LAND USE, SALINITY AND WATER QUALITY. THE CASE STUDY OF A COASTAL SYSTEM IN CENTRAL ITALY

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Abstract

In this study, the evaluation of soil and groundwater quality was coupled with a T-RFLP and real time qPCR analysis of 16S and 18S rRNA genes in order to investigate the soil microbial community structure and diversity in a coastal lagoon system of Central Italy. The main aim of the research was to assess the reciprocal effect of the lagoon salinity and of the different land uses both on the inland groundwater and quality, and on the soil microbial community structure and diversity. Results emphasize for the first time the diversity of the microbial communities in environments with a strong salinity gradient, as affected by land use, depth and spatial location.

Keywords: *coastal systems, land use, microbial community, salinity, water quality*

Introduction

Salinity is one of the most widespread soil degradation processes in both agricultural and native soils (Richards, 1954; Martinez-Beltran and Manzur, 2005). Saline soils are environments characterized by high concentrations of salts and by an uneven temporal and spatial water distribution. These excess salts change the availability of water and nutrients for both plants and microorganisms. A basic distinction must be made between primary and secondary salinisation processes. Primary salinisation involves salt accumulation through natural processes due to a high salt content of the parent material or in groundwater (Domínguez-Beisiegel et al., 2013; Canfora et al., 2014; 2015).

Secondary salinisation ("anthropogenic-induced") is caused by human interventions such as inappropriate irrigation practices, e.g. with salt-rich irrigation water and/or insufficient drainage (Tóth et al., 2008; Argaman et al., 2012; de la Paix et al., 2013; Mao et al., 2014). Salinity has been found to affect the size and the activity of soil microbioma biomass (Tripathi et al., 2006; Ventosa et al., 2015). Sea water intrusion is one of the most important and widespread processes in coastal lagoons, where the mixing of freshwater with marine influx occurs with large seasonal fluctuations (de Wit, 2011), which are also reflected in the microbial organisms that inhabit these environments (Singh et al., 2014; Srivastava et al., 2014; Drake et al., 2015; Iwai et al., 2015). In the present study, the evaluation of soil characteristics and of water quality was coupled with the analysis of soil microbial community structure and diversity, to investigate the relationships among

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land use, soil microbial communities, water quality and salinity. The main objectives of this work were: to assess the reciprocal effect of the lagoon salinity and of the different land uses on the inland soil and groundwater quality, to investigate differences in community structure and diversity of soil microbial assemblage along a salinity gradient, and to study the microbial community abundance and composition response to salinity due to the seawater intrusion and under different land use management.

Materials and methods

The study site

The coastal lagoon of Lake Fogliano (Fig.1) is located about 100 km south of Rome (Italy) nearby Latina, and is part of a shallow saline coastal system along the Tyrrhenian Sea. The lagoon of Fogliano (surface area of 4 km^2, 41º23'N, 12º54'E) has a perimeter of 11.2 km, its maximum width is 1.46 km, and the maximum depth is 2 m. The lake waters are brackish, tending towards salty, and the level of salinity shows a seasonal pattern linked to the rainfall regime. Being freshwater inputs avoided, lake salinity has increased in the last twenty years, up to the present average summer level above 40 g L^{-1}. The long term averages (1972-2012) of mean annual temperature and rainfall are15.5°C and 930 mm. The land surrounding the coast of the lake is under different land uses: arable and greenhouse crops, animal husbandry, recreational, and built-up areas mixed with agriculture

Figure 1
Location of the lagoon.

