Responses of bacterial community structure and diversity to soil eco-friendly bioremediation treatments of two multi-contaminated fields

Valeria Ventorino,[1,2] Vincenza Faraco,[2,3] Ida Romano,[1] Olimpia Pepe[1,2]

[1]Department of Agricultural Sciences, Division of Microbiology, University of Naples Federico II, Portici (NA); [2]Task Force on Microbiome Studies, University of Naples Federico II, Naples; [3]Department of Chemical Sciences, University of Naples Federico II, Naples, Italy

Abstract

Biodiversity of soil microbial populations could be altered and affected by anthropogenic pressures due to the release of organic and inorganic xenobiotic compounds and/or the application of remediation practices. Therefore, the assessment of the response of microbiota to environmental pollution and to bioremediation techniques is a critical issue in soil ecology. In this study a culture-independent approach was used to investigate the indigenous bacterial community structure in two contaminated soils of a National Interest Priority Site in Campania (southern Italy) and to monitor the impact of different remediation technologies. Our results show that bacterial populations shifted in the polluted soils over time after the application of compost and microbial inoculum. Statistical analyses based on the similarity of DGGE profiles show that the bacterial community structure and diversity was not affected by contamination. Hence the main change in similarity levels was induced by sampling time and by the interaction between soil eco-friendly bioremediation treatments.

Introduction

The harmful effects of environmental pollution do not impact only on human health, but also on ecosystems, landscape and soil biodiversity. Several studies have shown that in a polluted environment the number of animal, plant and microbial species could be greatly reduced (Øvreås *et al.*, 1998; Singh, 2003). Therefore, a contaminated site loses both ecological and economic value, and could become more vulnerable to other anthropogenic and natural pressures (Beier *et al.*, 2005). However, although organic pollutants reduce microbial biodiversity (Sutton *et al.,* 2013), they could stimulate the growth of some microbial species able to use them as a carbon source (Ventorino *et al.*, 2014). Among the cheapest technologies available for soil remediation, the use of compost (Chen *et al.*, 2015) as well as inoculation of selected microorganisms able to use organic xenobiotic compounds as a carbon source are considered eco-friendly and effective (Fiorentino *et al.,* 2013; 2017). Indeed, not only can compost improve soil quality but it is also a source of bacteria with putative suppressive effect and of nutrients for stimulating growth and activity of soil microbial populations able to degrade organic contaminants and promote plant fitness (Pepe *et al.*, 2013; Ventorino *et al.*, 2016; Taiwo *et al.*, 2016; Parillo *et al*., 2017). Therefore, the presence of specific pollutants, as well as the use of bioremediation techniques, could affect the microbial community structure of a soil (Ventorino *et al.*, 2018a). However, the high microbial biodiversity and the complex relationships among bacterial populations and biotic and abiotic processes influencing their activities in soil make it difficult to evaluate soil microbial response to contamination and remediation practices (Bastida *et al.*, 2016). In this context, it is necessary to use biomonitoring techniques for assessing soil microbial structure and diversity in order to establish and apply the best method for cleaning up contaminated soils. Since it is generally accepted that by using culture-dependent methods it is possible to recover less than 1% of the microbial populations living in environmental samples (Amann *et al.*, 1995), the use of culture-independent methods, such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), allows changes in the microbial community structure to be evaluated. In fact, molecular methods based on a metagenomic approach allow direct analysis of microbial populations in their natural habitat, thus avoiding the isolation and cultivation of the different microbial species according to their growth requirements. PCR-DGGE based on 16S ribosomal DNA and denaturing gradient gel electrophoresis fingerprinting technology is being increasingly used to assess changes in soil bacterial communities in a wide range of environments (Li *et al.*, 2006; Gupta *et al.*, 2016; Ventorino *et al.*, 2016, 2018a).

In this context, this study was carried out to assess the impact of contamination as well as the use of environmentally compatible techniques for soil remediation on diversity of bacterial communities in soil samples collected from two multi-contaminated fields of the area of the Litorale Domitio Agro Aversano (Giugliano and Trentola Ducenta), used as pilot fields in the LIFE-Ecoremed project.

