Spatial microbial community structure and biodiversity analysis in "extreme" hypersaline soils of a semiarid Mediterranean area

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ABSTRACT

In recent years specific attention has been paid on the biotechnological potential of microorganisms in extreme soils, in particular in saline soils. Salinity is one of the most widespread soil degradation processes on the Earth, and saline soils can be defined as extreme soils or border line habitats in which several factors, as high salt content, may limit the growth of organisms. In this study, the physical, chemical and microbiological soil properties were investigated in the shallower horizon of natural salt-affected soils in Sicily (Italy). The main aim of the research was to evaluate the structure and diversity of bacterial and archaeal communities by terminal-restriction fragment length polymorphism (T-RFLP) according to arbitrary different classes of vegetation and salt crust cover in soils. Furthermore, the structure of microbial communities was assessed considering the heterogeneity of physical–chemical properties of the habitat under investigation, as a function of vegetation, crust cover, and salinity classes. The results provided information on the type of distribution of different microbial community composition and diversity as a function of both vegetation and crust cover as well as salinity classes. In particular, the archaeal community showed a richness and diversity significantly affected by the spatial gradients of soil salinity, conversely, the bacterial one showed a decreasing trend with increasing gradient of soil salinity. The T-RFLP cluster analysis showed the formation of two groups for both bacterial and archaeal community, significantly ($p < 0.05$) influenced by sand and silt content, electrical conductivity (EC_e), vegetation cover percentage, salt crust and for by texture composition. In particular, the discriminant analysis obtained for the different salt crust classes for archaeal community stressed the membership of one of the two clusters to the class with the lower salt crust percentage (0–40%).

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1. Introduction

A definition of "extreme environment" completely acceptable for all ecosystems is not easy to find, but it is true in some habitats where environmental conditions such as pH, temperature, pressure, nutrients or saline concentrations can be extremely high or low and only limited numbers of species (that may grow at high cell densities) are well adapted to those conditions (Ventosa, 2006). Hypersaline ecosystems are distributed globally and represent a wide range of types (Terry et al., 2000; Oren, 2002; Ventosa et al., 2008; Hollister et al., 2010). The majority of studies published on the microbiology of hypersaline ecosystems focus on aquatic communities (Oren, 2002), whereas far fewer have attempted to characterize hypersaline soils and sediments (David et al., 2005; Dong et al., 2006; Ventosa et al., 2008; Hollister et al., 2010).

We can qualify soils as "extreme" when they support only colonization by organisms presenting a specific and common adaptation (Dion and Nautiyal, 2008). Microorganisms in these habitats may share a strategy and have developed multiple adaptations for maintaining populations and cope eventually to extreme conditions.

Salinity is one of the most widespread soil degradation processes and saline soils can be defined as extreme soils or border line soil in which uneven temporal and spatial water distribution and localized high concentration of salts occur, characterizing restricted habitats where most of the present micro-organisms are salt tolerant (halotolerant), or halophilic (that

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require salt for maintaining their membrane integrity and enzyme stability and activity). The differences between saline and hypersaline soils are not well defined by chemical parameters. Soils could be considered hypersaline when salt concentration exceeds certain thresholds (Dion and Nautiyal, 2008). According to Amoozegar et al. (2003) soil can be defined saline when the concentration of salt is higher than 0.2% (w:v), also a soil is saline when the electrical conductivity (EC_e) of a saturated paste is greater than $4\,dS\,m^{-1}$, (Richards, 1954). The two international soil classification systems introduced higher minimum thresholds of EC_e to classify a soil horizon as saline. In fact, the World Reference Base for Soil Resources (IUSS Working Group WRB, 2014) considers the reference value $15\,dS\,m^{-1}$ of EC_e in defining the salic horizon, while the USDA Soil Taxonomy (Soil Survey Staff, 2014) fixed the threshold at $30\,dS\,m^{-1}$.

Microorganisms are affected by the salt concentration and generally in hypersaline environment can be found from moderately halophilic Bacteria to extremely halophilic Bacteria and Archaea communities. Very little information exists regarding the diversity of microorganisms isolated by hypersaline soils (Dion and Nautiyal, 2008; Hollister et al., 2010). Halophilic and halotolerant Bacteria and Archaea communities are essential for the biogeochemical processes in soils of extreme environments (Ma and Gong 2013). The main mechanisms of tolerance of the microbial communities have been attributed to enzymes with unique structural features that provides them to sustain high salt conditions. Soil halophiles enzymes results to be potentially useful for a variety of applications, including restoration of conditions in salt-affected soils, remediation of pollutants, industrial bio catalysis, food processing, washing, biosynthetic processes, synthesis of exopolysaccharides, compatible solutes, carotenoids (Ventosa et al., 2008). Many studies have been focused on the isolation and characterization of halophilic Bacteria and Archaea communities in saline and hypersaline soils (Quesada et al., 1982; Ventosa et al., 1998) and biotechnological applications are under investigation (Ghazanfar et al., 2010; Keshri et al., 2013; Arora et al., 2014; Tsiamis et al., 2014; Canfora et al., 2014). However, more extensive studies on the ecology, structure, diversity, and functionality of organism occurring in natural saline soils are needed. Naturally salt-affected soils have a biotechnological potential in their microbial communities. These, in fact, represent not only a gene reserve for potential biotechnological applications in the improvement and conservation of saline environments, but they can serve as model systems for exploring relationships between diversity and activity at the soil level. The naturally saline soils differ from other saline soils because are not generated by any anthropic degradation processes, and the salinity is "genetic" (due to the parent material and/or pedogenesis).

In depth knowledge of autochthonous microbial community structure of natural hypersaline soils is basic to identify microbial candidate genes of target microorganisms that have evolved and adapted to live in saline soils. This could allow to detect, monitor and understand the effects of human-induced and/or climate changes induced by natural catastrophes. A naturally saline soil is also a mutable environment where precipitation and fluctuation of climatic conditions can strongly impact the distribution of salts creating sometimes a heterogeneous landscape. Space and scale are recognized as fundamental factors in studying microbial structure analysis.

In Italy, most of salt-affected soils are in the central-south and insular areas. Even if today a quantification of the total area with salt-affected soils is uncertain, recent exploratory surveys (Dazzi and Lo Papa, 2013) have put in evidence that these areas appear prevailingly distributed in the low Po Valley, in long stretches of the Tyrrhenian and Adriatic coasts, along the coast in Apulia, Basilicata, Calabria and Sardinia and in wide stretches in Sicily.