Soil and water sampling and analysis

The study is part of a wider monitoring activity carried out on soil and water quality in 2009-2010 along six transects approximately perpendicular to the lake shore. Soil cores have been sampled in each monitoring site with a manual auger, according to the depth of the pedologic horizons. Monthly monitoring of ground

water quality and water depth was performed through the installation of piezometers (300 cm of depth) in each sampling site. Four sites located at a spatial scale and affected by a salinity gradient were specifically selected considering the following land uses: 1. Recreational (lakeside), 2. Arable (biodynamic farming), 3. Arable (set-aside), and 4. Extensive grazing of buffalos and cattle (lakeside).
Soil characterization was performed according to the standard methods (SSSA, 1986; 1996; MiPA, 1997; MiPAF, 2000), and the following parameters were determined: pH, electrical conductivity, particle size distribution and texture according to USDA classification, total N, organic carbon C and soil organic matter (SOM = Cx1.724), and cation exchange capacity (CEC). Water quality parameters were determined by standard chemical methods (APHA, 1995; MiPAF, 2001; APAT and CNR-IRSA, 2003): electrical conductivity (ECw), chlorides, sodium, ammonium and nitrate nitrogen.

Extraction of genomic DNA and PCR amplification

Genomic soil DNA was extracted in duplicate from 0.6 g of soil using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's protocol. Nucleic acids were eluted in 100 µL of elution buffer (MoBio). Duplicates were then pooled for downstream analyses. The concentration of DNA crude extracts, was checked by a Qubit® 2.0 Fluorometer following manufacturer's instructions kit and stored to -20° C for PCR analysis. PCR reactions were repeated three times on each soil sample (technical replicates) and were performed in 30 µL volumes containing 3 µl 10-fold reaction buffer (10X PCR Buffer, Minus Mg, Invitrogen), 10 mM of dNTP mixture, 1.3 mM of each of the primers, 50 mM MgCl2, 0.2 U of Phusion hot start Taq DNA Polymerase (Platinum, Invitrogen), and 50 ng of isolated DNA as template.
For the amplification of the bacterial 16S rRNA gene, the primer 63f/ 1087r (Liu et al., 1997) was used. The amplification of the archaea was performed with the primers Ar3f and Ar927r and followed the same PCR condition of bacterial 16S rRNA gene amplification. The amplification of fungal 18S was performed with the primers ITS1/ITS4 (Gardes and Bruns, 1993). The quality of amplification products was confirmed by 1.5% agarose gels and ethidium bromide staining. PCR products resulting from three replicate reactions were pooled and purified with a Qiaquick PCR purification kit (Qiagen Inc. Chatsworth, CA, USA). Purified products were quantified by a Qubit® 2.0 Fluorometer following manufacturer's instructions kit.

Terminal Restriction Fragment Length Polymorphism (T-RFLP)

The digestion of fluorescently labeled PCR fragments was performed in duplicate using separately three restriction enzymes (TaqI, HaeIII e Hinf I), in the following reaction mixture: 20 U of restriction enzyme (Promega), 2µL of 10x Restriction Enzyme Buffer provided by the manufacturer, and 400–600 ng of purified PCR product, PCR-grade water up to a final volume of 20µL. The same protocol was followed for the digestion of fluorescent labeled PCR fungal fragments, but using HaeIII and HinfI (Promega) restriction enzymes. The samples were then incubated,

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respectively for 5h at 37°C and 65°C; the digestion was stopped heating at 95°C for 30 minutes. T-RFLP products (2µL) were mixed with 0.3µL of GeneScanTM 600 LIZ® internal size standard (Applied Byosystems, Darmstadt, Germany) and run on an ABI3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

qPCR

Quantification of bacterial, archaeal and fungal DNA sequences extracted from soil was carried out using the quantitative Polymerase Chain Reaction method (qPCR). The extracted DNA was diluted, to 2ng/µL and stored at -20°C for the following steps. Bacterial DNA was amplified using the primers that amplify a fragment of 193 bp, as referred by Muyzer primer pairs (Nadkarni et al., 2002). Fungal DNA was amplified by means of the 5.8S/ITS1f primer pair (Vigalys and Gonzales, 1990). Archaeal DNA was amplified using the primers Ar364/Ar964 (Ochsenreiter et al., 2003) that amplified a fragment of 550 bp. In brief, all qPCR reactions were carried out in 25 µL reactions containing 10 µL of template DNA (2ng/µL), 12.5µL of QuantiFast Sybr Green PCR Master Mix (Qiagen), 1.2 µM of primer, and PCR-grade water up to 15 µL.
The reactions were performed in a Stratagene Mx3000P qPCR (Agilent Technologies). Experiments were performed in duplicate. The results were processed using the program of the instrument (Stratagene Mx3000P qPCR, Agilent Technologies). The amplified products were purified after the qPCR reaction, quantified by Qubit® 2.0 Fluorometer kit, following manufacturer's instructions, and diluted to minimize the PCR bias (Towe et al., 2010).
The gene copy number was calculated using the following formula:

gene copy number = (ng∗number/mol)/(base pairs∗ng/g∗g mol base pairs)
(http://www.uri.edu/research/gsc/resources/cndna.html).

The standards were obtained in triplicate using a 10-fold dilution series, covering six orders of magnitude from 103 to 109 gene copies per qPCR reaction during each run. Bacterial, archaeal and fungal copy numbers were expressed as g^{-1} soil (dry weight) and the relative values were expressed as log2. The qPCR reactions were performed in duplicate within each DNA template.

Statistical analysis

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 T-RFLP profiles from the duplicate DNA samples, a derivative profile was created following the same criteria used by Dunbar et al. (2000). The analysis was carried out following the same criteria reported in Canfora et al. (2015).

Results

Groundwater

As expected, water depth during the monitoring was higher in sites 2 and 3 and different at $p<0.001$, lower in sites 1 and 4, and significantly different from sites 2 and 3 at $p<0.001$ (Fig. 2).

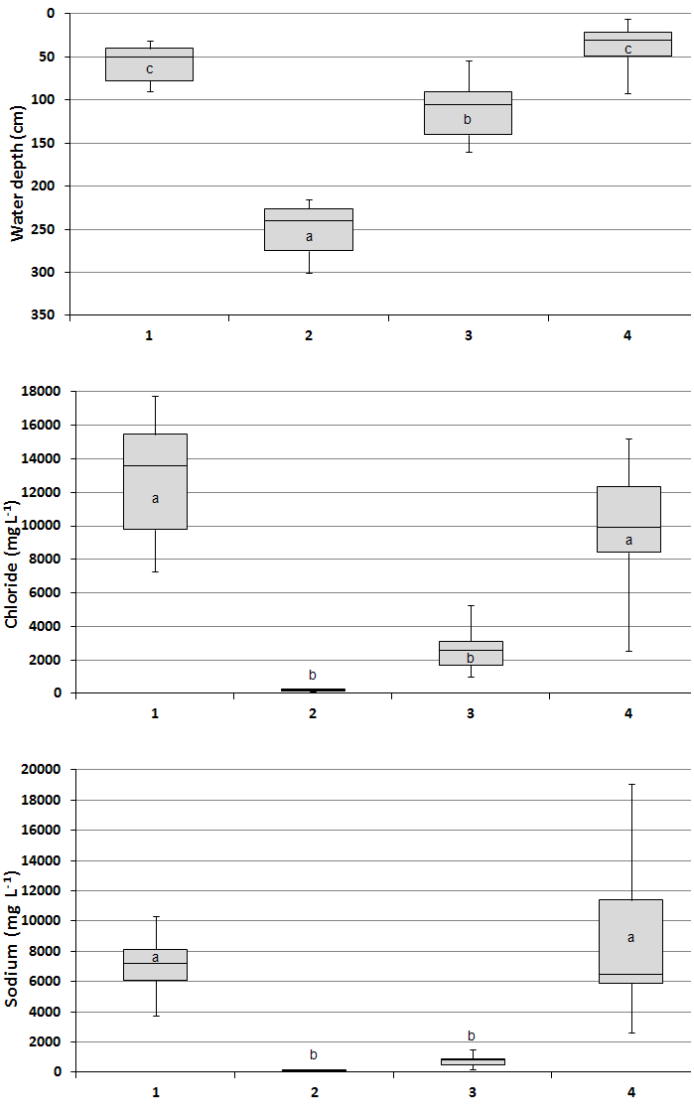

Figure 2
Water depth, chloride concentration and sodium concentration. Different letters indicate significant differences (Tukey's HSD post-hoc test).