Correspondence: Olimpia Pepe, Department of Agricultural Sciences, Division of Microbiology, Task Force on Microbiome Studies, University of Naples Federico II, Via Università 100, Portici, 80055 (NA), Italy. Tel.: +39.081.2539410.
E-mail: olipepe@unina.it

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Materials and methods

Study sites and soil sampling

The study sites were two fallow rural fields, Trentola Ducenta (TD) and Giugliano (G), contaminated both organically and with potentially toxic elements (PTEs) due to unauthorised waste disposal. These sites were used as pilot fields in the LIFE-Ecoremed project to validate environmentally compatible techniques for soil remediation (Ecoremed, 2017). In April 2014, some plots were amended with 20 t ha^{-1} of compost from the organic fraction of municipal soil waste. In addition, all plots were inoculated twice (October 2014 and April 2015) with a microbial consortium selected for its ability to use hydrocarbons as a carbon source (Ecoremed, 2017). Sixteen soil samples (10 from TD and 6 from G) were collected from the top soil (0-20 cm depth) at four sampling times: December 2013 (T0), after waste removal and before any bioremediation practices; May 2014 (TC), after compost addition; October 2014 (TIa), after the first inoculation of microbial consortium; April 2015 (TIb), after the second inoculation of microbial consortium (Tables 1 and 2 for sites TD and G, respectively). From each plot three 1-kg sub-samples were collected, homogenized and analysed to determine the diversity of bacterial communities.

Genomic DNA extraction and PCR-DGGE analysis

Total microbial DNA was extracted by using the FastDNA Spin Kit for Soil (MP Biomedicals, Illkirch Cedex, France) according to the manufacturer's specifications. DGGE analysis of bacterial communities was performed using the primers V3f (5'-CCTACGGGAGGCAGCAG-3') and V3r (5'- ATTACC GCG-GCTGCTGG -3'), spanning the 200-bp region of the 16SrDNA of *Escherichia coli* (Muyzer *et al.*, 1993). A GC-clamp was added to the forward primer according to Muyzer *et al.* (1993). The PCR mixture and conditions were performed according to Ventorino *et al.* (2017). DGGE analysis was performed in a polyacrylamide gel [8% (wt/vol) acrylamide-bisacrylamide (37:5:1)] with a denaturing gradient of 30-60% using a Bio-Rad DCode Universal Mutation System (Bio-Rad Laboratories, Milan, Italy) as previously described (Pepe *et al.*, 2011).

Statistical analysis

Phoretix 1 advanced version 3.01 software (Phoretix International Limited, Newcastle upon Tyne, England) was used to detect the DGGE bands automatically, to determine matching bands and to perform a cluster analysis as previously indicated by Ventorino *et al.* (2013). The correlation matrix of the band patterns was performed using the method described by Saitou and Nei

Table 1. Characteristics of plots and soil samples collected over time from each plot before and after the different bioremediation treatments applied in Trentola Ducenta.

Plot	Contamination*		Time°			
	C>12 (mg kg^{-1})	Zn (mg kg^{-1})	T0 Sample	TC Sample	TIa Sample	TIb Sample
6-3	93	-	1	11	21	31
6-7	106	-	2	12	22	32
21-1	132	163	3	13	23	33
21-5	150	-	4	14	24	34
21-9	206	-	5	15	25	35
32-3	109	-	6	16	26	36
32-4	329	-	7	17	27	37
32-5	176	-	8	18	28	38
32-7	541	228	9	19	29	39
32-8	250	-	10	20	30	40

*Organic and inorganic pollutant concentration (mg kg^{-1}) in soil samples measured after waste removal and before any bioremediation practices (T0), for more details please see Monaco *et al.*, 2015; Rocco *et al.*, 2016; Ventorino *et al.* (2018b). °Sampling time: T0, after waste removal and before any bioremediation practices in December 2013; TC, after compost addition in May 2014; TIa, after the first inoculation of microbial consortium in October 2014; TIb, after the second inoculation of microbial consortium in April 2015.

Table 2. Characteristics of plots and soil samples collected over time from each plot before and after the different bioremediation treatments applied in Giugliano.

Plot	Contamination*		Time°			
	C>12 (mg kg^{-1})	Cu (mg kg^{-1})	T0 Sample	TC Sample	TIa Sample	TIb Sample
1-3	533	219	1	7	13	19
1-8	75.7	110	2	8	14	20
6-7	79.5	33	3	9	15	21
8-2	705	91	4	10	16	22
8-5	401	96	5	11	17	23
8-8	590	53	6	12	18	24

*Organic and inorganic pollutant concentration (mg kg^{-1}) in soil samples measured after waste removal and before any bioremediation practices (T0), for more details please see Monaco *et al.*, 2015; Rocco *et al.*, 2016; Ventorino *et al.* (2018b). °Sampling time: T0, after waste removal and before any bioremediation practices in December 2013; TC, after compost addition in May 2014; TIa, after the first inoculation of microbial consortium in October 2014; TIb, after the second inoculation of microbial consortium in April 2015.

