis (DGGE) of total and extracellular DNA extracted from all the soil horizons. Microbial biomass C and basal respiration were also measured to assess changes in both microbial biomass and activity throughout the soil profile. The 16S rDNA-DGGE revealed composite banding patterns reflecting the high bacterial diversity as expected for a forest soil, whereas 18S rDNA-DGGE analysis showed a certain stability and a lower diversity in the fungal communities. The banding patterns of the different horizons reflected changes in the microbial community structure with increasing depth. In particular, the DGGE analysis evidenced complex banding patterns for the upper A1 and A2 horizons, and a less diverse microflora in the deeper horizons. The low diversity and the presence of specific microbial communities in the B horizons, and in particular in the deeper ones, can be attributed to the selective environment represented by this portion of the soil profile. The eubacterial profiles obtained from the extracellular DNA revealed the presence of some bands not present in the total DNA patterns. This could be interpreted as the remainders of bacteria not any more present in the soil because of changes of edaphic conditions and consequent shifting in the microbial composition. These characteristic bands, present in all the horizons with the exception of the A1, should support the concept that the extracellular DNA is able to persist within the soil. Furthermore, the comparison between the total and extracellular 16S rDNA-DGGE profiles suggested a downwards movement of the extracellular DNA.
© 2004 Elsevier Ltd. All rights reserved.

Keywords: Forest soil; Soil horizons; Bacterial and fungal microbial communities; Extracellular DNA; Denaturing gradient gel electrophoresis

1. Introduction

Soil is a biological system made of a solid phase consisting of mineral and organic (plant, living organisms, organic matter, undecomposed plant and animal debris) constituents, surrounded by water and air. The chemical composition of the soil, as other soil properties, depends on the parent rock, climate, relief, time and vegetation (Jenny, 1941). The presence of water and air, needed by plants and all the living organisms inhabiting soil, is function of the amount of porosity that, in turn, is due to the type of structure developed as a result of the presence of clay minerals and organic matter, and of the mixing and aggregation of mineral and organic phases. Hence, each soil, natural or cultivated, from the coldest to the hottest regions of Earth, has its own physical, chemical and biological characteristics that control the composition and activity of the microbial community, from which it is also feedback controlled. The soil microflora has been extensively studied in different environments, such as polar desert (Horowitz et al., 1972), high altitude (Ley et al., 2001), tundra (Zhou et al., 1997), boreal forest (Henckel et al., 2000), tropics (Gomes et al., 2001), desert mountain (Bailey et al., 2002), polluted and cultivated areas (Wenderoth and Reber, 1999; Kandeler et al., 2000). Within each soil some discrete microhabitats with high biological activity have been detected and called 'hot spots' (Nannipieri et al., 1990); for example, hot spots are aggregates, rhizosphere and preferential flow paths (Sextone et al., 1985; Joergensen, 2000; Bundt et al., 2001).

* Corresponding author. Tel.: +39-7122-04293; fax: +39-7122-04858.
E-mail address: a.agnelli@univpm.it (A. Agnelli).

0038-0717/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2004.02.004

Beside the presence of specific hot spots, soil has also a vertical diversity that reflects the combined effects of the pedogenetic factors responsible for its development; the result of this diversity is manifested by the formation of different horizons throughout the soil. From the surface to the underlying parent material, each horizon develops its own chemical, physical and mineralogical characteristics due to additions, losses, transfers, and transformations of energy and matter occurred during the formation and development of soil (Soil Survey Staff, 1999). This fact can determine various microbial environments within the same soil. For example, the organic matter, which represents the energy source for the prevailing heterotrophic microorganisms, changes its composition and structure throughout the profile of a forest soil, from the surface to the deeper horizons (Agnelli et al., 2002). In the same way, also the water:air ratio and the amount of oxygen vary. Such different conditions of the pedogenetic horizons could have sorted a segregation of the microbial communities in function of the variable availability of nutrients and energy.

Usually, only the composition of microflora of the surface horizon has been studied because it is supposed to be the most active one, whereas little attention has been paid to what happens further down the soil profile. Few papers take into account the subsurface soil layers (Zvyaginstev, 1994; Ekelund et al., 2001; Bundt et al., 2001; Fierer et al., 2003), and fewer, as far as we know, considered the pedogenetic horizons (Certini et al., 1999; Fritze et al., 2000; Agnelli et al., 2001) in assessing the soil inhabiting microorganisms.

Nucleic acid-based cultural independent methods, like targeting small subunit rRNA sequences (SSU rRNA) by PCR amplification coupled with the rDNA-fragment analyses by genetic fingerprinting approaches (e.g. DGGE, TGGE, ARDRA, SSCP), are considered the most accurate ones to assess the composition of soil microbial communities (Nannipieri et al., 2003). Denaturing and temperature gradient gel electrophoresis (DGGE, TGGE) are simple and rapid techniques, and they can be used to easily detect changes easily in the composition of soil microflora (Heuer et al., 2001). Whereas the assessment of bacterial diversity has not been a problem due to the availability of universal primers for bacteria, the determination of fungal communities has been impaired by the co-amplification of DNA from other eukaryotic organisms such as plants, algae and nematodes (Kowalchuk et al., 1997). This problem has now been solved because specific primers are available for fungal 18S rDNA (Kowalchuk, 1999).

The aim of this work was to study the distribution of bacterial and fungal communities in a forest soil profile by DGGE, after extraction and purification of total and extracellular DNA from all soil horizons. Extracellular DNA can persist in soil due to its adsorption by soil colloids, and its contribution to the total DNA should be discriminated for a correct assessment of microbial diversity in soil. Microbial biomass C and basal respiration were also measured to assess changes in both microbial size and activity along the soil profile.

2. Material and methods

2.1. Soil

The study site is located at 1100 m a.s.l in the Vallombrosa National Reserve, on the west flanks of the Apennine Mountains (Italy). The mean annual precipitation is 1340 mm and the mean annual temperature is 10.2 °C. The parent material is an Oligocene sandstone (Arenaria del Falterona) made of coarse turbidites intercalated with thin siltstone strata. An area of about 1200 m^2, named Cavalla, homogeneous in terms of soil, vegetal cover, slope and exposure was recognized within the forest. The homogeneity of the soil was assessed by digging several profiles and by auger cores. Soil, classified as fine loamy, mixed, mesic, Humic Dystrudept (Soil Survey Staff, 1999), was made of a modern soil (from A1 to Bw2 horizon) resting on a buried, truncate paleosol (Corti et al., 2001). Vegetal cover was a plantation of *Abies alba* Mill. about 80 years old. Slope was about 5% facing a NE exposure. In this area the soil was trenched to obtain an up-slope face of about 6 m wide. The profile description and the main soil characteristics are reported in Appendix 1. From the middle portion of each horizon three soil samples were collected as sketched in Fig. 1. The collected amount ranged from 2 to 3 kg *per* sample. All soil samples were sieved at 4 mm at field moisture and stored in plastic bags at 4 °C. For the DNA extraction, an aliquot of each sample was stored at − 20 °C.