Average chloride and sodium concentrations, and electrical conductivity (ECw) were inversely correlated to water depth (Fig. 2 and 3). Chloride concentration was higher in sites 1 and 4, and significantly different at $p<0.001$ in comparison with in sites 2 and 3. Sodium concentration was higher in sites 1 and 4, lower and

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significantly different ($p<0.001$) in sites 2 and 3. ECw in site 1 was significantly different from sites 2 and 3 at $p<0.01$ and $p<0.04$ respectively; site 4 was significantly different from site 2 at $p<0.02$ (Fig. 3).
NH_4-N was higher in site 4 used for extensive cattle grazing, NO_3-N was higher in site 2 under arable biodynamic farming, and was never detected in site 3 under set-aside (Fig. 3).

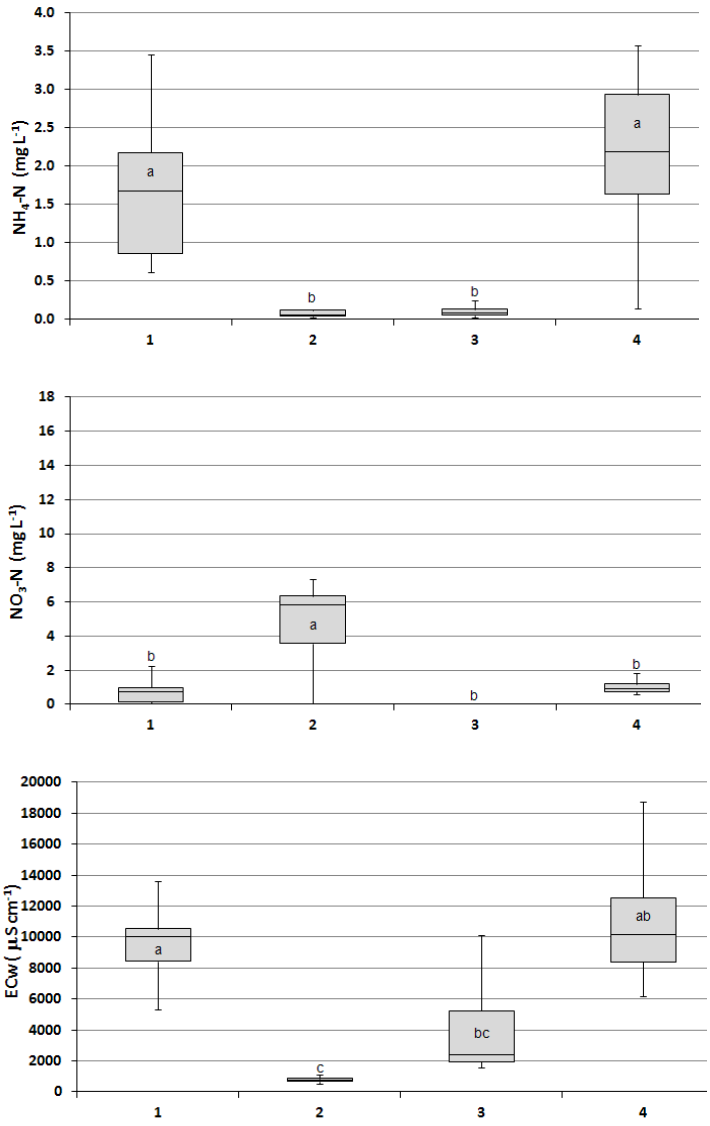

Figure 3
Ammonium concentration, nitrate concentration and ECw. Different letters indicate significant differences (Tukey's HSD post-hoc test).