Traditional microbiology using methods culture-dependent has been dealing only with a minor proportion of the actual soil microbiota. Only culture-dependent methods of molecular ecology of microorganisms have provided the possibility of analyzing microbial communities in their entirety. DNA-based technique has become a powerful tool for studying diversity and composition of soil bacterial communities in cultivation-independent ways.

Terminal-restriction fragment length polymorphism (T-RFLP) analysis is based on the detection of a single restriction fragment in each sequence amplified directly from the DNA soil sample and is capable of surveying dominant members comprising at least 1% of the total community. Terminal restriction fragment (TRF) patterns obtained by using T-RFLP technique are generated and analyzed in a series of steps that combine PCR, restriction enzyme digestion and electrophoresis on automated sequencer. T-RFLP can be used to examine microbial community structure and community dynamics in response to changes in different environmental parameters, or, to study bacterial population composition in natural habitats. It has been applied to studies of complex microbial communities in diverse environments such as soil (Lueders and Friedrich, 2003; Fierer et al., 2003; Kuske et al., 2003; Mengoni et al., 2006).

In this study, attention has been focused on the structure of soil microbial community, both bacterial and archaeal communities, in Mediterranean naturally salt-affected soils under semiarid climate. In particular, aims of this study were: (i) to evaluate the characteristics and the distribution of physical and chemical properties of the shallower soil horizons; (ii) to evaluate the genetic structure and diversity of bacterial and archaeal communities by T-RFLP approach and (iii) to correlate microbial genetic diversity with selected soil physical and chemical properties.

2. Materials and methods

2.1. Study area

It was considered a natural area in Piana del Signore (Italian for "Plain of Lord"), an alluvial flat land in Southern Sicily (Fig. 1S) where the geomorphology has been modeled by the river Gela. In the basin of this river the prevalent lithology is made up by Messinian evaporites belonging to the Gessoso-Solfifera geological formation, in which many types of saline rocks crop out (Gypsum, Carbonates and Marls, having frequently chloride and sulfide rock inclusions). The area we surveyed is 12.3 hectares wide and lies about 1 km far from the coastline and 1.2 km from the river Gela estuary.

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Vegetation is structured as a scattered mosaic of patches whose distribution is related to the flooding pattern and duration. The plant association is defined as Junco subulati-Sarcocornietum fruticosae Brullo, 1988 belonging to the Thero-Salicornietea Br.-Bl. & R.Tx. ex A. & O. Bolòs, 1950 class, in which most plants are halophilous pioneer swards typical of salt marshes. The community is constituted by Sarcocornia fruticosa (L.) A. J. Scott, Suaeda vera J. F. Gmelin, Juncus subulatus Forssk., Juncus bufonius L., Phragmites australis (Cav.) Steud subsp. australis, Symphyotrichum squamatum (Spreng.) G.L. Nesom, Polypogon monspeliensis (L.) Desf., Spergularia maritima (All.) Chiov., Monerma cylindrica (Willd.) Coss. & Durieu. Zones covered by vegetation alternate with zones having complete absence of plants. The area is temporarily flooded in the autumn and winter seasons, with longer permanence of water in those zones where salt crusts form on the soil surface afterwards it becomes drier. Salt crusts with thickness of 1–3 mm appear above the soil surface during the dry season, from June to September. Climate is semiarid

Mediterranean, characterized by an intense summer drought and a mean annual rainfall of 383 mm. Mean daily air temperature ranges from a maximum of 26.6 °C in August to a minimum of 4.9 °C in January. Soils develop on alluvial saline sediments (i.e., parent material) deposited by flooding of the River Gela over time.

2.2. Soil survey and sampling

In summer 2011, sixteen soils samples from upper horizons (at a depth of 0–10 cm) were collected according to a random simple sampling scheme (Fig. 1a). In positioning sampling sites authors try to distribute uniformly them considering a minimum distance between points of 50 m. Sites were accurately positioned in field with a GPS device. In each site, three soil samples were collected in the vertices of 1-m side equilateral triangle (Fig. 1b) and mixed together in a unique representative analytical sample. Each soil site was also characterized for vegetation and salt crust cover percent and other features.

Two soil profiles (Fig. 2) were surveyed: the first in a zone with 100% of vegetation and 0% salt crust and the second with 100% salt crust and 0% vegetation, both described according to Schoeneberger et al. (2012). They showed both a shallow Az horizon and very thick master Bz horizon subdivided in lithologic discontinuities. Further vertical subdivisions of the master Bz horizons were mainly functional to soil sampling and based on morphological features, such as structure, color and nature and abundance of salt concentrations described in field (gypsum, carbonates, chlorides).

Main chemical and physical analysis (Table 1) of soil horizons showed pH values around the neutrality in both profiles, while the high values of electrical conductivity of saturated paste (EC_e) were detected in the shallow Az and high values in the Bz with no significant variability through its depth. The assignment of the suffix "z" to both type of horizons found reason on the high presence of salt concentrations observed in field and it was confirmed by the EC_e values measured in laboratory. Particle size analysis and the variability of clay, silt and sand through soil depth confirmed the lithologic discontinuities observed in both soil profiles. Discontinuities of soil horizons correspond to the sequence of alluvial deposits that originated the soil. Considering the soil properties described in field, those measured in laboratory (shown in Table 1), plus many other analytical data we measured in laboratory on soil horizons (data not shown), the soil of the study area can be classified as Hypersalic Fluvisol (Gypsiric, Calcaric, Oxyaquic, Hyposodic, Hypereutric) according to the WRB international classification system (IUSS Working Group WRB, 2014).

The soil samples collected from sites were air dried and 2 mm sieved and divided in two different subsamples: one for the microbial molecular analysis using T-RFLP assay, the second for the chemical and physical soil analysis.

2.3. Soil analytical methods

Texture was determined by the pipette method without carbonate and organic matter removal and after complete removal of soluble salts by distilled water (US Salinity Lab Staff, 1954); pH was measured on 1:2.5 (w/v) soil to water mixtures. $EC_{1:5}$ was measured on 1:5 (w/v) soil to water mixtures at 25 °C. $EC_{1:5}$ was converted in electrical conductivity of the saturation paste extract (EC_e) using the correlation model proposed by (Khorsandi and Yazdi, 2011) for arid and semiarid environments. C_{org} was obtained using the Walkley and Black method (Walkley, 1947).