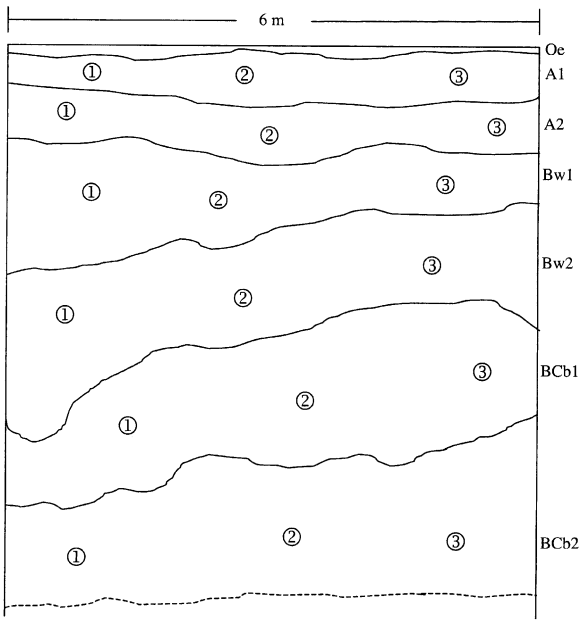

Fig. 1. Sketch of the soil trench with indicated numbers and positions of the collected samples, Vallombrosa forest (Firenze, Italy).

2.2. Determination of pH, organic C, total N, microbial biomass C, and basal respiration

The pH was measured potentiometrically with a sure-flow combine glass-calomel electrode in H_2O and 1 M KC1 solution (1:2.5 solid:liquid ratio).

The organic C content was determined, after washing soil with 0.2 M HCl solution, by Carlo Erba NA 1500 combustion analyser. The same instrument was also used to determine the total N content on sample aliquots without previous acid washing.

The microbial biomass C was determined by the fumigation-extraction method (Vance et al., 1987) after 14 days of conditioning at 25 °C and at 50% of their total water holding capacity, using a k_{EC} factor of 0.38 (Joergensen, 1996). Basal respiration was measured, as reported by Isermeyer (1952), by alkali (1 M NaOH solution) absorption of the CO_2 evolved during the incubation period (14 days), followed by titration of the residual OH with standardized acid solution.

2.3. Extraction of total and extracellular DNA

Total bacterial and fungal community structures were studied by the 16S and 18S rDNA-DGGE approaches, respectively.

Whole-community DNA was directly extracted from 0.5 g of sample (fresh weight of known moisture content) using a bead-beating method (FastDNA SPIN Kit for soil, Bio 101 Inc., USA). The quality of the extracted DNA was checked by horizontal electrophoresis on 1 × TAE (Tris Acetate EDTA buffer) agarose gel (1% w/v), and the amount of the extracted DNA was evaluated by fluorometer (Hoefer™ DyNA Quant™ 200) using bisbenzimide-dye (Hoechst H 33258).

Extracellular DNA was extracted by modifying the method of Ogram et al. (1987): 10 g of sample were sequentially washed three times with 10 ml of 0.12 M Na_2HPO_4 solution at pH 8.0 followed by centrifugation at 3700 *g* at 20 °C for 30 min. The raw DNA extracts recovered from the different horizons showed different colours due to organic contaminants (mostly humic substances): the extracts recovered from the A1 and A2 horizons resulted dark brown, those from Bw1 and Bw2 were yellow coloured, and those from BCb1 and BCb2 colourless. The DNA extracts were concentrated by precipitation with ethanol at −20 °C followed by phenol–chloroform–isoamilic alcohol purification. DNA pellet was resuspended in 1 ml of distilled water and the quality and quantity of extracted DNA were determined by agarose gel-electrophoresis and by fluorometer, respectively. Further, DNA was PCR-ready purified by following the Geneclean® procedure, according to the BIO101 protocol.

The extraction of total DNA was made from two specimens obtained from each of the three samples collected per horizon, with the exception of the A1 horizon where three specimens were obtained from samples 1 and 2 (Fig. 1). The extraction of extracellular DNA was made on a various number of specimens obtained from the three samples collected in the horizons: two specimens from sample 1 and one from samples 2 and 3 for A1 horizon, one specimen from samples 1 and 3 for the A2 horizon, two specimens from samples 1 and 2 and one from sample 3 for both the Bw1 and Bw2 horizons, three specimens from sample 1 and two from samples 2 and 3 for the BCb1 horizon, four specimens from sample 3 and five from samples 1 and 2 for the BCb2 horizon (Fig. 1). The total number of extractions of both total and extracellular DNA from each soil horizon is reported in Table 3.

2.4. PCR amplification

Both total and extracellular eubacterial 16S rDNA was amplified using the primer set GC-968f (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TA-3′) and 1401r (5′-GCG TGT GTA CAA GAC CC-3′), as described by Felske et al. (1997). DNA template (80 ng) was amplified with 5 U μl^{-1} PolyTaq (Polymed), 10 μM of each primer, 10 mM of each deoxynucleoside triphosphate, 10 mM of $MgCl_2$, 500 μg ml^{-1} of BSA and reaction buffer 1 × (Polymed, without $MgCl_2$) in a final reaction volume of 50 μl. The PCR was performed with a Perkin–Elmer 2400 thermocycler with the following reaction conditions: 94 °C for 90 s, followed by 33 cycles at 95 °C for 20 s, 56 °C for 30 s, 72 °C for 45 s, and a final extension step at 72 °C for 7 min. The PCR products, 5 μl sub-samples, were examined by electrophoresis on 1 × TAE agarose gel (0.8% w/v) with appropriate DNA size standards (Mass Ruler™, DNA Ladder Mix, Fermentas) to conform the size and approximate quantity of the generated amplicons.

