Functional and molecular responses of soil microbial communities under differing soil management practices

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Abstract

The effects of soil management on some microbiological properties and soil bacterial community structure were evaluated. Two field sites with the same soil type, located on the same geographic area adjacent to one another, have received different soil management practices and cultivation. One site has been subjected for 20 years to intensive horticulture under conventional tillage and irrigation with low quality salt-rich water; the second field site has been uncultivated for a long period and was turned to organic farming practices over the last 5 years and is currently cultivated with fruit orchard. Total bacterial counts, microbial ATP, microbial community metabolic (BIOLOG®) profiles, and DNA fingerprinting by PCR-DGGE were determined. Two-way ANOVA revealed that total bacterial counts were not significantly affected by the two different management practices; ATP content was consistently and significantly lower in salt-water irrigated soil than in organic soil at the three sampling times. The cluster analysis of community level physiological profiles indicated that microbial communities were much more uniform in organic soil than in irrigated one, suggesting that salt-water irrigation could have affected the size of the microbial population, its metabolic activities, as well as its composition. Molecular patterns fitted the BIOLOG® profile diversity. In particular, at any sampling time, PCR-DGGE patterns of bacterial DNA, extracted by an indirect method, significantly discriminated irrigated from organic soil samples. The PCR-DGGE patterns of total soil DNA, extracted by a direct method, showed a moderate to significant variation among irrigated and organic soil samples. Biochemical, microbiological and molecular data contributed to evidence a significantly different response of indigenous microflora to soil management by using saline water or organic farming.

Keywords: Soil salinization; Microbial communities; BIOLOG®; DGGE

1. Introduction

Soil ecosystems are highly complex, containing a tremendous amount of species. Indigenous microbial populations in soil are of fundamental importance for ecosystem functioning, through determining nutrient cycling, organic matter decomposition and energy flow (Doran and Zeiss, 2000). Despite all attempts to measure fluxes and gross microbial pools, the soil and its microbiota still remain a black box. Most soil microorganisms are still unknown, while very few have been isolated, cultured and identified, and directly related to their function in agroecosystems. Although new culture media have been recently developed to maximize the recovery of diverse microbial groups from soils (Balestra and Misaghi, 1997; Mitsui et al., 1997), the comparison between direct microscopic counts and plate counts indicates that as less than 0.1% of
agricultural soil microorganisms are culturable (Atlas and Bartha, 1998).

In recent years, the approaches for studying soil microbiota have moved from biochemical and microbiological determinations such as enzyme activities, microbial biomass and respiration coefficients towards the investigation of bacterial diversity and microbial communities structure (Hill et al., 2000). The BIOLOG® metabolic assay was originally developed for identification of microbial isolates on the basis of their ability to oxidize different carbon sources, and has been adapted to investigate functional diversity of soil microbial communities (Garland and Mills, 1991; Garland, 1997; Fließbach and Mäden, 1996; Di Giovanni et al., 1999). In the BIOLOG® approach Community Level Physiological Profiles (CLPPs) are obtained inoculating soil-extracted microbial populations in plates similar to the ones used for pure culture identification, and colorimetrically determined by assessing the color change of a redox indicator (tetrazolium violet) induced by the growth of aerobic heterotrophic microorganisms. Differences in the CLPPs of the microbial populations from various soil environments are evaluated, most commonly by multivariate statistical analysis. There are some considerations in the use of this method for community analysis. Density of the initial inoculum must be standardized because it affects the rate at which color develops. The commercially available BIOLOG® plates (GP, GN, Eco-MicroPlates and others) are not necessarily relevant and most likely they do not reflect the diversity of substrates found in the environment (Konopka et al., 1998), although changes to the design of BIOLOG® GN and BIOLOG® GP MicroPlates have recently been implemented (O’Connel and Garland, 2002). The method still suffers from bias problems similar to the ones encountered with culture plating method. Nevertheless, CLPPs have been used to provide useful information for assessment of soil microbial community diversity (Baudoin et al., 2002; De Fede et al., 2001; O’Connel and Garland, 2002; Smalla et al., 1998, Widmer et al., 2001).