2.4. Microbial diversity and community structure analysis

DNA was extracted in triplicate (technical replicates) from 0.3 g of soil using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's protocol. Triplicates were then pooled for downstream analyses. Nucleic acids were eluted in 100 μL of elution buffer (MoBio). The concentration of DNA extracts, was checked by Qubit® 2.0 Fluorometer following manufacturer's instructions kit. DNA was then purified from excess impurities with 5 μl of GeneReleaser® (Zidan et al., 2005), and stored at −20 °C for molecular downstream analysis. PCR reactions were repeated three times on each soil sample (technical repeats). 16S rDNA was amplified in a 25 μl volume with 30 ng of template DNA and 5 U of Taq DNA polymerase (Promega) using 63f primer labeled with VIC and 1087r primer (Liu et al., 1997) under the following conditions: 95 °C for 5 min; repeat of 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and a final extension of 10 min at 72 °C. The PCR products were separated on a 1.5% agarose.

The primer Ar3f and Ar927r labeled with fluorescent dye NED were used for the amplification of the 16S rDNA fragments for the Archaea. The PCR was performed under the following conditions: 95 °C for 5 min; repeat of 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and a final extension of 10 min at 72 °C. The PCR products were separated on a 1.5% agarose gel.

The amplified products were purified with a Qiaquick PCR purification kit (Qiagen Inc., Chatsworth, CA, USA), and 300–600 ng of amplified 16S rDNA was digested with 20 U of TaqI, AluI (Promega) for 3 h at 37 °C and 65 °C. A 200 ng aliquot of the digested products was resolved by capillary electrophoresis on an ABI3500 Genetic Analyzer (Applied Biosystems, Foster city, CA, USA) using LIZ600 Applied Biosystems as size standard for GeneMarker (SoftGenetics®) analysis. T-RFLP products (2 μl) was mixed with 0.3 μl of GeneScanTM 600 LIZ® internal size standard
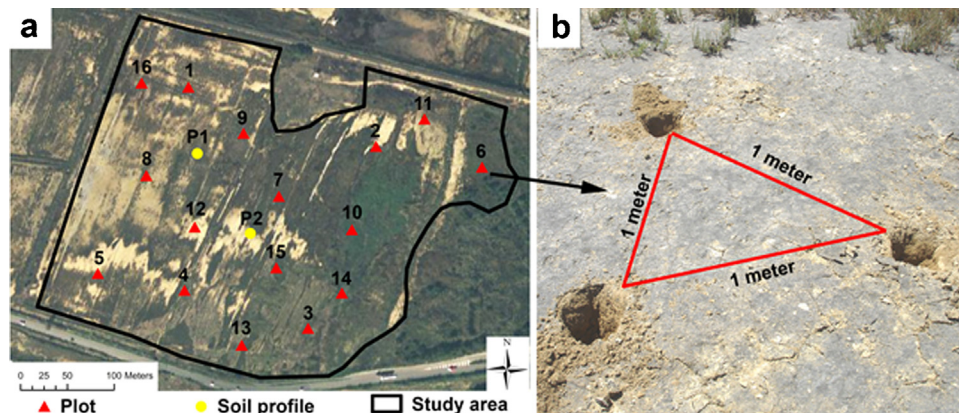

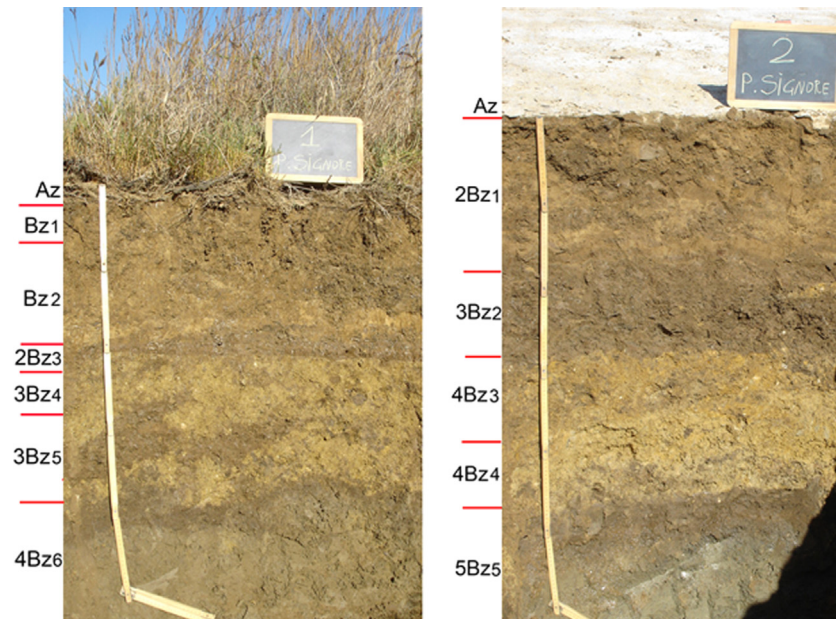**Fig. 1.** Distribution of the sampling sites (a) and sampling scheme (b).

Fig. 2. Representative soil profiles in the study area with horizon nomenclature and suffixes (Schoeneberger et al., 2012).

Table 1
Soil physical and chemical analysis.

Soil profile	Horizon	Depth (cm)	pH	EC_e (dS m^{-1})	Clay (g kg^{-1})	Silt (g kg^{-1})	Sand (g kg^{-1})
Soil profile 1	Az	0–3	7.4	295.23	51	183	766
	Bz1	3–13	7.2	37.01	285	291	424
	Bz2	13–37	7.1	37.52	275	256	469
	2Bz3	37–44	7.1	49.95	426	376	198
	3Bz4	44–58	7.3	44.69	402	296	302
	3Bz5	58–73	7.1	56.73	292	209	499
	4Bz6	>73	7.4	53.14	476	336	188
Soil profile 2	Az	0–1	7.7	282.91	57	89	854
	2Bz1	1–35	7.2	46.59	131	101	768
	3Bz2	35–56	7.3	65.74	395	376	229
	4Bz3	56–77	7.5	43.23	148	144	708
	4Bz4	77–94	7.6	37.41	113	98	789
	5Bz5	>94	7.3	51.52	543	317	140

(Applied Byosystems, Darmastadt, Germany) and run on an ABI3500 Genetic Analyzer (Applied Biosystems, Foster city, CA, USA).