Both total and extracellular fungal 18S rDNA was amplified following the nested PCR strategy reported Kowalchuk (1999), using the primer set NS1/NS8 and the GC-clamped primer set NS1-GC/NS2 + 10 for the first and the second round of the PCR assay, respectively. The PCR conditions were those described by Kowalchuk (1999). The products of both the two PCR-rounds were checked and quantified by agarose gel-electrophoresis as illustrated above.

2.5. Denaturing gradient gel electrophoresis

16S rDNA-DGGE was performed by using the Dcode System (Universal Mutation Detection System, Biorad). An amount of 300 ng of amplicons was loaded in duplicate (top filling method) on a 6% polyacrylamide gel (Acrylamide/-Bisacrylamide 37.5:1, Biorad) containing a denaturant gradient of 46–56% parallel to electrophoresis direction made of urea and formamide (100% denaturant contains 7 M urea and 40% formamide). Gels were electrophoresed at constant temperature (60 °C) and voltage (75 V) for 16 h, followed by 2 h colouration using SybrGreen I nucleic acid

gel stain 1 × (FMC Bio Products, Rockland, ME USA). Bands were detected from digital images (Polaroid Gel Cam, Elect; Polaroid Type 667 Film ISO 3000) by UV light gel transillumination.

18S rDNA-DGGE was run by using the Dcode System. According to Kowalchuk (1999), 350 ng of the amplicons were loaded in duplicate on a 6% (w/v) polyacrylamide gel with a denaturant gradient of 25–45% parallel to electrophoresis direction. Gels were electrophoresed at constant temperature (60 °C) and voltage (85 V) for 16 h prior to SybrGreen staining and band detection was carried out as described above.

Amplicons of *Bacillus subtilis* (16S rDNA-DGGE) and *Saccharomyces cerevisiae* (18S rDNA-DGGE), generating known lane profiles, were loaded on the first and on the last position of the gel, respectively. These marker bands were used to facilitate the interpretation of the generated banding patterns.

For each horizon, except the A2, the whole 16S rDNA- and 18S rDNA-PCR-DGGE assays based on total and exctracellular DNA were repeated four times using two single DNA aliquots of those extracted from sample 1 and one aliquot of those extracted from samples 2 and 3. For the A2 horizon, the 16S rDNA- and 18S rDNA-PCR-DGGE assays based on extracellular DNA were repeated two times, using one DNA aliquot of those extracted from samples 1 and 3.

2.6. Statistical analysis

The analyses were run at least in triplicate and the standard error calculated. The error propagation technique (Skoog and West, 1987) was used for computing the standard errors of the calculated values.

3. Results

3.1. General characterisation

The pH values (Table 1) of the samples were in the acidic range, both in water and in KCl. The pH in water tended to rise in the deepest horizons, ranging from 4.5 at the surface to 5.1 in the BCb2 horizon, while those in KCl increased only slightly with depth, from 3.5 to 3.8.

Table 1
pH, organic C and total N contents of the horizons of a soil of Vallombrosa forest (Firenze, Italy)

Horizons	$pH_{(H_2O)}$	$pH_{(KCl)}$	Organic C (g kg^{-1})	Total N (g kg^{-1})
A1	4.5	3.5	91.0 (2.2)	5.4 (0.0)
A2	4.6	3.7	31.4 (0.8)	2.0 (0.0)
Bw1	4.8	3.7	12.7 (0.1)	0.9 (0.0)
Bw2	4.7	3.8	5.9 (0.2)	0.5 (0.0)
BCb1	4.9	3.8	4.3 (0.2)	0.3 (0.0)
BCb2	5.1	3.7	3.1 (0.5)	0.4 (0.0)

Numbers in parentheses are the standard errors ($n = 3$).

The organic C content, as expected, decreased with depth, with a large amount of organic C in the A1 and A2 horizons (Table 1). The organic C concentration showed a steep decrease from the A1 (91.0 g kg^{-1}) to the Bw2 horizon (5.9 g kg^{-1}) and a further slight decrease in the BCbl and BCb2 horizons (4.3 and 3.1 g kg^{-1}, respectively). The total N ranged from 5.4 in the A1 to 0.3 in the BCb1 horizon, following the same trend of the organic C. The C/N ratio decreased slightly with increasing depth and the little variations among the horizons can be ascribed, according to Stevenson (1994), to the presence of fixed NH_4^+–N in the deeper horizons. Corti et al., (1999) found in the same soils that the fixed NH_4^+–N contributed to the total N content for the 0.25% in the A1 horizon and from 36 to 97% in the rest of the profile, with the highest values in the BCb horizons.

3.2. Microbial biomass C, basal respiration and metabolic quotient

The amount of microbial biomass C (C_{mic}) decreased with increasing depth from 338.9 μg g^{-1} in the A1 horizon to 39.9 μg g^{-1} in the Bw2 horizon, then it increased to about 53 μg g^{-1} in the BCb horizons (Table 2). The proportion of organic C present as the microbial biomass (C_{mic}/C_{org}) became larger in the deeper horizons, with the highest value in the BCb2 horizon (1.7%). The CO_2–C evolved during 14 days of incubation (Table 2) decreased, following the same trend of the microbial biomass C, until the Bw2 horizon. At the level of the BCb horizons, the respired CO_2–C showed an inverse depth-trend, increasing from BCb1 to BCb2 (448.1 and 521.1 μg g^{-1}, respectively). In the same way of the C_{mic}/C_{org}, the ratio between the cumulative value of the evolved CO_2–C and the organic C content (ΣCO_2–C/C_{org}) displayed higher values with increasing depth. In this case, the values found in the BCb horizons were up to 20 times than that of the other horizons.

The metabolic quotient (qCO_2), that expresses the CO_2–C evolved per unit of microbial biomass and time (Anderson and Domsch, 1993), increased with depth, showing a sharp rise at the level of the Bw2 horizon (23.5).

3.3. Total and extracellular DNA yield

The amounts of both total and extracellular DNA directly extracted from each horizon of the soil profile showed a decreasing trend with depth, ranging from 92.6 to 20.4 μg g^{-1} for the total DNA, and from 41.1 to 2.2 μg g^{-1} for the extracellular DNA (Table 3). The extracellular DNA had the highest value in the A2 horizon (41.11 μg g^{-1}), about 2-folds higher than that in the A1 horizon (18.86 μg g^{-1}) and from 4- to 20-folds higher than the horizons below (Table 3).