New molecular techniques, based on characterization of soil-extracted nucleic acids, offer great potential for investigating the vast portion of soil bacterial communities, which is not culturable. Of the various genetic approaches used to estimate the microbial composition and diversity, the PCR amplification of conserved and variable regions of 16S rRNA gene may give either a rough overview of taxonomically distant groups or a deeper insight into selected eco-physiological groups. Amplified DNA can be cloned into vectors, sequenced and analyzed for similarity to other known sequences in public-domain databases (Maidak et al., 1997), or resolved by denaturing gradient (DGGE) or temperature gradient (TGGE) gel electrophoresis (Heuer and Smalla, 1997; Muyzer and Smalla, 1998) and non-denaturing gel electrophoresis (Griffiths et al., 2001; Liu et al., 1997). Although DGGE/TGGE represents a rapid and suitable technique for resolving PCR-amplified products from complex microbial communities, the major limit of the technique is that in soil ecosystems the number of different genomes is so high that the complexity of rDNA fragments can exceed the resolving power of denaturing gels (Nakatsu et al., 2000; Øvreås and Torsvik, 1998).

Since no single method is available, at present, to describe the total community, a combined approach offers the opportunity to correlate information, and to overcome and minimize drawbacks arising from culture-dependent and independent methods.

Little information is available about the contribution of soil microbial diversity in soil ecosystem functioning. An important benefit of such diversity may be to provide greater resistance to environmental stresses and external disturbance. Several studies have found decreased levels of microbial biomass, diversity and altered community structure due to disturbance mostly caused by pesticides, heavy metals, sludge amendments (Bååth, 1989; Engelem et al., 1998; Jonsen et al., 2001; Smit et al., 1997). Anyway, it is not known for sure whether a decreased diversity of soil organisms will cause declines in resistance to external stresses and how it affects soil capacity to function as a vital living system (Degens et al., 2001), largely because soil microbes have greater functional redundancy than higher organisms (Othonen et al., 1997). This would mean that, even though anthropogenic activities affect the genetic composition of soil microbial communities, gross microbial processes and their potential role in maintaining soil quality might remain unaffected. On the other hand, natural or human-induced perturbations may influence the level of soil microbial activities without causing compositional shifts in soil communities structure (Giller et al., 1997).

Soil salinization of anthropogenic origin is becoming a worldwide problem of major concern that affects especially Mediterranean areas. A significant contribution to this problem is derived by the improper use of salt-rich waters in agriculture (Szabolcs, 1998).

The aim of the present study was to evaluate the impact on soil microflora of salt-rich water irrigation in intensive horticulture. Soil bacterial functional and genetic diversity was herein investigated by using a multiple technique approach, that is a combination of cultivation-based and cultivation-independent molecular methods.

2. Materials and methods

2.1. Soil and soil sampling

The study was conducted in two field sites located on the same geographic area near Bari (Ripagnola) in southern Italy. These fields are located adjacent to one other, have the same soil type and topographical features, but have received different soil management practices and cropping systems. The first field site has been subjected for 20 years to intensive horticulture under conventional tillage
and irrigation with low quality salt-rich water (76.4 F; salinity, 3410 mg L\(^{-1}\); EC\(_w\), 1.5–4.0 dS m\(^{-1}\); chloride, 1590 mg L\(^{-1}\)) (intensive horticulture-salt rich water, IH-SW). The second field site was kept fallow for several consecutive years and was turned for 5 years to a crop production system that reduces, avoids or largely excludes the use of synthetic compound fertilizers, pesticides, growth regulators, and livestock feed additives (organic farming, OF), according to the European Union legislation (2002/91), and is currently being cultivated with fruit orchard.

Five composite samples, each resulting from four soil cores pooled together, were collected from the surface (0–15 cm) of both OF and IH-SW soils according to a systematic sampling design across a W-shaped transect 1 × 1 m grid size (van Elsas and Smalla, 1997). Field-moist soils were sieved to 2-mm particle size and stored at +4 °C before analysis. In order to assess seasonal variations, soil sampling was repeated three times over a 1-year period. Sampling dates were 14 April 2000 (I), 25 October 2000 (II), and 24 April 2001 (III).