Fragment sizes from 55 to 600 bp were considered for profile analysis. The quality of T-RFLP data was first visually inspected in GenMapper Software v4.1 (Applied Biosystems) and then transferred to GeneMarker software (SoftGenetics). By comparison of TRFLP profiles from the duplicate DNA samples, a derivative profile was created following the same criteria used by Dunbar et al. (2000). Only fragments with fluorescence intensity >55 arbitrary units were considered, and the total DNA quantity represented by each profile was checked by summing all peak areas. Alignment of the profiles was performed directly on the output table of the software GenMarker, and ±0.5 bp was allowed to discriminate peaks of consecutive sizes. Derivative T-RFLP profiles of the different enzymes were then combined together and a binary vector, in which intensity areas of peaks were scored as strings, was prepared.

2.5. Microbial diversity index and statistical analyses

Diversity indexes and boxplots were performed using Past software available at http://folk.uio.no/ohammer/past/.

Significance was tested by T-test. The analysis was carried out on T-RFLP profiles. TRF numbers, corresponding to OTU (operational taxonomic units) numbers, were calculated counting the numbers of TRFs. The Shannon–Wiener index (H') (also referred to Shannon index), is a measure of the order or disorder in a particular system widely applied in ecology to evaluate the biodiversity (Hill et al., 2003). In soil ecology, the H' index takes into account both species richness and the proportion of each species within a community. We calculate H' for Archaea, fungi and Bacteria in each site using the binary vector profiles, according to the following formula:

$$H' = -\Sigma \left(\frac{n_i}{N}\right) \ln \left(\frac{n_i}{N}\right)$$

where n_i is the peak area belonging to the ith vector profile; N is the sum of all peak areas in a given T-RFLP profile pattern. Higher values of H' indicate either higher number of T-RFLP or a relatively higher evenness (Jeffery et al., 2010).

Discriminant analysis (DA) allowed to study the differences between two or more groups of objects with respect to several variables simultaneously. DA was preliminary performed using all chemical and physical characteristics (pH, EC_e, C_{org} and texture), microbial diversity (Shannon index) and community structures (sum of TRFs per site) as grouping variables. The groups have been

formed by arbitrary classes of percentage of both vegetation and crust cover (0–40, 40–70, 70–100%). The statistical significance of the DA was checked by Wilk's lambda test. The standardized canonical discriminant coefficient (SCDC) was used to rank the importance of each variable.

The similarity between both Bacteria and Archaea communities was evaluated by cluster analysis (CA) of T-RLFP profiles of different soil samples using the Ward's hierarchical clustering method with Euclidean distance for Bacteria and dissimilarity of Bray–Curtis for Archaea.

3. Results

3.1. Physical and chemical properties of soil sites

Physical and chemical properties of soil sites are shown in Table 2 together with soil surface properties estimated in field (vegetation and salt crust covers in percentage of site soil surface).

The variability of pH values was low even if they ranged from 6.4 to 8.0. In fact, the soil reaction was mostly neutral in all sites, except in site 1 (6.4, slightly acidic) and site 14 (8.0, subalkaline), with a standard deviation of 0.43 and a coefficient of variation of 5.9%. Electrical conductivity of saturation paste extract (EC_e) was very variable, ranging from 5.37 to 169.96 dS m^{-1} with average of 58.57 dS m^{-1}, standard deviation of 43.62 and all soil sites in Piana del Signore are saline because their EC_e value exceeds the threshold of 4 dS m^{-1}. Only the EC_e values of the soil sites 3, 6, 10, 14 and 15 do not exceed the WRB salinity threshold of 30 dS m^{-1} and, among these, only 10, 14 and 15 are above the salinity threshold fixed by the Soil Taxonomy (Soil Survey Staff, 2014), while most of the soil sites showed EC_e values abundantly over all the thresholds, even two or three times greater than the highest threshold (i.e., Hypersaline).

Particle size analysis showed a certain variability of the three classes considered (clay, silt and sand). Most frequent texture class was loamy sand (in seven sites), with a rather low content in clay and silt, following silty clay loam class (in four sites) where clay and silt contents are around 300 g kg^{-1} both. Only the site 14 showed a very low content in clay and silt and high content in sand (908 g kg^{-1}), therefore classified as sandy.

C_{org} showed the lowest values in sites 14 and 5 with the amount of 3.8 and 6.7 g kg^{-1}, respectively, while the maximum was recorded in site 6 (42.6 g kg^{-1}). In the first case, the low amount of organic C was associated to loamy sand and sand texture classes for sites 5 and 14, respectively, while no trend was found between C_{org} content and both vegetation and salt crust cover percentage. The highest content of C_{org} was detected in silty loam class with 100% of vegetation cover.

Values in percentage of vegetation and salt crust cover indicates a substantial difference of surface characteristics among sites, i.e., sites having full vegetation cover and no salt crusts, sites completely covered by salt crust and without any plant, some cases (sites 7, 9 and 16) with a high cover of both vegetation and salt crust. For this reason three arbitrary classes for both vegetation and salt crust covers were chosen (0–40, 40–70, 70–100%, lower, intermediate and highest classes, respectively) to simplify the experimental scheme and for subsequent statistical analyses.

3.2. Genetic diversity and structure of the microbial community

Data relative to site 4 are not available, since the extraction yielded insufficient amounts of DNA (0.5 ng μl^{-1}) for downstream analyses. The amounts of bacterial and archaeal communities in soil sites were estimated from the results of T-RFLP analysis considering TRFs numbers. Each peak, in fact, on the T-RFLP profiles, corresponds to a certain anonymous taxon referred to as operational taxonomic unit (OTU) and the peak area corresponds to the proportion of this OTU in the microbial community. In particular, 146 and 422 OTU for Bacteria and Archaea, respectively, were detected in T-RFLP profiles and in Table 1S the numbers of OTUs and Bacteria/Archaea, characterizing the different sites, were also shown (Table 1S). The average amount of bacterial community is significantly lower than that of archaeal community and differences among the sites were detected in which the ratio between Bacteria/Archaea OTU number was higher than 1 for 3, 8, 11 and 14 sites. The genetic heterogeneity of microbial communities estimated with Shannon–Wiener index (H'), which characterize the evenness in the distribution of microbial abundance among the taxa, rather than the number of taxa in the population as shown in Table 1S. Shannon–Wiener index (H') for archaeal community ranged from 2.16 to 3.99 (average 2.83), and from 1.05 to 2.80 (average 2.16) for Bacteria. In both communities, however, the coefficient of variation of this index is almost the same (~~21%). In terms of diversity between the two communities

Table 2
Physicochemical properties of the soil sites and summary of performed statistical analyses.