The highest percentage of extracellular DNA related to the total DNA was recovered for the A2 horizon, where it

Table 2
Microbial biomass C (C_{mic}), percentage of organic C present as microbial biomass (C_{mic}/C_{org}), CO_2–C evolved during 14 days of incubation (ΣCO_2–C), percentage of organic C evolved as CO_2–C (ΣCO_2–C/C_{org}) and qCO_2 from the horizons of a soil of Vallombrosa forest (Firenze, Italy)

Horizons	C_{mic} ($\mu g\ C_{mic}\ g^{-1}$)	C_{mic}/C_{org} (%)	ΣCO_2–C ($\mu g\ CO_2$–C g^{-1})	ΣCO_2–C/C_{org} (%)	qCO_2 ($\mu g\ CO_2$–C $mg^{-1}\ C_{mic}\ h^{-1}$)
A1	338.9 (35.2)	0.4 (0.0)	760.6 (8.6)	0.8 (0.0)	6.8 (2.6)
A2	151.7 (24.5)	0.5 (0.1)	399.2 (10.4)	1.3 (0.0)	7.8 (3.0)
Bw1	98.6 (12.6)	0.8 (0.1)	302.6 (35.4)	2.4 (0.3)	9.1 (3.7)
Bw2	39.9 (0.1)	0.7 (0.0)	315.1 (47.0)	5.3 (0.8)	23.5 (8.7)
BCb1	53.0 (3.4)	1.2 (0.1)	448.1 (42.1)	10.4 (1.0)	25.2 (9.9)
BCb2	53.0 (3.8)	1.7 (0.1)	521.1 (27.2)	16.9 (0.9)	29.3 (11.3)

Numbers in parentheses are the standard errors ($n = 3$).

represented 60% of the total extractable DNA. The percentages in the A1 (20.4%) and Bw1 horizons (24.1%) were similar, whereas in the horizons below about 10% of the total DNA was made of extracellular DNA (Table 3).

3.4. DGGE

DGGE was applied to both types of DNAs to analyse the fragments generated from eubacterial and fungal specific 16S rDNA- and 18S rDNA-PCR, respectively.

For each horizon, because of the high similarity among the PCR-DGGE replicates (performed on DNA separately extracted from different specimens), only one of each DGGE gel was presented and, according to Kowalchuk (1999), was also reported as schematic drawing.

3.4.1. DGGE profiling of eubacterial 16S rDNA

For both total and extracellular DNA, the DGGE of 16S rDNA (Fig. 2a,b) revealed complex banding patterns and indicated distinct shifts in the bacterial community structure of each horizon. The total DNA patterns of the A1 and A2 horizons were characterised by higher diversity than that of the deeper ones, as indicated by the large number of bands; in the B horizons the number of bands remained rather constant up to the bottom of the profile (Fig. 2a). Two evident bands were present throughout the soil profile (one of them showed the same electrophoretic behaviour of the marker strain *B. subtilis*), whereas many of the other bands, characteristic of the organic C-rich A horizon, disappeared in the patterns of the deeper horizons. The DGGE profile of both total and extracellular DNA from the BCb horizons, showed bands attributable to a specific bacterial species only dominant in the deep soil (Fig. 2a,b, indicated by not-dashed arrows).

By comparing total and extracellular DNA it was evident that several bands detected in the DGGE profile of the total DNA were absent in the profiles of the extracellular DNA, and, conversely, in this latter appeared bands not present in the total DNA profile (Fig. 2a,b). Further, only few bands of the total DNA of the A1 horizon could be found in the profile of the respective extracellular DNA. The DGGE profile of the extracellular DNA of the A2 horizon showed two additional bands (Fig. 2b, indicated by dashed arrows), not present in the total DNA profile of the same horizon, but characteristic of that of the A1. In the B horizons, the extracellular DNA patterns were more similar to those of the respective total DNA.

3.4.2. DGGE profiling of fungal 18S rDNA

In contrast to the complex banding patterns of the eubacterial 16S rDNA-DGGE, those from the total and extracellular 18S rDNA showed a low fungal diversity (Fig. 3a,b).

The total 18S DNA fingerprinting indicated the presence of six bands in the A1 horizon (Fig. 3a). The two bands of the isolated marker strain *S. cerevisiae* showed the same electrophoretic behaviour of the two highest bands that were present throughout the whole soil profile. According to

Table 3
Amounts of total DNA, total DNA/C_{mic} ratio, extracellular DNA, and percentage of extracellular DNA on total DNA from the horizons of a soil of Vallombrosa forest (Firenze, Italy)

Horizons	Total DNA ($\mu g\ g^{-1}$)	Total DNA/C_{mic} ratio	Extracellular DNA ($\mu g\ g^{-1}$)	Extracellular/total DNA (%)
A1	$n = 8$ 92.6 (23.6)	0.27 (0.08)	$n = 4$ 18.9 (2.9)	20.4 (6.1)
A2	$n = 6$ 68.5 (21.4)	0.450 (0.16)	$n = 5$ 41.1 (0.9)	60.1 (18.9)
Bw1	$n = 6$ 44.9 (18.3)	45.5 (0.20)	$n = 5$ 10.8 (1.5)	24.1 (10.4)
Bw2	$n = 6$ 39.2 (12.9)	0.98 (0.32)	$n = 5$ 5.6 (2.2)	14.3 (7.4)
BCb1	$n = 6$ 36.8 (13.8)	0.69 (0.26)	$n = 7$ 3.8 (0.7)	10.4 (4.3)
BCb2	$n = 6$ 20.4 (9.0)	0.39 (0.17)	$n = 14$ 2.2 (0.3)	10.5 (4.8)

Numbers in parentheses are the standard errors (n = number of extractions).