Soil

Soil pH was mainly neutral or moderately alkaline in site 1, 2 and 3, and moderately acid in site 4. Soil texture along the profiles was mainly sandy-loam,

sandy-clay-loam up to 140 cm, sandy-loam, sandy and loamy-sand. Cation Exchange Capacity was generally medium, high and very high. Soil electrical conductivity (Fig. 4) was from moderate to excessive in site 1, neglectable in site 2, from neglectable to strong in site 3, and from neglectable to excessive in site 4. Total N and soil organic carbon have been evaluated through the C/N ratio (Fig. 5). Site 1 and 4 showed C/N values higher than 12 since the anoxic conditions mainly induced by the high groundwater levels did not allow the decomposition of the organic materials. Site 2 and 3 showed generally similar C/N ratio values (C/N=9-12), and mineralization and immobilization processes were in equilibrium.

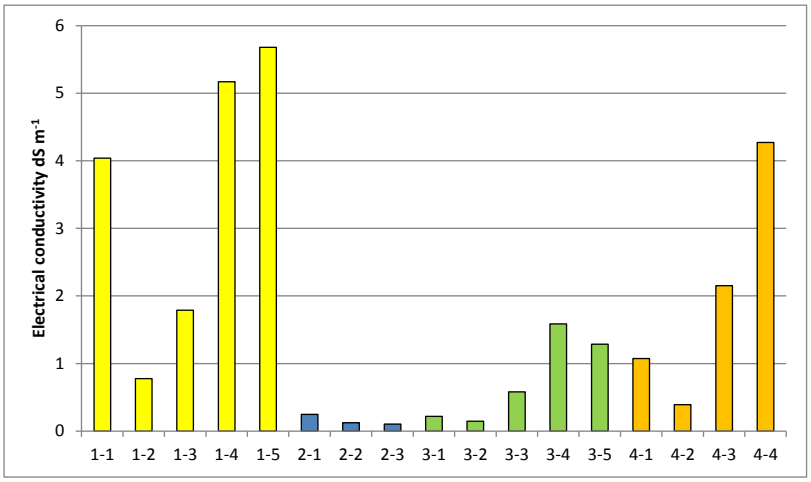

Figure 4
Soil electrical conductivity.

Excessive >4,
Very strong 2-4
Strong 1-2
Moderate 0.5-1.0
Neglectable <0.5
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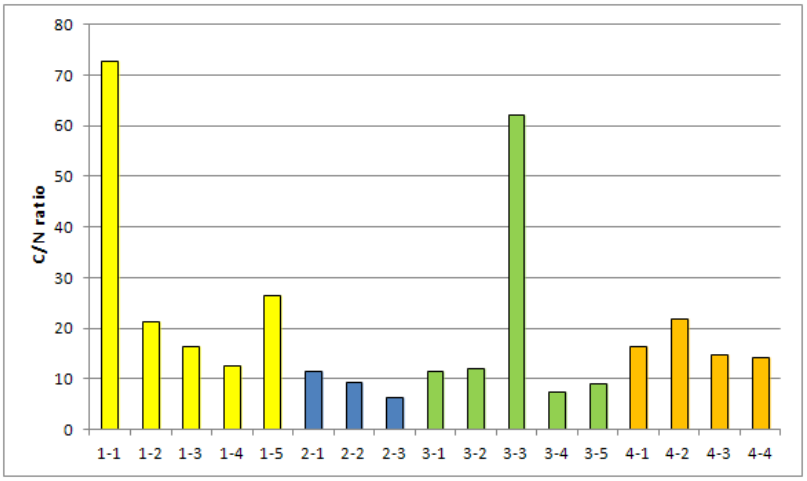

Figure 5
C/N ratios.