Site	pH	EC_e (dS m^{-1})	C_{org} (g kg^{-1})	Clay (g kg^{-1})	Silt (g kg^{-1})	Sand (g kg^{-1})	Texture class	Vegetation cover (%)	Salt crust cover (%)
1	6.4	102.48	2.96	52	54	894	Loamy sand	60	50
2	7.9	50.45	2.27	189	177	634	Loam	90	70
3	7.0	15.06	1.32	80	98	822	Loamy sand	85	5
4	6.9	77.78	1.54	64	56	880	Loamy sand	0	100
5	7.2	92.73	0.67	76	74	850	Loamy sand	40	100
6	7.4	28.00	4.26	245	294	461	Silty loam	100	0
7	7.1	51.69	2.45	291	267	442	Silty clay loam	95	95
8	7.0	90.77	1.14	293	406	301	Silty clay loam	0	100
9	7.0	74.65	2.18	305	350	345	Silty clay loam	90	100
10	7.8	5.82	1.4	83	145	772	Loamy sand	100	0
11	6.9	63.50	1.46	369	263	368	Silty clay loam	90	0
12	7.2	169.96	2.35	188	217	595	Loam	3	100
13	7.0	46.93	1.36	75	118	807	Loamy sand	90	35
14	8.0	5.37	0.38	54	38	908	Sand	70	0
15	7.6	7.11	1.4	70	65	865	Loamy sand	100	40
16	7.5	54.88	1.27	107	105	788	Sandy loam	90	100
Min	6.4	5.37	0.38	52	38	301		0.0	0.0
Max	8.0	169.96	4.26	369	406	908		100	100
Mean	7.2	58.57	1.78	159	170	671		69	56
Median	7.2	53.29	1.43	95	131	780		90	60
SD	0.43	43.62	0.95	109	116	220		37	44
CV (%)	5.90	74.47	53.26	69	68	33		54	79

the H′ index of Archaea is almost greater than Bacteria in all sites, except in the site 3 in which is slightly lower and in site 10 where the values are almost equal (Table 1S). To confirm this observation and to show both richness and diversity of the bacterial and archaeal community along a salinity gradient, box plots of richness and diversity were performed (Fig. 3). The three groups of salinity gradient were discriminated by arbitrary classes analysis of salinity (0–40, 40–70, and >70 dS m^{-1}, lower, intermediate and highest, respectively). Groups obtained from this arbitrary classes discriminated diversity and richness of archaeal and bacterial communities. Both richness and diversity of archaeal community increased with salinity, particularly within the highest salinity. Intermediate and lower salinity classes supported a significantly higher diversity of archaeal community than bacterial community, but highest salinity gradient supported the most significant high diversity of archaeal community. The large difference between lower, intermediate and highest groups in terms of diversity, and the large heterogeneity in terms of significant differences ($p < 0.05$, ranking: a, b/a, b/a, c), suggests the dominance of archaeal populations in the hypersaline sites.. The archaeal community composition exhibited a large heterogeneity with salinity gradient, the highest salinity sites were dominated by archaeal population, whereas bacterial seems to follow an opposite trend decreasing in the hypersaline sites, as well as along a salinity gradient. Grouping by salinity revealed that bacterial population diversity decreased within the highest salinity, showing clear and significant differences of diversity from lower and intermediate salinity groups. Richness of the dominant archaeal community detected from the number of T-RFLP profiles, showed a decrease within the intermediate salinity, with a significant increase within the highest salinity class and this is consistent with the diversity

Table 3
Results of the linear regression analysis between Shannon diversity indices of Archaea and Bacteria and some soil properties.

Shannon diversity index	Variable	r	r^2	t	$p(>|t|)$
Archea	pH	−0.2908	0.0846	−1.0959	n.s.
	EC$_e$	0.5168	0.267	2.1763	*
	Clay	0.1718	0.0295	0.6286	n.s.
	Vegetation cover	−0.1343	0.018	−0.4887	n.s.
	Salt crust cover	0.1665	0.0277	0.6087	n.s.
	C$_{org}$	0.7314	0.5349	3.8666	**
Bacteria	pH	−0.311	0.0967	−1.18	n.s.
	EC$_e$	0.0101	0.0001	0.0365	n.s.
	Clay	0.438	0.1918	1.7565	n.s.
	Vegetation cover	0.2039	0.0416	0.7509	n.s.
	Salt crust cover	−0.1363	0.0186	−0.4961	n.s.
	C$_{org}$	0.3167	0.1003	1.204	n.s.

n.s. = not significant.
* $p \leq 0.05$.
** $p \leq 0.01$.