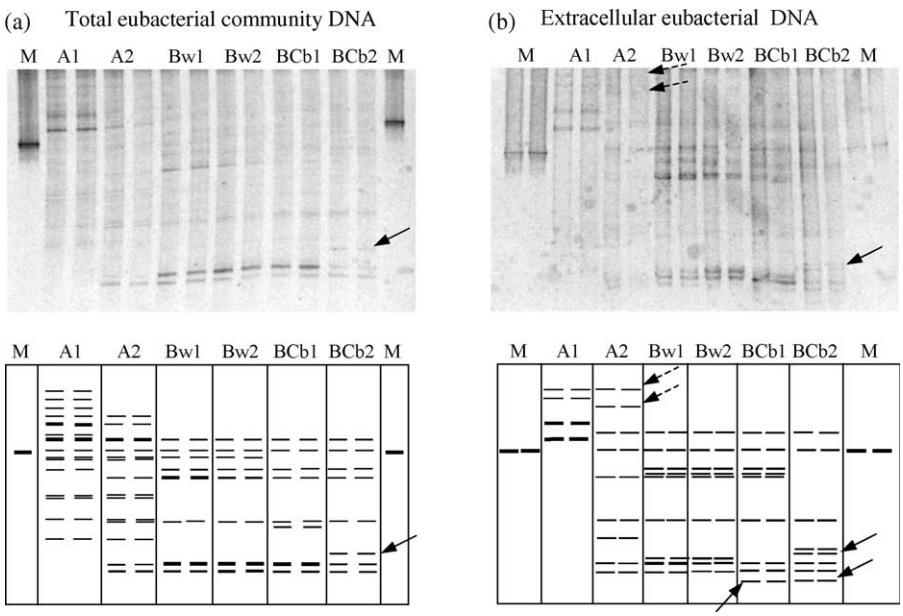

Fig. 2. 16S rDNA-DGGE profiles, based on (a) total and (b) extracellular DNA extracted from the horizons of a soil of Vallombrosa forest (Firenze, Italy), and their respective schematic drawings.

the trend of the eubacterial communities, the complexity in the fungal community structure decreased with increasing depth. As for the eubacteria, the deepest BCb2 horizon was characterised by the appearance of new bands, one of which was particularly intense and probably due to a numerically significant population (Fig. 3a, indicated by the arrow). This band also characterised the extracellular DNA profiles of the BCb2 horizon (Fig. 3b, indicated by the arrow).

4. Discussion

It is well established that microbial biomass C content decreases as the organic C content by increasing soil depth (Anderson and Domsch, 1989; Van Gestel et al., 1992; Fritze et al., 2000; Bundt et al., 2001; Ekelund et al., 2001; Fierer et al., 2003). The relatively large amount of the microbial C found in the BCb horizons, according to Wolters

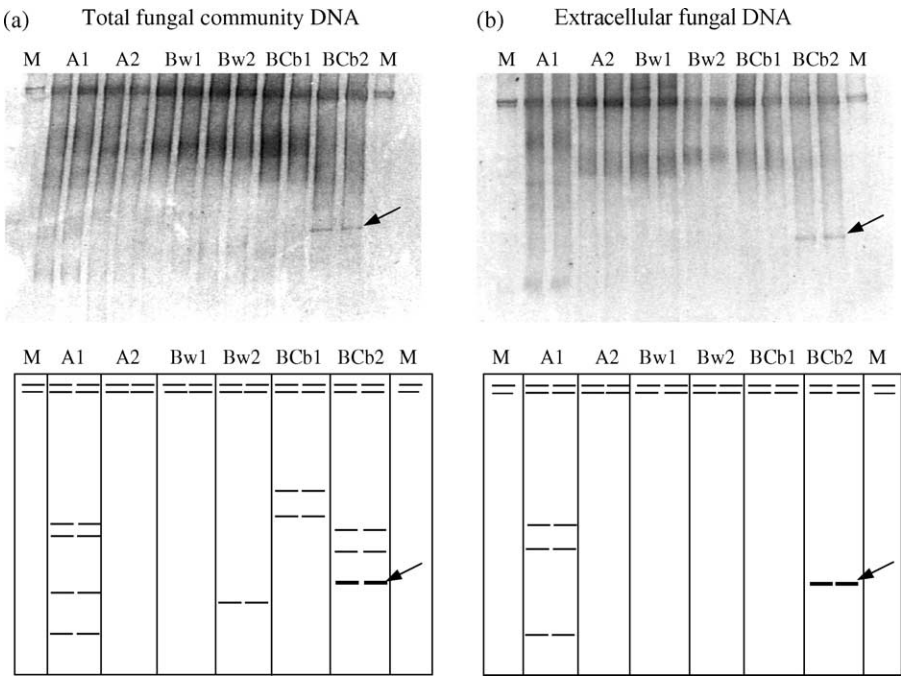

Fig. 3. 18S rDNA-DGGE profiles, based on (a) total and (b) extracellular DNA extracted from the horizons of a soil of Vallombrosa forest (Firenze, Italy), and their respective schematic drawings.

and Joergensen (1991), can be related to some edaphic conditions such as pH and amount of exchangeable Ca, both higher in the BCb than in the horizons above (Agnelli et al., 2001). However, the high C_{mic}/C_{org} ratio of the BCb horizons may also be due to the prevalence of microbial species capable of utilising the soil organic matter as energetic substrate (Lavahum et al., 1996).

The cumulative CO_2–C evolved after 14 days of incubation followed the same trend of the microbial biomass C content. Further, the reverse depth-trend of the evolved CO_2–C and the high value of the CO_2–C/C_{org} ratio of the BCb horizons could confirm the higher use of the native organic matter by the microflora inhabiting the deepest soil horizon. According to Kaiser et al. (1992), an alternative hypothesis, explains the high basal respiration of the BCb horizons as due to the presence of a relatively large content of total N per unit of organic C. However, the high values of the basal respiration of the BCb horizons could be also ascribed to elevated stress conditions of the habitat (Killham and Firestone, 1984). The qCO_2 values seemed to support this latter hypothesis, indicating that the energy required by the microbial community for its growth and maintenance (Anderson and Domsch, 1993) increased from the surface to the deeper horizons. In other words, the microorganisms inhabiting the BCb horizons appear to spend more energy to metabolise the available organic substrate than those of the upper A–Bw horizons. However, in the deeper horizons strictly oligotrophic microorganisms may generate more energy by activation of preferential electronic transfer through the respiratory chain, although they should be a scarcely represented population. In the deeper horizons the microbial community should satisfy its energy-demand using the relatively easy degradable C resources, enriching the organic pool of progressively more recalcitrant C compounds. This enrichment could be gained by the scarce replacement of fresh organic matter in the deeper soil horizons. In time, according to Wolters and Joergensen (1991), these conditions could bring to ‘a shift in the population structure of the microbial community’ of the deeper horizons.

The high amount of the total extracted DNA (Table 3) with respect to the microbial biomass C (Table 2) could be due to the use of a K_{EC} value which underestimated the partial lysis of the microbial cells with chloroform (Toyota et al., 1996; Marstorp and Witter, 1999), and/or to the presence of extracellular DNA (from 10 to 60% of the total DNA), which can also derive from non-microbial sources. This latter hypothesis might be true in the upper A–Bw1 horizons, where the soil harbours, besides the microflora, a high number of organisms, such as micro- and mesofauna, roots, etc. Marstorp and Witter (1999) found a negligible contribution of the extracellular DNA to the total DNA. However, except for the Bw2 horizon, the ratios between the quantity of total DNA and microbial biomass C of the different horizons of the soil understudy were comparable to those obtained by calculation from the data reported for eight different soils by Marstorp et al. (2000), who used similar bead-beating methods for the DNA extraction.