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Soil microbial community structure and diversity along depth

DNA Yields were significantly higher at 0-10 cm depth in site 1 (hereafter referred as the control, located near the shore), with similar average lower values along the horizons (4-2 ng μL^{-1}), but intermediate high yields at 60-80cm.
Uniform and low values were observed in site 2, arable land under biodynamic farming (3-4 ng μL^{-1}). The taxonomic genes investigated were 16S rRNA for bacteria and archaea, and 18S rRNA gene ITS specific primers for fungi. The total amounts of the bacterial, archaeal, and fungal biomass were estimated from the results of qPCR and expressed as Log2 of the copy number of rRNA operons per gram of soil (Tab.1). Fungi were consistently the most abundant of all of the genes analysed, in all the sites selected and along the whole profile, followed by bacteria and archaea. The Bacteria/Fungi ratio B/F reflects this trend, and the fact that Fungi were one order of magnitude higher than Bacteria, without significant differences among the four sites (Tab. 1).

Table 1. *DNA yields, Log2 Bacteria, Archaea and Fungi gene abundances, and bacteria/fungi ratios.*

Depth (cm)	Total DNA (ng μL^{-1})	Log2 Bacteria template abundance (g^{-1} soil)	Log2 Fungi template abundance (g^{-1} soil)	Log2 Archaea template abundance (g^{-1} soil)	Bacteria/Fungi Ratio
			Site 1 Recreational		
0-10	28.9	19.9	32.5	10.5	0.61
10-45	4	15.5	30.0	10.5	0.52
45-60	4	15.4	28.9	9.8	0.53
60-80	14	22.18	26.8	6.3	0.83
80-120	2				
			Site 2 Arable biodynamic		
0-50	4	15.7	32.4	11.4	0.49
50-80	4	13.6	29.5	7.2	0.46
80-120	3				
			Site 3 Arable set-aside		
0-30	5.7	16.24	30.91	10.43	0.53
30-60	2.4	16.71	30.38	10.97	0.55
60-140	2	12.93	26.53	7.39	0.49
140-180	2.9				
180-200	3.7				
			Site 4 Extensive grazing		
0-30	7	16.25	30.27	7.45	0.54
30-70	4	14.67	27.70	5.61	0.53
70-140	7	13.58	29.45	5.00	0.46
140-180	3				

The amounts of bacterial, archaeal and fungal phylotypes in soil samples, were estimated also from the results of T-RFLP analysis considering TRFs numbers (Fig. 6). T-RFLP profiles showed a total number of 349 OTU for Bacteria in site 1, 34 in site 2, 110 in site 3, and 202 in site 4 (for which we observed also the higher

OTU number for Fungi). The average number of Fungi was high in site 1 (control) and 4 (extensive grazing), the investigated sites near to the coastal lagoon, intermediate in the site 3 (set-aside), but quite very low in site 2 (biodynamic farming).

The genetic heterogeneity of microbial communities was estimated with Shannon-Wiener index (H'). H' index for bacterial, archaeal and fungal communities were consistent with the previous parameters of quantification by Real Time qPCR. In terms of diversity between the two communities the H' index of Bacteria is greater than Archaea in all investigated sites, with the highest value in the top layer of the site 4 where the values are very close to the values of site 1, in agreement with the results showed above (data not shown).