The main physico-chemical properties of IH-SW and OF soils, which were determined using the standard methods recommended by SSSA (Sparks, 1996), are shown in Table 1.

### 2.2. Total bacterial count

Soil microorganisms were extracted by shaking 10 g of soil in 100 ml of one-quarter strength Ringer solution (Oxoid). After 10-fold serial dilution in sterile Ringer solution, suspensions (1 ml) were inoculated in Petrifilm Total Aerobic Count (Oxoid). After 10-fold serial dilution in sterile Ringer solution, suspensions (1 ml) were inoculated in Petrifilm Total Aerobic Count (Oxoid). The plates were incubated at 30 °C in the dark and colonies counted after 3 days. Data from triplicate readings were expressed as Colony Forming Units (CFU) g\(^{-1}\) dry soil.

### 2.3. Microbial ATP

Microbial adenosinetriphosphate (ATP) was extracted from each soil sample according to Arnebrant and Bååth (1991) and bioluminometrically titrated with Lumac (The Netherlands) equipment and reagents. An internal standard procedure, as suggested by Ciardi and Nannipieri (1990), was used in order to express the results as μg ATP g\(^{-1}\) dry soil.

### 2.4. Microbial community metabolic (BIOLOG\(^{\circledR}\)) profiles

Carbon source utilization patterns of soil microbial communities (CLPPs) were assessed by using BIOLOG\(^{\circledR}\) 96-well Eco-Microplates (Biolog Inc., USA), which contained 31 different carbon sources, consisting of eight carbohydrates, eight carboxylic acids, four polymers, six amino acids, two amines and three miscellaneous substrates, three times replicated on each microplate. Soil microorganisms were soil-extracted according to Zak et al. (1994) with minor modifications. Briefly, a 10\(^{-1}\) (w/v) soil suspension in one-quarter strength Ringer solution was shaken on a reciprocal shaker (30 min, 90 rpm) and then paper filtered. Serial 10-fold dilutions (up to 10\(^{-5}\)) in sterile Ringer solution were dispensed (150 μl) into each of the 96 wells of the BIOLOG\(^{\circledR}\) Eco-Microplates. The microplates were incubated at 30 °C in the dark for 5 days, and the color development (carbon utilization) in the wells was measured as absorbance at 590 nm every 24 h using a Microplate E-Max Reader (Bio-Rad, USA). Monitoring every 24 h the absorbance evolution in the plates inoculated with different dilutions of the microbial suspension, permitted to standardized inoculum density and reading time at 10\(^{-3}\) and 96 h, respectively.

### 2.5. Soil DNA extraction and purification

A direct and an indirect method were used, respectively, for total and bacterial community DNA extraction from soil. Total soil DNA was obtained by a direct lysis method (Tsai and Olsen, 1991) followed by purification steps (Smalla et al., 1993) with slight modifications. Briefly, soil samples (1 g) were initially washed (three times) with 4 ml 120 mM sodium phosphate buffer (pH 8.0), shaken at 150 rpm for 15 min, and centrifuged for 10 min at 6000 g. The pelletted soil was resuspended in 2 ml of lysis solution (0.15 M NaCl–0.1 M Na\(_2\)EDTA, pH 8.0) containing 15 mg lysozyme ml\(^{-1}\), incubated at 37 °C with occasional shaking for 2 h, and then added with 1 ml of 0.2 M NaCl–1 M Tris–HCl (pH 8.0) and 1 ml 20% SDS. After 10-min resting at room temperature, cells were lysed by three cycles of freezing/thawing (−70 °C dry ice–ethanol/+65 °C water bath). The soil slurry was then sequentially extracted with Tris-buffered (pH 8.0) phenol, phenol–chloroform–isoamylalcohol (25:24:1), and chloroform–isoamylalcohol (24:1). DNA in the aqueous phase was precipitated with an equal volume of isopropylalcohol (overnight at −20 °C), pelleted by centrifugation (10,000 g, 10 min) and lyophilyzed. Further purification was done by sequential CsCl and KAc precipitation steps (Smalla et al., 1993) followed by spin
column filtration through Wizard® DNA Clean-up System (Promega, USA).