(1987). Finally, the percentage of similarity (S) of the bacterial community was estimated by analysing the resulting matrix using the average linkage method in the cluster procedure of Systat 5.2.1.

Results and discussion

The PCR-DGGE culture-independent approach was employed to obtain a qualitative fingerprint of the bacterial communities due to the effect over time of the environmentally compatible restoration treatments on the resident soil bacterial community of multi-contaminated soil sites TD and G (Figures 1 and 2).

In TD, the comparison of DGGE patterns showed important changes in bacterial community structure over time, revealing complex profiles and hence a high diversity of bacteria in all soils. In fact, the number of distinct DNA bands ranged from 21 at the beginning of the bioremediation treatment (T0, Figure 1A; TC, Figure 1B) whereas a considerable increase in the number of bands (up to 34) was observed after inoculation treatments (Figure 1C and D).

In site G, high bacterial diversity was also observed in all soil samples showing a number of bands of 28-29 (Figure 2). Although the number of bands remained constant during the experiment, their position and intensity strongly varied over time (Figure 2A-D) as also observed in TD. Improvement in the biodiversity of the bacterial populations after remediation treatments could be correlated with a disappearance of a stress factor, such as depletion of pollutants, to an increment in the abundance of taxonomic units and a redistribution of the bacterial specimen in the soil interpreted as the recovery of the resilience of the matrix (Ruffini Castiglione *et al.*, 2016).

As shown in Figure 3, statistical analysis of the DGGE profiles revealed that approximately 25-30% of the bacterial populations of the two soils remained stable during the experimental period without microbial perturbations (Figure 3A and B, cluster 1). These persistent bacteria could represent autochthonous populations whose growth and activity are not affected by anthropogenic activity