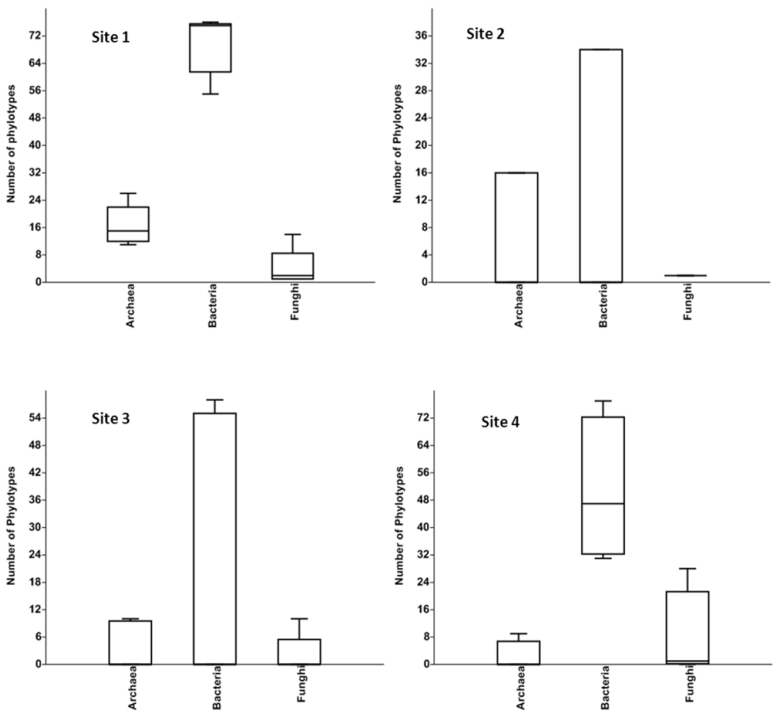

Figure 6
Box plots of phylotypes numbers. The black line in the box represents the median value, the box contains 50% of the data, upper and lower whiskers are respectively the 75th and 25th percentile of the dataset.

To investigate the effects of salinity on the species richness, XY plot of species richness and salinity values along the depths, were drawn (Fig. 7). The combination of species richness and salinity along the depths indicated that species richness shows first an increase at high levels of salinity, and then a decline at increasing depths in site 2 and 3.

In particular, data show an opposite situation where top layers of sites 1 and 4 are more diverse than top layer of site 2 and 3, and presented a slight decreasing trend along the depth, maintaining high species richness at high salinity levels.

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Discussion

Both groundwater parameters linked to salt intrusion and soil salinity have shown higher values in the sites near the lake shore in comparison with the inland sites under arable land use. Soil C/N ratio plays a further important role in affecting soil microbial dynamics (Marin, 2004), very important where the intrusion of salts by groundwater increase the salinity levels. The inland sites under arable land use showed optimum C/N values only in the topsoil as could be expected.

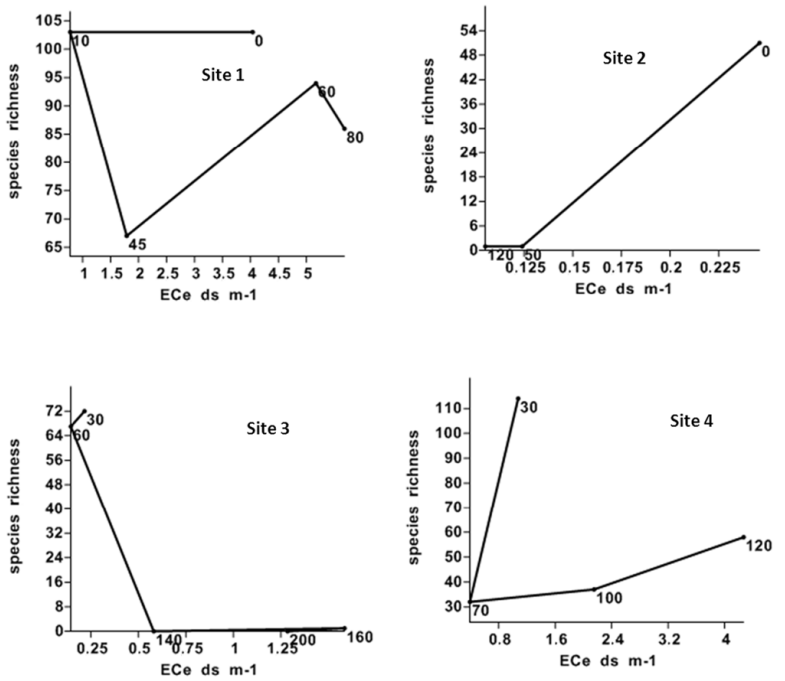

Figure 7
Plots of species richness and salinity values along the investigated depths. The numbers indicates the soil depths and the line show the trend of salinity and species richness along the depth.