index analysis. Richness of bacterial population showed the same trend but the differences in terms of richness of Archaea and Bacteria are very high. Ratio Bacteria/Archaea showed a clear dominance of archaeal population (Table 1S). Correlation matrix of Shannon–Wiener diversity index, both for Archaea and Bacteria, and selected soil properties (Table 3) proved a significant positive correlation between H′ of Archaea and C$_{org}$ and a weak positive relationship between H′ of Archaea and EC$_e$. A significant positive correlation was found also between EC$_e$ and salt crust cover (0.7353, $p < 0.001$), while a significant negative correlation was
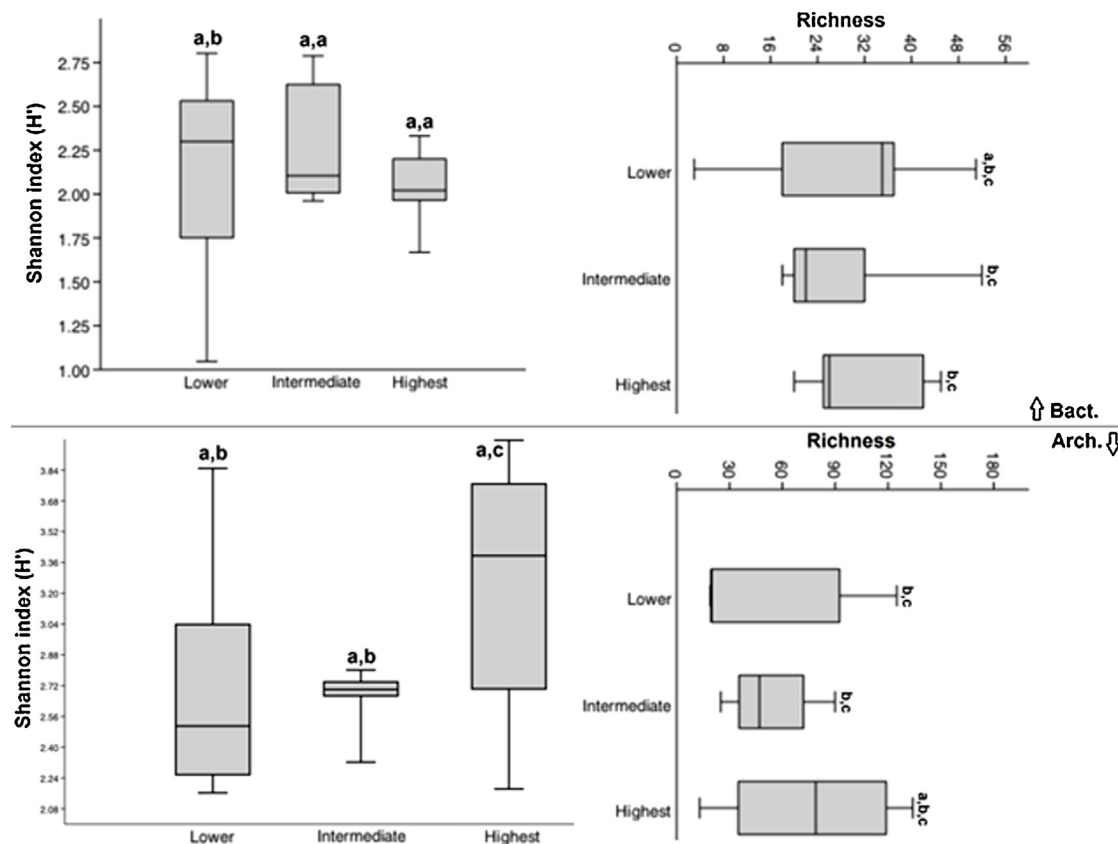

Fig. 3. Box plots of diversity and richness for Bacteria and Archaea. The three groups were discriminated by arbitrary classes analysis of salinity (0–40, 40–70, and >70 dS m^{-1}, lower, intermediate and highest, respectively). a, b, c indicate significant differences ($p < 0.05$). Richness and H′ diversity indices were calculated from the number and the relative peak area of bands on T-RFLP profiles.
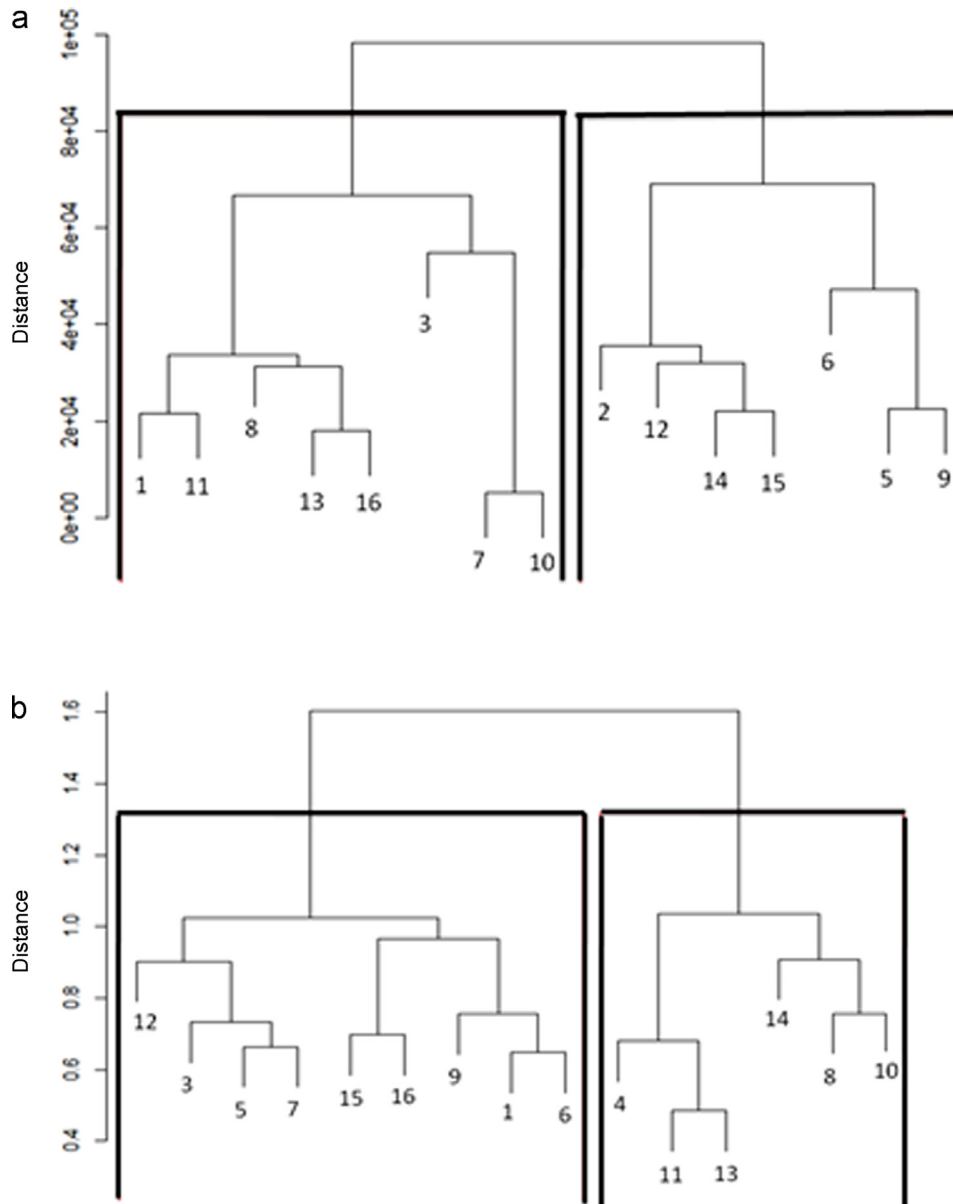
Fig. 4. Dendograms obtained by cluster analysis of T-RFLP profiles of Bacteria (a) and Archaea (b) in sites. Ward's hierarchic clustering with Euclidean distance for Bacteria, dissimilarity of Bray–Curtis for Archaea.

found between EC_e and vegetation cover (-0.6639, $p < 0.001$). Data showed that the high presence of salts in the soil or the concomitant high amounts of both salt and soil organic matter represents conditions favoring the predominance of Archaea community.