The 16S rDNA-DGGE analysis (Fig. 2a,b) revealed composite banding patterns reflecting the high microbial diversity, as expected for a forest soil (Henckel et al., 2000). Conversely, the 18S rDNA-DGGE analysis (Fig. 3a, b) showed certain stability and a lower diversity in the fungal communities. The banding patterns for the different horizons reflected changes in the microbial community structure with increasing depth, as found by other authors mainly using PLFA-assays (Henckel et al., 2000; Fritze et al., 2000; Fierer et al., 2003; Griffiths et al., 2002). In particular, the DGGE analysis evidenced very complex banding patterns for the upper A1 and A2 horizons, indicating a high diversity and density of microorganisms; on the contrary, the deeper horizons harboured a less diverse microflora. The low diversity and the presence of specific microbial communities in the B horizons, and in particular in the BCb ones, can be attributed to the selective environment represented by this portion of the soil profile. According to Fierer et al. (2003), the scarce availability and the partly kerogen-like nature of the organic matter in the deeper horizons in this soil (Agnelli et al., 2002) can be considered as the main factor in controlling the structure and activity of the microbial communities.

The eubacterial profiles obtained from the extracellular DNA revealed the presence of some bands not shown in the total DNA patterns (Fig. 2a,b). In all horizons, and in particular in the A2, this could be due to several causes: (a) limitation of the cultural-independent molecular biology techniques; (b) the amount of the extracted extracellular DNA was only in small part of microbial origin and affected by the presence of DNA from other organisms (fungi, plants, soil fauna); (c) the specific sequences that originated those bands were few and, although visible in the extracellular DNA patters, were hidden in the total DNA patterns. However, the presence of bands in the extracellular DNA patterns not revealed in those obtained from the total DNA, could be interpreted as the remainders of bacteria no longer present in the soil because of changes of edaphic conditions with consequent shift of the microbial composition. These characteristic bands, that were present in all the horizons with the exception of the A1, should support the concept that the free DNA is able to persist within the soil. The presence and persistence of extracellular DNA in soil for extended periods (Paget et al., 1998; Frostegård et al., 1999; Niemeyer and Gessler, 2002), occur thanks to protection against nucleases hydrolysis due to adsorption of DNA molecules on soil components like clay minerals, sand particles, humic substances and polysaccharides (Alvarez et al., 1998; Crecchio and Stotzky, 1998; Pietramellara et al., 2001).

The comparison between the total and extracellular 16S rDNA-DGGE profiles showed the presence of bands in the extracellular DNA profile of the A2 horizon not revealed in the profile of total DNA of the same horizon. The same bands, however, were present in the profile of total DNA of the A1 horizon. This might be the result of the movement, from the A1 to the A2 horizon, of the extracellular DNA released in soil by organisms inhabiting the superficial horizon. The potential movement throughout the soil of free, non-degraded, and biologically active DNA has been observed in a microcosm study by Potè et al. (2004), who hypothesised a possible transport of DNA in water saturated soil and groundwater.

The movement of extracellular DNA from the surface downwards to the deeper horizons and its persistence in soil should be considered also in the light of the gene transfer by natural transformation. Thus the uptake of extracellular DNA by competent bacteria in soil (Gallori et al., 1994; Nielsen and van Elsas, 2001) is a normal process to mix genetic information among the horizons throughout the profile, and it also represents a potential environmental risk. In this latter case, natural transformation mediating horizontal gene transfer in soil (Lorenz and Wackernagel, 1994; England et al., 1997), with the increasing use of genetically modified organisms, might cause changes in the functional community structure of the native microflora inhabiting deep soil horizons. Since microbial communities of subsurface horizons are important to maintain natural ecosystem, contributing on key-processes within the biogeochemical cycles (Hiebert and Bennet, 1992; Richter and Markewitz, 1995), changes in their functional structure might affect the stability of natural soil system.

Appendix A

Descriptions of the soil of Vallombrosa forest (Firenze, Italy)

Altitude: 1100 m; exposure: N-NE; slope: $<5\%$; parent material: 'Arenaria del Falterona' (Oligocene)

Vegetation: plantation of *Abies alba* Mill. about 80 years old; understory: *Hieracium murorum, Prenantes purpurea, Luzula nivea, Sanicula europaea, Senecio fuchsii, Rubus idaeus, Fragaria vesca, Geranium robertianum, Cardamine* sp., *Galium* sp., seedlings of *Abies alba*

Soil: Humic Dystrudept, fine loamy, mixed, mesic (Soil Survey Staff, 1999).

Horizons	Depth (cm)	Munsell colour[a]	Texture[b] (USDA)	Structure[c]	Consistency[d]	Plasticity[e]	Roots[f]	Thickness (cm)	Boundary[g]	Other observations[h]
Oe	0–1	–	–	–	–	–	–	1–2	cw	Partially decomposed needles of fir
A1	0–11	10YR3/2	cl	2–3 f-m cr	mfr, wss	wps	3f, 2m	9–11	cw	
A2	11–23	10YR3/3	g sil	3–2 m-f sbk	mfr; wss	wps	2f	12–14	cw	
Bw1	23–42	10YR5/4	g sil	2–3 m-f abk-sbk	mfr-mfi, wss	wps	1f	19–40	ci	
Bw2	42–71	10YR5/5	g sil-g l	2–3 f sbk	mfi, wss	wps	1f	25–42	ci	wd silt caps; xp
BCb1	71–109	10YR5/4	vst l	2 c–m abk	mfi, wss	wps	1f	24–40	ci	wd silt caps; xp; some clasts are saprolitic
BCb2	109–149 +	10YR4/4	st l-sl	2 c-m abk	mfi, wss	wps	1m			wd silt caps; xp; some clasts are saprolitic

[a] Moist and crushed.
[b] v, very; g, gravelly; st, stony; c, clay; si, silt; s, sandy; l, loam.
[c] 1, weak; 2, moderate; 3, strong; f, fine; m, medium; c, coarse; pl, platy; abk, angular blocky; sbk, subangular blocky; cr, crumb.
[d] m, moist; w, wet; fr, friable; fi, firm; ss, slightly sticky.
[e] w, wet; ps, slightly plastic; p, plastic.
[f] 1, few; 2, plentiful; 3, abundant; mi, micro; vf, very fine; f, fine; m, medium; co, coarse.
[g] c, clear; g, gradual; s, smooth; w, wavy; i, irregular; a, abrupt.
[h] cf, compressed fines; vw, very weak; w, weak; wd, well developed; pe, poorly expressed; xp, fragic properties.