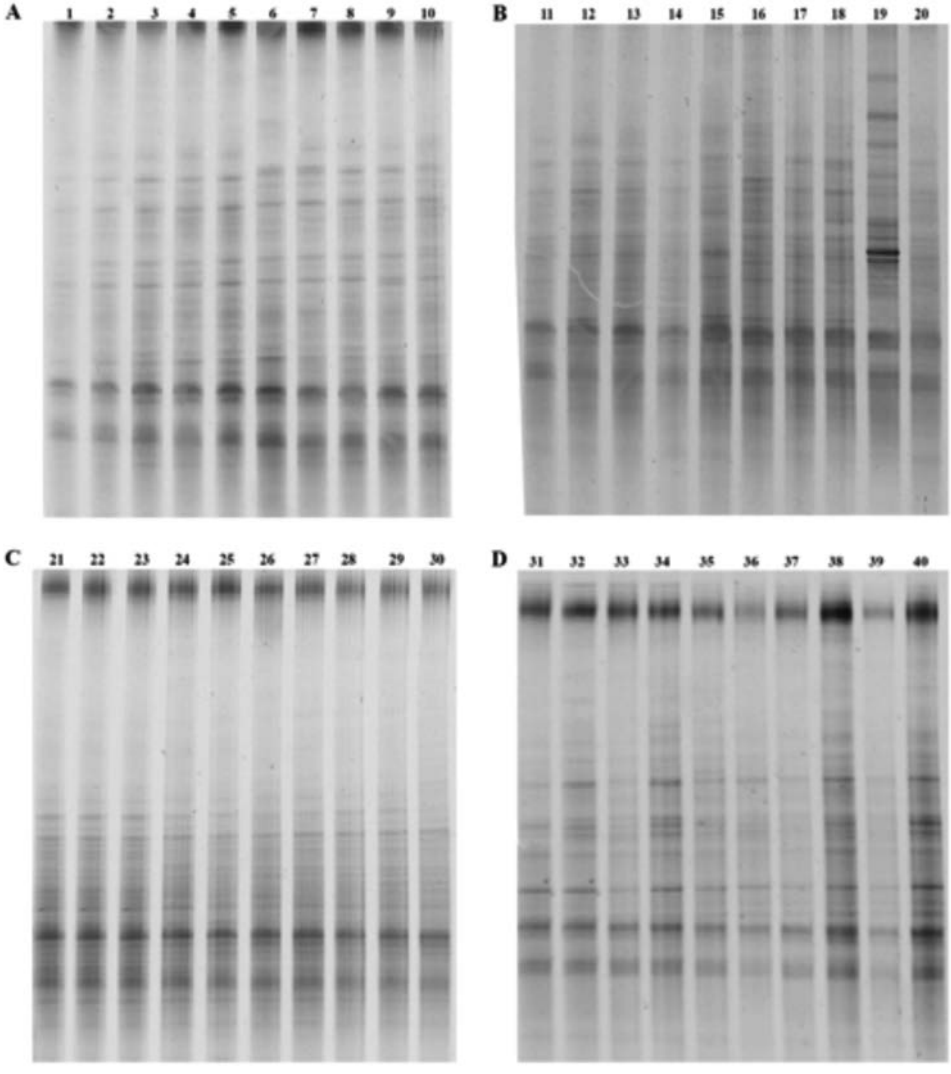

Figure 1. DGGE profiles of bacterial populations from soil samples of the Trentola Ducenta site collected before any bioremediation practices (A), after compost addition (B), after the first inoculum addition (C) and the second inoculum addition (D). See the text and Table 1 for the details of each sample.

(xenobiotic compounds and bioremediation techniques) and environmental pressures. Autochthonous naturally occurring bacteria possess some traits that may be used to survive and grow in specific habitats, enhancing their environmental survival (Søborg *et al.*, 2013). Information related to responses of autochthonous microbiota to pollution and to remediation treatment could help to assess the impact of environmental perturbation (Islam *et al.*, 2011) and to detect putative biomarkers (Ventorino *et al*., 2018b. DGGE analysis revealed important microbial shifts that accounted for about 70% of bacteria in both TD and G (Figure 3A and B, cluster 1). This behaviour is a typical fluctuation observed in the zymogenous population selected by environmental conditions. Interestingly, cluster analysis of TD (Figure 3A) and G (Figure 3B) soils detected two main groups in both sites: cluster 2 (Figure 3A and B) grouped the soil samples collected before any bioremediation practices (T0) and after compost addition (TC); cluster 3 (Figure 3A and B) included soils sampled after the first (TIa) and second (TIb) inoculum addition. In both sites a dramatic shift in bacterial community structure was detected after inoculation treatments, since cluster 2 shared only about 30 % of similarity with cluster 3 (Figure 3). Interestingly, within each of the major clusters delineated, the subgroupings of the prokaryotes (Figure 3A; cluster 4, 5, 6 and 7) were always similar and clearly associated to sampling times (T0, TC, TIa and TIb). Indeed, in TD soils, clusters 4 and 6 shared a 60% similarity with clusters 5 and 7, respectively. Within each subgroup low alterations in the bacterial community structure were observed, showing a similarity level ranging from 80 to 100%. Similarly, in site G four subclusters were identified on the basis of sampling time in which slight changes within the bacterial populations were observed with similarity level from 90 to 100% (Figure 3B; clusters 4, 5, 6 and 7). It is well known that the resident microbiota is able to adapt and acclimate to soil pollutants and/or to bioremediation treatments (Haritash and Kaushik, 2009) even if allochthonous organisms could affect fluctuations of the

