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Prolonged irrigation with municipal wastewater promotes a persistent and active soil microbial community in a semiarid agroecosystem

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ABSTRACT

The use of treated wastewater (WW) for irrigation is a common practice, especially in arid and semiarid agroecosystems. We aimed to evaluate the influence of long-term (up to 45 years) irrigation with WW on the soil microbial community structure, microbial activity and physicochemical properties, in comparison with soil irrigated with fresh water (FW), in a semiarid orange-tree orchard. Phospholipid fatty acid (PLFA) analysis was used to assess the shifts in the soil microbial community in response to the application of WW. Total organic carbon and available P increased significantly, by about 49% and 37%, respectively, due to WW irrigation. The urease, β-glucosidase, alkaline phosphatase and dehydrogenase activities and aggregate stability were higher in the soil irrigated with WW than in that irrigated with FW. The PLFA analysis showed a significant increase in bacterial abundance, particularly in G+ bacteria. The relative abundances of fungi, G− bacteria and actinobacteria were similar in the two soils. Principal components analysis of the PLFAs showed discrimination between the FW-irrigated soil and the WW-irrigated soil, which was enriched in actinobacterial PLFA 10Me18:0. The prolonged use of treated WW for irrigation in a semiarid agroecosystem promoted the establishment of a specific and persistent microbial community that was functionally more active.

1. Introduction

Water shortage has been rising as a consequence of population growth and increased incidence of drought in many areas, thus becoming a major global concern. The decline in fresh water availability has necessitated the search for alternative water sources of lower quality, and diverse levels of treatment, to satisfy the demands of agriculture (Toze, 2006; Adrover et al., 2012). Irrigation with treated wastewater (WW) is a practice used extensively when natural resources are scarce, especially in arid and semiarid agroecosystems around the world (Pereira et al., 2002). Treated WW contains considerable amounts of inorganic substances, such as heavy metals, boron and salts, which may have negative effects on plant growth and the environment (Frenk et al., 2014). In addition, irrigation with treated WW may increase soil organic matter and nutrients (Angin et al., 2005). In this respect, the use of recycled water for irrigation in sustainable ecosystems should contemplate its effectiveness for maintaining and improving soil fertility and quality.

Traditionally, the effects of agricultural management on soil quality have been assessed by measuring soil C and N contents. However, biological properties are much more sensitive to soil management than is soil organic matter as a whole (Caravaca et al., 2002). Particularly relevant are the effects on the soil microorganisms, which have been shown to be an early warning signal of ecosystem perturbations (Friedel et al., 2000). Microorganisms play vital roles in nutrient and energy cycling, and are thus a critical component of any functioning ecosystem. The addition of organic and inorganic compounds with the irrigation water would be expected to affect the soil physicochemical properties which, in turn, would impact on the soil microbial structure community (Mechri et al., 2007; Frenk et al., 2014). The organic content of the WW provides additional organic C that can stimulate the growth of the microbial biomass (Morugán-Coronado et al., 2011). In contrast, some organic molecules can be toxic and therefore likely to reduce microbial growth (Barbera et al., 2013). Long-term application of municipal

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WW has been shown to reduce the diversity of arbuscular mycorrhizal fungi (Ortega-Larrocea et al., 2007; Alguacil et al., 2012) and some types of WW, such as olive mill WW, have been found to impact on the microbial community structure (Saadi et al., 2007; El Hassani et al., 2007; Mechri et al., 2007; Mechri et al., 2014). Most research has focused on the effect of irrigation with treated WW on the enzymatic activity of soil microorganisms, generally recording an increase in microbial activity in the irrigated soils (Filip et al., 1999; Li et al., 2010). However, relatively-few studies have examined its influence on soil microbial structure, these being limited to short- or medium-term WW irrigation (Oved et al., 2001; Frenk et al., 2014).