Bacterial community DNA was extracted from 4 g of fresh-sampled soil by using the indirect DNA extraction method described by van Elsas et al. (1997), with the following modifications: after removal of bacterial cells from soil matrix by blending in 0.1% (w/v) sodium pyrophosphate and differential centrifugation (114g, 3 min), the pelleted bacterial fraction (18,000g, 20 min) was resuspended in 4 ml 120 mM sodium phosphate buffer (pH 8.0), added with 3 g of glass beads (0.1 mm diameter), 500 μl 20% SDS and 3.5 ml Tris-buffered phenol (pH 7.6) and lysed by bead beating (B. Braun, Melsungen, D, cell homogenizer, 2000 rpm, 60 s × 3, rest 20 s in between). Bead mill homogenization in a lysis mixture containing buffered phenol and SDS was found to greatly improve the efficacy of DNA extraction procedure (Miller et al., 1999). After chloroform-extraction the nucleic acids were precipitated with 0.1 vol 5 M NaCl and 2 volumes ice-cold ethanol. Purification was obtained by CsCl precipitation followed by GeneClean Spin Kit (Bio 101; cat no. 1101–400) column filtration, according to manufacturer’s instructions.

Yield and quality of soil-extracted DNA were assessed by 0.7% agarose gel electrophoresis and staining with ethidium bromide (Sambrook et al., 1989).

2.6. Community fingerprinting by PCR-DGGE

The 16S rRNA genes from soil microbial communities were amplified by PCR by using the primer pair F984GC/R1378 described by Heuer et al. (1999). A GC-rich sequence was attached to the 5' end of the F984GC primer in order to prevent complete melting during the separation in the denaturant gradient (Muyzer et al., 1993). Soil DNA was amplified in a PCR Sprint thermocycler (Hybaid, Middlesex, UK). The 100-μl PCR reaction mixture contained: 100 ng of DNA template, 100 pmol of each of the two primers, 20 nmol of each of the dNTPs, 2.5 Units /100 μl of Taq DNA Polymerase (High Fidelity, Roche Diagnostics) in a buffered final volume of 100 μl, according to the manufacturers. BSA (4 μg/100 μl) was added to avoid inhibition of amplification by organic compounds co-extracted from soil. The PCR conditions were: a hot start of 4 min at 94 °C; 10 cycles consisting of 94 °C for 10 s, 68–58 °C for 20 s decreasing the temperature by 1 °C every cycle (‘touchdown’), 72 °C for 40 s; 30 cycles consisting of 94 °C for 10 s, 58 °C for 20 s, 72 °C for 40 s; a final step of 10 min at 72 °C. Amplification products were analyzed by electrophoresis in 1.0% agarose gels stained with ethidium bromide. DGGE was performed using a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). A 15-μl amount of PCR samples were loaded onto 6% (w/v) polyacrylamide gels containing a linear chemical gradient ranging from 40 to 60% denaturant (100% denaturant corresponds to 7 M urea plus 40% (v/v) of deionized formamide) in a 1× TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM disodium EDTA, pH 8.3). The electrophoresis was run in a 1× TAE buffer at 60 °C at a constant voltage of 100 V for 7 h. After the run the gels were stained with SYBR® Gold nucleic acid gel stain (Molecular Probes, Eugene, OR, USA) and photographed under UV light (λ = 254 nm) with a Bio-Rad Gel Doc documentation system.