Taking into account the internal changes among the investigated sites, the results indicate two gradients: the salinity gradient at vertical scale and the different land use at spatial horizontal scale. In fact, the studied sites represent a strong environmental patchwork, with different anthropic agricultural activity and an increasing seawater salinity intrusion, shaping the microbial community assemblage. The most important differences through the sites were the significant changes and fluctuations with depth and space, a pattern that in parallel reflects the pronounced impact of land use management. At the same time, the results of the present study highlight also the magnitude of the depth-related changes, as reported in other studies (Fierer et al., 2003; Eilers et al., 2012).

It was observed that the two sites near the lake shore, the site 1 and 4, respectively the site here referred as the 'control', and the site under extensive grazing, were most similar in comparison with the two inland sites, both chemically and ecologically. A dominance of Fungi was observed, followed by Bacteria, and

finally by Archaea, and this trend was present in all sites with similar patterns of abundance. In the present study, the population size of fungi estimated by the quantitative PCR was found to be within the range observed in other soil systems (Bach et al., 2002; Ochsenreiter et al 2003), but did not support the typical B/F ratio usually reported in agricultural soils (Bossuyt et al., 2001; Bailey et al., 2002). Interestingly, the microbial abundance did not respond to the microbial community structure analysis by the T-RFLP approach. Because of the limitations of the culture-independent approach with Fungi (Avis et al., 2006), the T-RFLP approach in contrast, showed a different pattern where Bacteria were higher, followed by Archaea and finally by Fungi. Therefore, we argue that our results can be ascribed to the fluctuations observed among the different samples rather than to a pure analytical noise. Results showed also that the diversity of Bacteria was higher in comparison to the Archaea diversity; however, Fungi diversity index was very low given the difficulty to perform the T-RFLP approach for screening Fungi in this environment. We may reasonable suppose that the occurrence of increasing salt levels along the soil layers as affected by the groundwater chemical parameters, the concomitant impact of the land use, and the consequent different soil properties, could lead to a decreasing number of species richness. As a fact, agronomic practices cause frequent disturbance that can result in declining species richness (Goodfriend, 1998).

On the other hand an increasing number of species richness was observed in conjunction with higher salinity levels linked to groundwater chemical parameters, and increasing depths. The species richness is an indicator of microbial species with an adaptation mechanism in response to high salinity levels. In agreement with other studies reporting that salinity alters microbial community structure (Pankhurst et al., 2001; Chowdhury et al., 2011), this study showed that microbial community structure is influenced by salinity but in a different way in comparison with other environments. In a previous study, it was observed that in a naturally salt-affected soil under semi-arid Mediterranean climate, Archaea showed a greater diversity in comparison with Bacteria, with a strong and positive correlation with soil organic matter and salinity (Canfora et al., 2015).

Microbial communities in this peculiar transition environment are believed to be more diverse than those of a natural saline soil (Jiang et al., 2007; Rousk et al., 2011; Canfora et al., 2014; Canfora et al., 2015).

Conclusions

The present data emphasize for the first time the diversity of the microbial communities in environments with a strong salinity gradient, as affected by land use, depth and spatial location. Microbial communities in agro-ecosystems are functionally diverse in comparison with natural systems, although the variety and distribution of micro-organisms clearly depend on very complex dynamics, and are in correlation with many environmental parameters. It is well known, for example, that the response of microbial activity to salinity varies with C organic form (Elmajdoub and Marschner, 2013). A recent meta-analysis of soil microbial

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communities of naturally salt-affected soil, reported that soil pH and other chemical parameters seemed to have a minor impact on bacterial group distribution when analysed at the considered spatial scale (Canfora et al., 2014).
During our research, we realised that the problem of increasing salinity in agricultural areas is still relatively little explored, and not all aspects are covered evenly. Additionally, assessing the impact of land use and management practices in a natural area on soil parameters can provide useful indicators of environmental sustainability.

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