Phospholipid fatty acid (PLFA) analysis has been used to measure changes in the soil microbial community. These analyses use the lipids of the microbial membranes as biomarkers for specific groups of microorganisms, besides creating a profile or fingerprint of the community structure. As a consequence, rapid changes in the soil microbial community structure can be detected by changes in the PLFAs pattern (Zelles, 1999). In addition, the total concentration of PLFAs can be used as a measure of the viable microbial biomass, since phospholipids are rapidly degraded after cell death (Zelles, 1997). Analysis of PLFAs has been used to study changes in the soil microbial community structure caused by different perturbations or management practices (Hedlund, 2002; DeGrood et al., 2005; Van der Wal et al., 2006; Zornoza et al., 2009; García-Orenes et al., 2013). The PLFA method is a rapid and inexpensive way of assaying the biomass and composition of microbial communities in the soil (Frostegård et al., 2011). To the best of our knowledge, there are no reports describing the use of this technique for the assessment of shifts in the soil microbial community in response to long-term irrigation with treated WW.

We hypothesised that irrigation with treated WW could have a significant effect on the soil microbial community structure. The objective of this work was to compare the influence of the long-term irrigation with treated WW and fresh water on the soil microbial community structure (evaluated as the abundance of phospholipid fatty acids, PLFAs), microbial activity and soil physicochemical properties, in a semiarid orange-tree orchard. In addition, we wished to ascertain the relationships between any shifts in the soil microbial populations and biochemical and microbiological variations induced by the type of irrigation.

2. Materials and methods

2.1. Study site

This research was conducted in an area located in Alicante (southeast Spain) (coordinates 38°17′38″N, 0°33′50″W). The climate is typically Mediterranean with a mean annual precipitation of 301 mm and a mean annual temperature of 17.9 °C. The soil of this study is classified as a Xerorthent. For 45 years, an experimental *Citrus aurantium* L. (orange-tree) orchard has been drip-irrigated with water from an urban wastewater plant with secondary treatment by activated sludge (WW), while control plots subjected to drip irrigation with freshwater (FW) were also established during all of the experimental period. The amount of water applied for the irrigation during the study was 650–700 l m^{-2} per year, according with the meteorological conditions of the area. Both irrigation water sources were sampled monthly during the last 5 years of study taking integrated samples for the analyses. Analysis of biological oxygen demand (BOD_5), chemical oxygen demand (COD) and physical–chemical parameters in water samples were conducted according to protocols recommended in the standard methods (APHA, 2005). The quantification of helminth eggs was made by sedimentation and observation by an optical microscope with a McMaster camera. Determination of total coliform bacteria and salmonella were made by previous filtration of the sample and following growth in EMB-agar and Hektoen-agar culture media, respectively. The main characteristics of the two types of water used for the drip irrigation are shown in Table 1.

Table 1
Characteristics of fresh water (FW) and wastewater (WW) used for the irrigation. Average and SD of monthly samplings conducted during the last 5 years of the study ($n = 60$).

Parameters	Average of FW	Average of WW
pH	7.8 ± 0.5	8.2 ± 0.5
EC (μS cm^{-1})	1800 ± 15	3088 ± 16
BOD_5 (mg O_2 l^{-1})	–	13 ± 1
COD (mg O_2 l^{-1})	–	52 ± 1
SS (mg l^{-1})	–	22 ± 1
Total N (mg l^{-1})	bd	43 ± 1
Ca^{2+} (mg l^{-1})	82 ± 2	125 ± 3
Na^{+} (mg l^{-1})	132 ± 2	275 ± 2
Mg^{2+} (mg l^{-1})	41 ± 1	42 ± 1
SAR	2.97 ± 0.01	5.43 ± 0.02
K^{+} (mg l^{-1})	3 ± 0	20 ± 1
B (mg l^{-1})	bd	0.5 ± 0.1
P (mg l^{-1})	bd	2.5 ± 0.5
Fe (mg l^{-1})	bd	0.07 ± 0.01
Mn (mg l^{-1})	bd	0.02 ± 0.01
Zn (mg l^{-1})	bd	0.01 ± 0.01
Cd (mg l^{-1})	bd	bd
Cr (mg l^{-1})	bd	bd
Ni (mg l^{-1})	bd	bd
Hg (mg l^{-1})	bd	bd
Cl^{-} (mg l^{-1})	300 ± 5	460 ± 5
SO_4^{2-} (mg l^{-1})	150 ± 4	320 ± 3
NO_3^{-} (mg l^{-1})	bd	0.25 ± 0.05
Total coliform (cfu/100 ml)	0	$12 \times e^{+5} \pm 1 \times e^{+2}$
Salmonella detection	Negative	Negative
Helminth eggs (n° l^{-1})	0	<1