2.7. Data analysis

The bacterial CFU and ATP data are mean values from five replicates and are expressed on an oven-dry soil basis (105 °C, 24 h). For the BIOLOG® data the average well color development (AWCD) of all 31 carbon sources for each sample were calculated prior to any statistical analysis in order to eliminate variation in well color development caused by different cell densities (Garland and Mills, 1991; Garland, 1997). Functional diversity from BIOLOG® data was evaluated by calculating Shannon’s substrate diversity index (H'), substrate richness (S) and substrate evenness (E) according to Zak et al. (1994). Microbial data (ATP and CFU) and diversity indices (H', S, E) were treated statistically by two-way analysis of variance (ANOVA) by using a SigmaStat v2.0 (Jandel Scientific, San Rafael, CA) software. Soil management and sampling time were used as factors. Pairwise multiple comparison between all treatment means were made by using the Tukey test at P < 0.05. The data from BIOLOG® were further analyzed by cluster analysis with a SPSS software.

Banding patterns of DGGE profiles were analyzed by the Bio-Rad Quantity One™ software to obtain percentages of similarity among lanes and by the Diversity Database™ Fingerprinting software (Bio-Rad Laboratories) to generate similarity dendrograms (Dice coefficient of similarity) via an unweighted pair group method average (UPGMA) analysis.
using arithmetic averages (UPGMA) clustering method (Rademaker et al., 1999).

3. Results

Lower numbers of culturable heterotrophic bacteria (CFU) were isolated from salt-water irrigated soil (IH-SW) than from organic farmed soil (OF) soil both at the first and the second sampling date, while a reversed trend was found in samples from the third sampling (Fig. 1). Total CFU ranged from $0.38 \times 10^6$ to $1.14 \times 10^6$ g$^{-1}$ dry weight in IH-SW soil and from $0.68 \times 10^6$ to $0.93 \times 10^6$ g$^{-1}$ dry weight in OF soil. Two-way ANOVA of CFU data run by considering soil management as sole source of variation evidenced that total bacterial counts were not significantly ($P > 0.05$) affected by the two different management practices (Table 2), while significantly ($P < 0.01$) differed with sampling time, and also management × sampling time interaction had a significant effect. Multiple comparison procedure (Tukey-HSD test) indicated that only CFU of IH-SW at III sampling time were significantly different ($P < 0.05$) when comparing mean values among different sampling dates within each management system (Fig. 1). Averaged values of microbial ATP content of tested soils at the three sampling dates are reported in Fig. 2. Two-way ANOVA revealed that ATP value was significantly affected by differing management strategy, being lower in IH-SW soil than in OF soil. Moreover, soil ATP content was influenced significantly by both sampling time and by sampling time × management interaction (Table 2). Multiple comparison procedure (Tukey-HSD test) showed that within each treatment samples from III sampling date were significantly different from other sampling dates (Fig. 2). It is worth noting that sampling time exerted a strong and a significant influence either on CFU or on ATP analysis.

Values of diversity ($H'$), substrate richness ($S$) and evenness ($E$), obtained from BIOLOG$^\text{®}$ analysis of IH-SW and OF soil samples, are reported in Table 3. $H'$, $S$ and $E$ values were generally higher in IH-SW than in OF soil, with a slight increasing trend from I to III sampling time. Two-way ANOVA of data (Table 2) evidenced a significant effect on diversity, substrate richness, and substrate evenness due to either differing management systems and sampling times. Once again, when comparing the variance within each group, the means at the third sampling time resulted in significant differences between management treatments. In other words, tillage with salt-rich water seemed to increase the functional diversity of bacteria by increasing both substrate richness and evenness. Cluster analysis of CLPPs obtained after 96 h incubation are reported in Fig. 3. In the first sampling (Fig. 3a), the cluster analysis showed great similarity for the profiles of OF soils, which were linked together at a low Euclidean distances. On the other hand, only a moderate similarity was found for two of IH-SW soil profiles, which were linked at a higher Euclidean distance, and no similarity at all was found for the third triplicate, which was linked to OF soil profiles. In the second sampling (Fig. 3b), four out of five OF profiles were found very similar, linked together at low Euclidean distances, and no similarity was found for the two IH-SW soil profiles.