References

Agnelli, A., Ugolini, F.C., Corti, G., Pietramellara, G., 2001. Microbial biomass-C and basal respiration of fine earth and highly altered rock fragments of two forest soils. Soil Biology and Biochemistry 33, 613–620.

Agnelli, A., Celi, L., Degl'Innocenti, A., Corti, G., Ugolini, F.C., 2002. The changes with depth of humic and fulvic acids extracted from fine earth and rock fragments of a forest soil. Soil Science 167, 524–538.

Alvarez, A.J., Khanna, M., Toranzos, G.A., Stotzky, G., 1998. Amplification of DNA bound on clay minerals. Molecular Ecology 7, 775–778.

Anderson, T.H., Domsch, K.H., 1989. Ratios of microbial biomass carbon to total organic carbon in arable soils. Soil Biology and Biochemistry 21, 471–479.

Anderson, T.H., Domsch, K.H., 1993. The metabolic quotient for CO_2 (qCO_2) as a specific activity parameter to assess the effects of environmental conditions, such as pH, on the microbial biomass of forest soils. Soil Biology and Biochemistry 25, 393–395.

Bailey, V.L., Smith, J.L., Bolton, H. Jr., 2002. Fungal-to-bacterial ratios in soils investigated for enhanced C sequestration. Soil Biology and Biochemistry 34, 997–1007.

Bundt, M., Widmer, F., Pesaro, M., Zeyer, J., Blaser, P., 2001. Preferential flow paths: biological hot spots in soils. Soil Biology and Biochemistry 33, 729–738.

Certini, G., Dilly, O., Ugolini, F.C., Corti, G., 1999. Distribution of the microbial biomass in forest soils of the Tuscan Apennines. Agrochimica 43, 10–17.

Corti, G., Agnelli, A., Ugolini, F.C., 1999. A modified Kjeldahl procedure for determining strongly fixed NH_4^+–N. European Journal of Soil Science 50, 523–534.

Corti, G., Agnelli, A., Certini, G., Ugolini, F.C., 2001. The soil skeleton as a tool for disentangling pedogenetic history: a case study in Tuscany, central Italy. Quaternary International 78, 33–44.

Crecchio, C., Stotzky, G., 1998. Binding of DNA on humic acids: effect on transformation of *Bacillus subtilis* and resistance to DNAse. Soil Biology and Biochemistry 30, 1061–1067.

Ekelund, F., Rønn, R., Christensen, S., 2001. Distribution with depth of protozoa, bacteria and fungi in soil profiles from three Danish forest sites. Soil Biology and Biochemistry 33, 475–481.

England, L.S., Lee, H., Trevors, J.T., 1997. Persistence of Pseudomonas aureofaciens Strains and DNA in soil. Soil Biology and Biochemistry 29, 1521–1527.

Felske, A., Rheims, H., Wolterink, A., Stackebrandt, E., Akkermans, A.D.L., 1997. Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. Microbiology 143, 2983–2989.

Fierer, N., Schimel, J.P., Holden, P.A., 2003. Variations in microbial community composition through two soil depth profiles. Soil Biology and Biochemistry 35, 167–176.

Fritze, H., Pietikäinen, J., Pennanen, T., 2000. Distribution of microbial biomass and phospholipid fatty acids in Podzol profiles under coniferous forest. European Journal of Soil Science 51, 565–573.

Frostegård, A., Courtois, S., Ramisse, V., Clerc, S., Bernillon, D., Le Gall, F., Jeannin, P., Nesme, X., Simonet, P., 1999. Quantification of bias related to extraction of DNA directly from soils. Applied and Environmental Microbiology 65, 5409–5420.

Gallori, E., Bazzicalupo, M., Dal Canto, L., Fani, R., Nannipieri, P., Vettori, C., Stotzky, G., 1994. Transformation of *Bacillus subtilis* by DNA bound on clay in non-sterile soil. FEMS Microbiology Ecology 15, 119–126.

Gomes, N.C.M., Heuer, H., Schönfeld, J., Costa, R., Mendonca-Hagler, L., Smalla, K., 2001. Bacterial diversity of rhizosphere of maize (Zea mays) grown in tropical soil studied by temperature gradient gel electrophoresis. Plant and Soil 232, 167–180.

Griffiths, R.I., Whiteley, A.S., O'Donnell, A.G., Bailey, M.J., 2002. Influence of depth and sampling time on bacterial community structure in an upland grassland soil. FEMS Microbial Ecology 1450, 1–9.

Henckel, T., Jäckel, U., Schnell, S., Conrad, R., 2000. Molecular analyses of novel methanotrophic communities in forest soil that oxidize atmospheric methane. Applied Environmental Microbiology 66, 1801–1808.

Heuer, H., Wieland, G., Schonfeld, J., Schnwalder, A., Gomes, N.C.M., Smalla, K., 2001. Bacterial community profiling using DGGE or TGGE analysis. In: Rochelle, P.A. (Ed.), Environmental Molecular Microbiology: Protocols and Applications. Horizon Scientific Press, Wymondham, UK, pp. 177–190.

Hiebert, F.K., Bennet, P.C., 1992. Microbial control of silicate weathering in organic-rich ground water. Science 258, 278–281.

Horowitz, N.H., Cameron, R.E., Hubbard, J.S., 1972. Microbiology of the dry valleys of Antarctica. Science 176, 242–245.

Isermeyer, H., 1952. Eine einfache Methode zur Bestimmung der Bodenatmung und der Karbonate im Boden. Zietschrift für Pflanzenernährung und Bodenkunde 56, 26–38.

Jenny, H., 1941. Factors of Soil Formation: A System of Quantitative Pedology. McGraw-Hill, New York, 281 pp.

Joergensen, R.G., 1996. The fumigation-extraction method to estimate soil microbial biomass: calibration of the k_{EC} value. Soil Biology and Biochemistry 28, 25–31.

Joergensen, R.G., 2000. Ergosterol and microbial biomass in the rhizosphere of grassland soils. Soil Biology and Biochemistry 31, 647–652.