EC: electrical conductivity; BOD_5: biological oxygen demand; COD: chemical oxygen demand; SS: suspended solids. bd: below detection limit.

2.2. Soil sampling

In June 2013, rhizosphere soil samples from individual trees were collected in a randomised design with three replicates, composed by six subsamples, for each irrigation treatment: irrigation with treated wastewater (WW) and irrigation with fresh water (FW). Field-moist soil samples were sieved at 2 mm and stored at environmental temperature to determine the physicochemical analysis. Soil sample aliquots were sieved between 0.25 and 4 mm to determine the percentage of stable aggregates. Also an aliquot of every soil sample was kept in cool (4 °C) to carry out the PLFA analysis.

2.3. Soil physicochemical, microbiological and biochemical analyses

Soil pH and electrical conductivity were measured in a 1:5 (w/v) aqueous solution. In soil aqueous extracts, water soluble carbon (WSC) was determined with an automatic carbon analyser for liquid samples (Shimadzu TOC-5050A). The microbial biomass carbon (Cmic) was obtained by fumigation-extraction method (Vance et al., 1987), determined with an automatic analyser for liquid samples (Shimadzu TOC-5050A) and calculated as Cmic = 2.22 (fumigated soil C—unfumigated soil C). Total soil organic carbon (TOC) was determined using potassium dichromate oxidation method (Nelson and Sommers, 1982). Aggregate stability (AS) was

Table 2
Phospholipid fatty acid biomarkers of bacteria, fungi and actinobacteria.

Group designation	Biomarkers
Bacteria	i14:0, i15:0, a15:0, i16:0, 16:1ω7, 17:0, i17:0, a17:0, cy17:0, cy19:0, 10Me16:0, 10Me17:0, 10Me18:0
Fungi	18:2ω6
G− bacteria	cy17:0, cy19:0, 16:1ω7
G+ bacteria	i14:0, i15:0, a15:0, i16:0, i17:0, a17:0
Actinobacteria	10Me16:0, 10Me17:0, 10Me18:0

measured according to Roldán et al. (1994), based on Benito et al. (1986). This method examines the proportion of aggregates that remain stable after a soil sample is subjected to an artificial rainfall of know energy (279 J m^{-2}). Available phosphorus was extracted with acetic acid, sulphuric acid and carbonates at pH 3.3 according to the Burriel–Hernando method and measured colorimetrically (Díez, 1982). Water holding capacity (WHC) was assayed by the method exposed by Forster (1995). The water-holding capacity of the soil was determined by placing 20 g field-moist soil samples in funnels fitted with folded Whatman 2 V filter paper on the inside and mounted on preweighed 250 ml flasks as described by Forster (1995).

Urease activity (EC 3.5.1.5) was assayed according to the method of Tabatabai (1994), using 1 M urea as substrate. Two milliliters of 0.1 M phosphate buffer at pH 7and 0.5 ml of substrate were added to 0.5 g of sample, which was incubated at 30 °C for 90 min. The activity was determined as the NH_4^+ released in the hydrolysis reaction.