![Fig. 2. Changes in ATP content in IH-SW and OF soils at differing sampling times. Values are the means (n = 5) with ± standard error of the mean (SEM). Different letters indicate a significant difference at $P < 0.05$ when comparing the variance among times within each treatment according to Tukey test.](image-url)
distances. The profiles of IH-SW soils were in part assimilated to these (two out of five) and in part grouped in two different distinct clusters. In the third sampling (Fig. 3c), all the OF soil samples and one IH-SW sample presented microbial profiles strictly linked together at a low Euclidean distance, while similarities for the others were found at much higher distances.

Genetic fingerprinting by DGGE of eubacterial 16S rDNA amplified fragments from soil DNA obtained with the indirect extraction method showed a few strong dominating bands (8–10) appearing across all the samples, regardless of the differing sampling time and till/cropping systems (Fig. 4). Together with these strong signals, a greater number of fainter well-resolved bands appeared in the profiles, which were all considered when clustering method was applied. In fact, whereas the genetic profiles share a high number of common strong bands, differences found in less numerous weaker bands may consistently affect clustering of the profiles. In other words, a striking diversity was observed in DGGE patterns of both tested soils due to numerous faint bands in the background, indicating that the structure of microbial communities was rather complex. Profiles of replicates were generally highly repetitive and this greatly facilitated image analysis of DGGE banding patterns by using the Dice similarity coefficient directly applied to the array of densitometric values forming the fingerprints. The UPGMA was used to

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**Table 3**

Effects of management and sampling date on microbial functional diversity evaluated by Shannon’s diversity index ($H'$), substrate richness ($S$) and substrate evenness ($E$) for BIOLOG® Eco-Microplates

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sampling$^a$</th>
<th>$H'$</th>
<th>$S$</th>
<th>$E$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IH-SW</td>
<td>I</td>
<td>4.13±0.18</td>
<td>27±2</td>
<td>0.87±0.03</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>4.15±0.27</td>
<td>28±3</td>
<td>0.86±0.04</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>4.44±0.20</td>
<td>30±1</td>
<td>0.90±0.03</td>
</tr>
<tr>
<td>OF</td>
<td>I</td>
<td>3.74±0.19</td>
<td>24±2</td>
<td>0.83±0.04</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>3.72±0.33</td>
<td>24±4</td>
<td>0.82±0.04</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>4.37±0.15</td>
<td>29±1</td>
<td>0.90±0.04</td>
</tr>
</tbody>
</table>

$^a$ See Section 2. Values are the means ($n=5$) with standard error of the mean.

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**Fig. 3.** Cluster analysis of BIOLOG® profiles of IH-SW and OF soil replicates from differing sampling times. Scale indicates Euclidean distance. (a) I sampling; (b) II sampling; (c) III sampling. Sampling time, see Section 2.
cluster the data. The resulting similarity dendrogram (Fig. 5) shows that at any sampling time molecular patterns of IH-SW soils could be well discriminated from patterns of OF soils, within a range of similarity from 52 to 60%.

Soil DNA was also extracted by a direct method and PCR-amplified 16S rDNA fragments used to generate genetic fingerprinting. As well as for soil DNA obtained with the indirect extraction method, DGGE patterns (Fig. 6) showed few strong dominating bands and a great number of faint unresolved bands: a kind of background very difficult to analyze, likely due to the higher heterogeneity of directly extracted total soil DNA compared to bacterial DNA. Once again the DGGE profiles of replicate samples were generally highly repetitive. The DGGE patterns were transformed into graphs by the Bio-Rad Quantity One™ software: each resolved band of all gel lanes was converted to a trait whose intensity is related to the amount of the corresponding DNA fragment. The software calculated the percentage of similarity among lanes taking into account the migration distance and the relative intensity of all bands. Averaged values with standard errors and coefficients of variability of calculated similarities are reported in Table 4. Statistical analysis (Student’s t-test) of similarity data indicated that between IH-SW and OF soil samples there was no significant difference ($P > 0.05$) in the first sampling, a moderate variation ($P < 0.01$) in the second sampling, and a highly significant difference ($P < 0.001$) in the third sampling.