The cluster analysis returned two distinct groups for both Bacteria and Archaea and the dendrograms are shown in Fig. 4a and b, respectively. The discriminant analysis, obtained for salt crust and vegetation cover obtained for the arbitrary classes (lower, intermediate and highest classes for <40, 40–70 and >70%, respectively), showed no clear trend for both Bacteria and Archaea. However, it was interesting to note that a cluster of Archaea belonged completely to the group characterized by lower values of crust cover (Fig. 5). LD2 separated the sampling sites with lower crust (triangle) cover from the intermediate (plus) and highest (per) classes. The variables ranking these different groups were

linked to texture of the sites. For this reason, to identify which soil physical and chemical properties were determinant for the clustering of archaeal and bacterial communities, boxplots were obtained with the soil properties of sites selected by two differently derived clusters (Fig. 6a and b).

Statistical T-test is performed on the boxplot data of Bacteria clusters which stressed significant difference ($p < 0.05$) for salt crust cover percentage, sand, silt, vegetation cover and EC_e, while data of Archaea stressed significant difference for salt crust cover, vegetation cover percentage, sand, silt, clay and EC_e.

4. Discussion

The EC_e values of the soil sites confirmed that soils can be classified saline (IUSS Working Group WRB, 2014; Soil Survey Staff, 2014) while some sites can be considered "Hypersalic". These
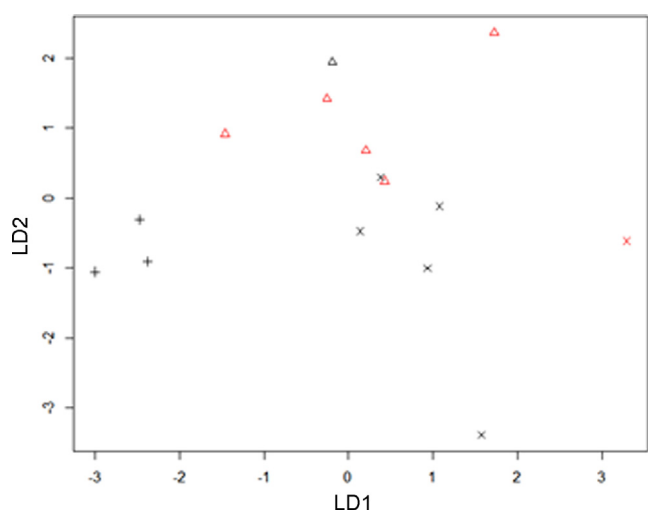
Fig. 5. Discriminant analysis obtained with arbitrary class of crust cover and physicochemical properties of soils, microbial biodiversity and sum of microbial T-RFL as variables for archaeal communities.

features do not seem to influence the percentage of both vegetation and salt crust cover, which are apparently independent from soil properties.

The variability of the considered site properties shows therefore certain heterogeneity of the topsoil in Piana del Signore at the working scale adopted in this study. The variability of soil texture among sites is explained by the alluvial genesis of the soil as highlighted by the pedological survey. Thus, the texture variability of the upper horizons is a direct consequence of the variability of fluvial deposition processes in space and time. Other different factors linked to spatial variability, physical and chemical characteristics and microbial diversity are the percentage of vegetation and salt crust cover.

The genetic analysis of the microbial communities based on T-RFLP profiles proved that the archaeal community composition exhibited a large heterogeneity with salinity gradient, the highest salinity sites were, in fact, dominated by archaeal population, whereas bacterial seems to follow an opposite trend decreasing in the hypersaline sites, as well as along a salinity gradient. Archaea are historically and phylogenetically more closely associated with extreme habitats than bacteria, more heterogeneous and gain the ecological niches. By definition, Archaea, are microorganisms that have better genetically suitable to live in habitats with extreme conditions (Ventosa et al., 1982; Ventosa et al., 1998; Walsh et al.,
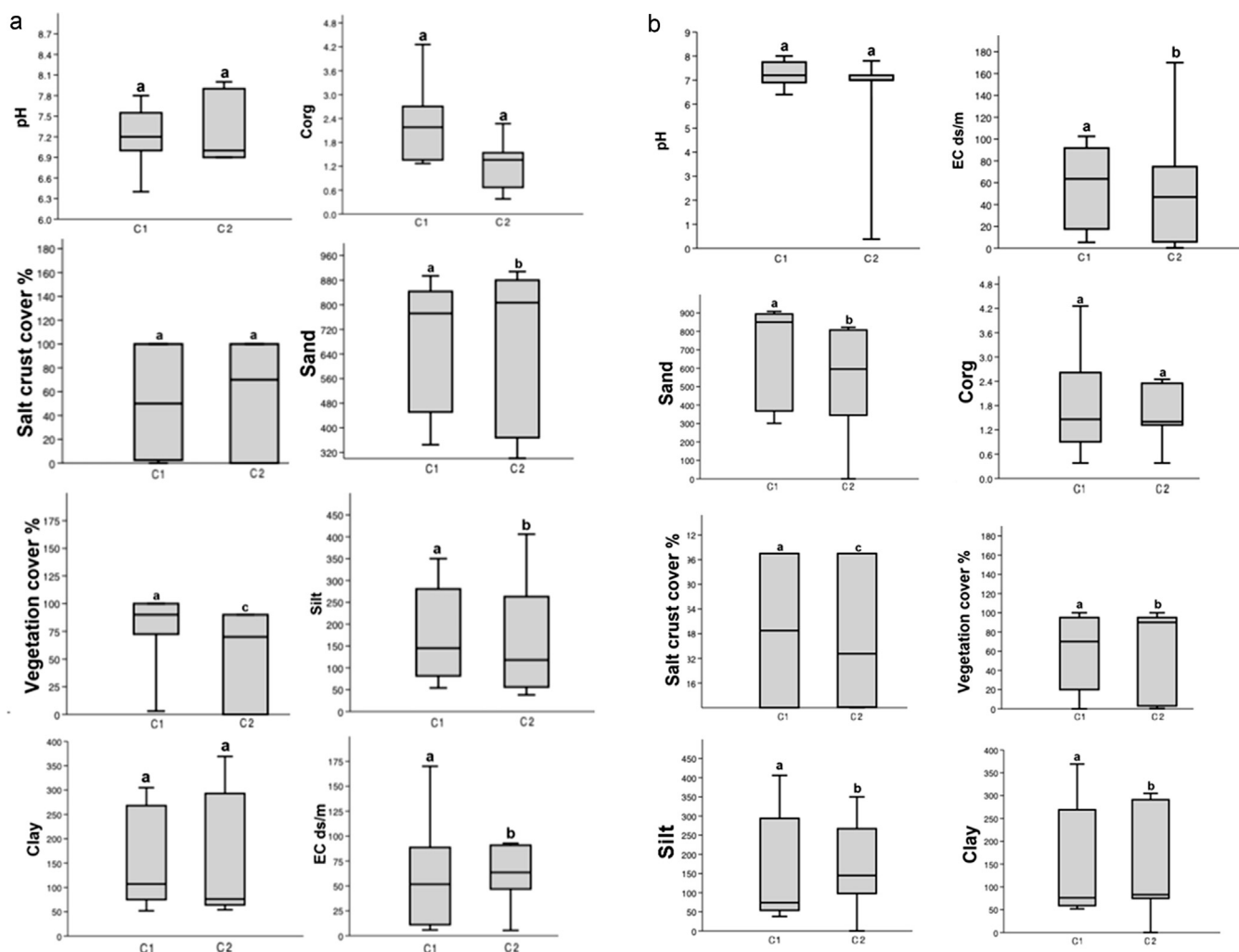