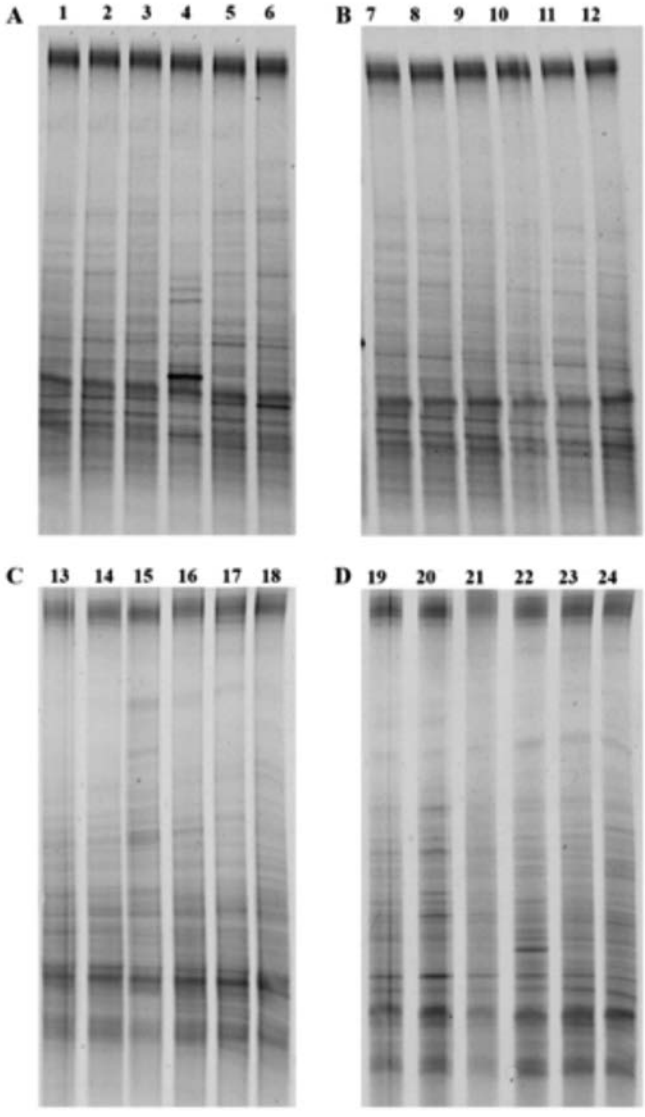

Figure 2. DGGE profiles of bacterial populations from soil samples of the Giugliano site collected before any bioremediation practices (A), after compost addition (B), after the first inoculum addition (C) and the second inoculum addition (D). See the text and Table 2 for the details of each sample.

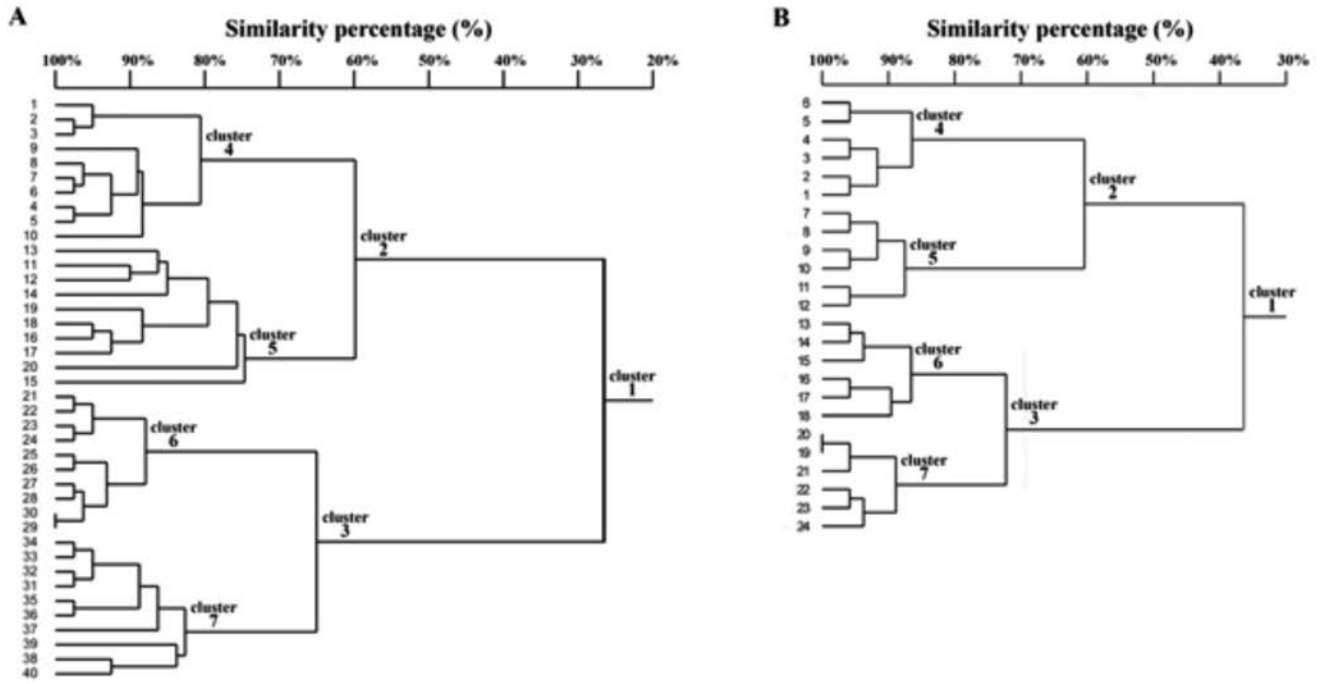

Figure 3. Dendrogram showing the degree of similarity (%) of PCR-DGGE profiles of the bacterial populations from soil samples of Trentola Ducenta (A) and Giugliano (B) site. See the text, and Tables 1 and 2 for the description of each sample.

autochthonous microbial groups in capturing an important part of the overall energy influx during bioaugmentation of soils (Dejonghe *et al.*, 2001).

Conclusions

In conclusion, the responses of bacterial community structure and diversity were not affected by contamination and hence the main change in similarity levels was induced by sampling time and by interaction between soil environmentally friendly bioremediation treatments. This behaviour suggests that a processes of acclimatization (Lladó *et al.*, 2015) to the polluted environment occurred especially with regard to allochthonous and/or zymogenous bacterial populations.

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