4. Discussion

The inconsistent relationship found between total bacterial counts and different management practices is likely to be the consequence of large-scale heterogeneity within samples taken across the experiment in the same test-site, due to an irregular distribution of microbial population sizes in the soil. Thus, the criticisms often addressed to this microbiological index as a way of accounting for soil health in general, seems here to be confirmed.

Average values of microbial ATP content of tested soils suggest a strong metabolic depression due to the nature of anthropogenic disturbance, i.e. irrigation with salt-rich water. Microbial ATP evaluation appears a much more reliable indicator under such respect, at least where the disturbance is able to influence microbial metabolism, as is the case with salt-water irrigation. While total bacterial count is affected by the differential capacity of extracted cells to grow on laboratory cultural media, ATP evaluation is based on its extraction from all bacterial cells occurring in soil and for this reason the assay is able to better represent the factual situation at the time of the analysis. Moreover, the ATP content not only reflects the microbial biomass size, but also metabolic activity changes as affected by management and anthropogenic inputs (Horwath and Paul, 1994).

As BIOLOG® Eco-Microplates were used, which allow metabolic characterization on the basis of 32 carbon sources only, a guild analysis of the CLPPs as suggested by some authors (Zak et al., 1994; Siciliano and Germida, 1998) was
not possible in this case. Nevertheless, BIOLOG® methodology was able to discriminate between IH-SW and OF soil microbial populations. $H'$, $S$ and $E$ indices were found to be consistently higher in IH-SW than in OF soils and cluster analysis of CLPPs indicated that the microbial communities are qualitatively much more uniform in OF soil samples than in the irrigated ones. These findings, along with the lower ATP production in IH-SW soil, suggest that the disturbance induced by salt-water irrigation could have affected the size of the microbial population, its metabolic activities, as well as its composition. It seems that soil communities in tested soils shared a rather low level of genetic similarity, according to Duineveld et al. (1998) who recognized a different similarity > 18% to indicate a significant change in community structure. There was also some apparent evidence of slight temporal shifts showing a lowering genetic diversity between IH-SW and OF samples moving from I to III sampling date. However, whether this finding is due to an artifact, to environmental factors related to season, cropping effect, or year, could only be answered by repeated sampling taken across a long-term experiment.

Molecular data after DGGE fingerprinting of indirectly extracted soil DNA agree with the cluster analysis of BIOLOG® metabolic profiles in discriminating between IH-SW and OF soil bacterial community. A similar finding, although in two of the three samplings, was obtained when total DNA was used as template for 16S rDNA DGGE fingerprinting. This seems to indicate that salt-rich water irrigation induced significant changes in both phenotypic and genetic diversity of microbial populations. Such findings seem also to indicate that the lower ATP production found in irrigated soils is not simply due to a quantitative reduction of the microbial population, but to an alteration involving microbial species and their ratios.

The two extraction–purification protocols, targeting bacterial community DNA obtained after centrifuge separation of bacteria from soil particle, or total DNA, that is extracellular soil DNA and plant debris DNA, besides DNA from living microbiota, may affect yields, composition, extractability, and amplificability (Lloyd-Jones and Hunter, 2001; Roose-Ansaleza et al., 2001). At this regard, Kozdrorj and van Elsas (2000) evidenced that differing soil DNA extraction methods would result in differing yields and in divergent fingerprints generated by PCR-DGGE. It is also likely that the indirect extraction method may preferentially target only major, easily desorbed bacterial fractions, giving low cell yields, i.e. 20–30% for clay-loam agricultural soils. Our molecular data seem therefore to substantiate the statement that comparison of bacterial communities are dependent on the DNA recovery method used (Martin-Laurent et al., 2001).