Fig. 6. Box plots of derived cluster by cluster analysis with physical–chemical characteristics for Bacteria (a) and Archaea (b). Different letters indicate significant difference as tested by T-test ($p < 0.05$).

2005; Petrova et al., 2010). In this study, the site of bacteria diversity grouping by salinity, revealed that population decreased within the highest salinity, showing clear and significant differences of diversity from those of lower and intermediate salinity. On the contrary, the archaeal community composition exhibited a large heterogeneity with salinity gradient, the highest salinity sites were dominated by archaeal population, whereas bacterial seems to follow an opposite trend decreasing in the hypersaline sites, as well as along salinity gradients.

Salinity may reduce soil respiration (Setia et al., 2011; Asghar et al., 2012) and, just because of this reason, strongly affects microbial community composition favoring Archaea (Rousk et al., 2011) and halophilic Bacteria. Hence, the fact that abundance, composition and diversity of the microbial community shift to Archaea is obvious. Jiang et al. (2007) found very low abundance, composition, diversity, and metabolic functions of microbial communities in saline and hypersaline terrestrial environments. Furthermore, the abundance, composition, and diversity of microbial communities within soils in natural environments are significantly strongly influenced by pH (Eichorst et al., 2007), texture, organic carbon content (Hansel et al., 2008) and this is particularly true for the saline soils where osmotic potential and pH play a key role.

In our study, results obtained by plotting the soil chemical and physical factors with the clusters of Bacteria and Archaea showed a positive relationship between Archaea composition and distribution and C_{org}, suggesting that the presence of high contents in organic matter in the soil favors the diversity of these microorganisms. The discriminant analysis for Archaea community underlines the influence of texture linked to lower crust cover percentage in their distribution. Bacterial community seems to show a different behavior in terms of diversity, suggesting that the soil spatial variability favors Archaea rather than Bacteria. The high increase of both diversity and richness of archaeal community could be likely consistent with a strategy of multiple adaptations for resisting high salt concentrations. From the genetic point of view, these species display an under- or over-expression of peculiar genes and metabolites, as well as osmolytes, which is an important mechanism to counteract the high osmotic pressure in saline soil. It likely seems that the high salinity leads to a dominance of community, which are more tolerance to high salinity gradients.

Furthermore, it is well known that the response of microbial activity to salinity varied with C_{org} form (Elmajdoub and Marschner, 2013). In saline soils, addition of C increases the tolerance of microbes to low osmotic potentials by providing the cells with the energy needed for tolerance mechanisms, because adaptation to osmotic stress requires a high amount of energy to synthesize organic osmolytes (Hagemann, 2011). A recent meta-analysis of soil microbial communities of ten sites of these soils, reported that soil pH and other chemical properties seemed to have a minor impact on bacterial group distribution when analysed at the considered spatial scale (Canfora et al., 2014). Our observations suggests that pH, C_{org} represented a determinant factor to the clustering of both Archaea and Bacteria, whereas salinity, as suggested by the boxplots (Fig. 4b), seems to be determinant for the clustering of Archaea.

The results of this study suggested that soil spatial variability correlates with the microbial spatial diversity; in this environment some limiting factors favor some microbial groups and not others separated for the physical and chemical factors, and in particular for the discontinuity and variability of these factors at spatial scale. However, our findings also suggested that pH and other chemical properties (C_{org}, texture) are important parameters shaping microbial community structures in terms of composition and distribution.. The richness of microbial

community changed significantly between soil sites. Furthermore, the Archaea dominated the microbial community composition and distribution both in terms of diversity and richness, and the abundance seemed to be strongly influenced by the hypersaline character of this soil.

5. Conclusions

Very little information exists regarding the diversity of microorganisms isolated by hypersaline soil, and very few studies address the diversity of microbial species structure, distribution, and diversity according to the different salt concentration and, at a spatial scale in relation to salinity gradient. Nevertheless, microbial community in the soils are not distributed randomly; therefore, understanding the specific composition and distribution of microbial community as function of spatial gradients of salinity, but also others factors such as soil composition, organic matter, pH, vegetation cover, and crust cover, is important to gain more detailed insight on microbial ecology and in particular on microbe-environment pattern and interactions occurring in natural saline soils.

This study contributes to understand relationships between hypersaline soils and genetic diversity and structure of microbial communities in natural salt-affected soil, showing as a function of the spatial variability of soil properties, the structure and diversity of bacterial and archaeal community. The high presence of salts in the soil or the concomitant high amounts of both salt and soil organic matter, together with the spatial variability of some properties favors Archaea rather than Bacteria.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2015.04.014.

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