Kaiser, E.-A., Mueller, T., Joergensen, R.G., Insam, H., Heinimeyer, O., 1992. Evaluation of methods to estimate the soil microbial biomass and the relationship with soil texture and organic matter. Soil Biology and Biochemistry 24, 675–683.

Kandeler, E., Tscherko, D., Bruce, K.D., Stemmer, M., Hobbs, P.J., Bardgett, R.D., Amelung, W., 2000. Structure and function of the soil microbial community in microhabitats of a heavy metal polluted soil. Biology and Fertility of Soils 32, 390–400.

Killham, K., Firestone, M., 1984. Salt stress control of intracellular solutes in streptomycetes indigenous to saline soils. Applied and Environmental Microbiology 47, 301–306.

Kowalchuk, G., 1999. Fungal community analysis using denaturing gradient gel electrophoresis (DGGE). In: Akkermans, A.D.L., van Elsas, J.D., De Bruijn, F.J. (Eds.), Molecular Microbial Ecology Manual, vol. 3.4.6. Kluwer Academic Publisher, Dordrecht, pp. 1–16.

Kowalchuk, G.A., Gerards, S., Woldendorp, J.W., 1997. Detection and characterization of fungal infection of *Ammophila arenaria* (Marran grass) roots by denaturing gradient gel electrophoresis of specifically amplified 18S rDNA. Applied Environmental Microbiology 63, 3858–3865.

Lavahum, M.F.E., Joergensen, R.G., Meyer, B., 1996. Activity and biomass of soil microorganisms at different depths. Biology and Fertility of Soils 23, 38–42.

Ley, R.E., Lipson, D.A., Schmidt, S.K., 2001. Microbial biomass levels in barren and vegetated high altitude talus soils. Soil Science Society of America Journal 65, 111–117.

Lorenz, M.G., Wackernagel, W., 1994. Bacterial gene transfer by natural genetic transformation in the environment. Microbiological Reviews 58, 563–602.

Marstorp, H., Witter, E., 1999. Extractable dsDNA and product formation as measures of microbial growth in soil upon substrate addition. Soil Biology and Biochemistry 31, 1443–1453.

Marstorp, H., Guan, X., Gong, P., 2000. Relationship between dsDNA, chloroform liable C and ergosterol in soils of different organic matter contents and pH. Soil Biology and Biochemistry 32, 879–882.

Nannipieri, P., Ceccanti, B., Grego, S., 1990. Ecological significance of the biological activity in soil. In: Stotzky, G., Bollag, J.M. (Eds.), Soil Biochemistry, vol. 6. Marcel Dekker, New York, pp. 293–355.

Nannipieri, P., Ascher, J., Ceccherini, M.T., Landi, L., Pietramellara, G., Renella, G., 2003. Microbial diversity and soil functions. European Journal of Soil Science 54, 655–670.

Nielsen, K.M., van Elsas, J.D., 2001. Stimulatory effects of compounds present in the rhizosphere on natural transformation of *Acinetobacter* sp. BD413 with homologous cell lysates in soil. Soil Biology and Biochemistry 33, 345–357.

Niemeyer, J., Gessler, F., 2002. Determination of free DNA in soil. Journal of Plant Nutrition and Soil Science 165, 121–124.

Ogram, A., Sayler, G.S., Barkay, T., 1987. The extraction and purification of microbial DNA from sediments. Journal of Microbiological Methods 7, 57–66.

Paget, E., Lebrun, M., Freyssinet, G., Simonet, P., 1998. The fate of recombinant plant DNA in soil. European Journal of Soil Biology 34, 81–88.

Pietramellara, G., Franchi, M., Gallori, E., Nannipieri, P., 2001. Effect of molecular characteristics of DNA on its adsorption and binding on homoionic montmorillonite and kaolinite. Biology and Fertility of Soils 33, 402–409.

Potè, J., Ceccherini, M.T., Tran Van, V., Rosselli, W., Wildi, W., Simonet, P., Vogel, A.M., 2004. Fate and transport of antibiotic resistance genes in saturated soil columns. European Journal of Soil Biology 39; 65–71.

Richter, D., Markewitz, D., 1995. How deep is soil? BioScience 45, 600–609.

Sextone, A.J., Revsbech, N.P., Parkin, T.B., Tiedje, J.M., 1985. Direct measurement of oxygen profiles and denitrification rates in soil aggregates. Soil Science Society of America Journal 49, 645–651.

Skoog, D., West, D., 1987. Analytical Chemistry: An Introduction, second Italian ed. Saunders, Philadelphia, PA, USA, 751 pp.

Soil Survey Staff, 1999. Soil Taxonomy. A Basic System of Soil Classification for Making and Interpreting Soil Surveys, second ed, Agriculture Handbook Number 436, US Department of Agriculture and Natural Resources Conservation Service, US Gov. Print. Office, Washington, DC, 869 pp.

Stevenson, F.J., 1994. Humus Chemistry: Genesis, Composition and Reactions. Wiley, New York, 496 pp.

Toyota, K., Ritz, K., Young, I.M., 1996. Survival of bacterial and fungal popu-lations following chloroform-fumigation: effects of soil matrix potential and bulk density. Soil Biology and Biochemistry 28, 1545–1547.

Vance, E.D., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring microbial biomass C. Soil Biology and Biochemistry 19, 703–707.

Van Gestel, M., Ladd, J.N., Amato, M., 1992. Microbial biomass responses to seasonal change and imposed drying regimes at increasing depths of undisturbed topsoil profiles. Soil Biology and Biochemistry 24, 103–111.

Wenderoth, D.F., Reber, H.H., 1999. Correlation between structural diversity and catabolic versatility of metal-affected prototrophic bacteria in soil. Soil Biology and Biochemistry 31, 345–352.

Wolters, V., Joergensen, R.G., 1991. Microbial carbon turnover in beech forest soils at different stages of acidification. Soil Biology and Biochemistry 23, 897–902.

Zhou, J., Davey, M.E., Figueras, E., Rivkina, E., Gilichinsky, D., Tiedje, J.M., 1997. Phylogenetic diversity of a bacterial community from Siberian tundra soil DNA. Microbiology 143, 3913–3919.

Zvyaginstev, D., 1994. Vertical distribution of microbial communities is soils. In: Ritz, K., Dighton, J., Giller, K. (Eds.), Beyond the Biomass. Wiley, UK, pp. 29–37.