Genetic fingerprinting of PCR-amplified 16S rDNA fragments was used to profile complex microbial community inhabiting the same soil type under two differing management systems: intensive horticulture combined with long-term irrigation with saline water vs. organic fruit tree culture. One of the main tasks of the present work was to assess whether irrigation with salt-rich water could determine valuable shifts in soil bacterial functioning and composition. Most soils are considered slightly saline

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**Table 4**

Similarity among digitalized DGGE profiles of PCR-amplified 16S rDNA sequences from directly extracted soil DNA after Bio-Rad Quantity One software comparison

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sampling</th>
<th>Similarity (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IH-SW</td>
<td>I</td>
<td>64.45±5.02</td>
<td>24.62</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>71.34±4.27</td>
<td>18.91</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>72.56±2.93</td>
<td>12.77</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>59.32±4.19</td>
<td>22.17</td>
</tr>
<tr>
<td>OF</td>
<td>II</td>
<td>78.44±1.54</td>
<td>6.21</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>65.67±3.19</td>
<td>15.37</td>
</tr>
</tbody>
</table>

*a* See Section 2.

*b* Values are the means ($n=5$) with ± standard error of the mean.
if the EC of a saturated past extract exceeds 2 dS m\(^{-1}\). However, the threshold above which microbial processes are impaired by increased salinity can vary as a result of many factors, i.e. organic amendments, crops and crop management (Smith and Doran, 1996). In IH-SW soil EC\(_e\) reached a value of 1.9 dS m\(^{-1}\), which is higher than EC\(_e\) in OF soil (0.9 dS m\(^{-1}\)) even though not enough to make the IH-SW a saline soil. This would mean that, in spite of being irrigated for a long term with salt-rich water, IH-SW soil still has a certain drainage, which avoids excess salt accumulation in the profile, and supports crop production. Nevertheless, addition of soluble salts given with irrigation can greatly influence physical, chemical and biological properties in soil. This is evidenced by changing in bulk density from OF (1.31 kg dm\(^{-3}\)) to IH-SW (1.88 kg dm\(^{-3}\)), accompanied by a reduction in soil porosity (50% in OF and 20–30% in IH-SW soil) (Table 1). Changes in physical properties were possibly due to an increased dispersion of clay particles linked to the addition of Na ions. So, the use of saline water appears as the main factor which markedly affected soil bacterial composition, possibly by changing soil physico-chemical properties. Appearance of less numerous strong signals in DGGE profiles would substantiate the hypothesis that a limited number of dominant, ubiquitous and ecologically well-adapted bacterial types are present in either soils. Conversely, a great number of faint bands indicated that many equally abundant populations characterized each soil site, giving a rather high level of bacterial diversity. We can therefore argue that different long-term irrigation systems may be viewed as the main factor exerting a strong influence over the development of distinct microbial communities. Nevertheless plant type (Greyston et al., 2001; Marschner et al., 2001) and root zone effects (Yang and Crowley, 2000) are also important variables in determining the bacterial species composition. However, this was not the case as bulk soil instead of rhizosphere soil was investigated under the present investigation.

5. Conclusions

Assessment of genetic and functional diversity of the microbial communities is central to understanding the potential effects of environmental perturbations, i.e. soil salinization. Our study shows that microbial ATP determination and CLPPs (BIOLOG\(^\circ\)) appear to be more reliable and useful indicators than total bacterial counts to describe the effects of saline water irrigation on soil microorganisms. However, both methods are quite insufficient to account for quantitative and qualitative changes in the composition of soil microbial community, and to determine which microorganisms are mainly affected by the soil management under investigation. Genetic fingerprinting by DGGE analysis of PCR amplified 16S rDNA provides a deeper insight of the effects of soil salinization on the composition of soil microbial communities. Although these findings seem to be affected by the soil DNA extraction method, a relationship with ATP and BIOLOG\(^\circ\) data was pointed out. So, a multitechnique approach that combines traditional biochemical and cultivation-based methods with molecular-based techniques, along with some methodological improvements, may represent a promising tool to broaden our knowledge on the role of microbial diversity in soil ecosystem functioning.

This strategy showed that saline water irrigation has induced a genetic and metabolic alteration of soil microbial communities, corresponding to a lower microbial activity. Further investigation is in order to establish which microbial functions are involved in such alteration and their effects on long-term soil fertility.

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