Alkaline phosphatase (EC 3.1.3.1) and β-glucosidase (EC 3.2.1.21) activities were determined using *p*-nitrophenyl phosphate disodium (PNPP, 0.115 M) and *p*-nitrophenyl-β-D-glucopyranoside (PNG, 0.05 M) as substrates, respectively. The assay is based on the release and detection of *p*-nitrophenol (PNP) in a spectrophotometer at 398 nm according to Tabatabai (1994).

Dehydrogenase activity was determined according to García et al. (1997). For this, 1 g of soil, at 60% of its field capacity, was exposed to 0.2 ml solution of 0.4% INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride) for 20 h, at 22 °C in darkness. The INTF formed (iodo-nitrotetrazolium formazan) was extracted with 10 ml of methanol by shaking vigorously for 1 min and filtration through a Whatman No. 5 filter paper. The INTF was measured spectrophotometrically at 490 nm.

Phospholipid fatty acid (PLFA) analysis was performed as described by Bossio et al. (1998). Briefly, fatty acids were extracted from 8 g of soil samples using a chloroform:methanol:phosphate buffer. PLFAs were separated from neutral and glycolipid fatty acids on a solid phase extraction column (0.58 Si; Supelco Inc., Bellafonte, PA, USA). After mild alkaline methanolysis, samples were analysed using a Hewlett Packard 6890 Gas Chromatograph with a 25 m Ultra 2 (5% phenyl)-methylpolysiloxane column (J and W Scientific, Folsom, CA, USA). Fatty acids were quantified by comparison of the peak areas with those of an internal standard 19:0 peak. The peaks were assigned using bacterial standards and identification software from the Microbial Identification System (Microbial ID, Inc., Newark, DE, USA). We identified 48 fatty acids. The fatty acid nomenclature used was described by Frostegård et al. (1993). PLFA were grouped into bacterial, fungal, Gram-negative [G−] bacteria, Gram-positive [G+] bacteria and actinobacterial following Federle, (1986), Zelles et al. (1994) and Olsson et al. (1995). Thereby iso/anteiso methyl-branched fatty acids are considered as markers for Gram-positive and cyclic forms and the monoenoic 18:1ω7 fatty acid for gram-negative bacteria, whereas 10-methyl-substituted fatty acids are characteristic for actinobacteria. The PLFA 18:2 ω6.9 represents saprotrophic fungi. A detailed grouping is given in Table 2. Two ratios were also calculated: fungal to bacterial PLFAs (fungi:bacteria) and Gram-negative: Gram-positive bacteria (G−:G+ bacteria). The total biomass was estimated as the sum of all the extracted PLFAs (total PLFAs).

2.4. Statistical analyses

The fitting of the data to a normal distribution for all soil properties was checked with the Kolmogorov–Smirnov test at $P < 0.05$. Statistical differences ($P < 0.05$) between mean values were assessed by using Student's *t* test.

The concentrations of individual fatty acids were analysed by principal component analysis (PCA) in order to determine the changes in soil microbial community structure among the types of irrigation water (Guisande et al., 2006). In addition, PCA was used to evaluate impact of WW irrigation on soil physico–chemical and biochemical properties. PCA can be influenced by rare fatty acids. Fatty acids that only appear in a few samples are usually unreliably represented because they have values near the detection limit. Hence, fatty acids that were present in less than 25% of the samples were omitted. The relative abundance (nanomole of total PLFA) of distinct microbial groups such as bacteria, actinobacteria, G+ and G−bacteria and fungi was calculated. Pearson's correlation coefficients (*r*) were calculated to assess the relationship between soil physico–chemical, biochemical properties and the relative abundance of microbial groups. Mean comparisons and correlations were performed with the SPSS program (Statistical Program for the Social Sciences 18.0). PCA was performed using CANOCO for Windows v. 4.5.

3. Results

3.1. Characteristics of the treated WW used for irrigation

The treated WW had an alkaline pH of 8.2 (Table 1) being considered suitable for its use on agricultural lands. Water salinity measured as electrical conductivity (EC) was very high in the treated WW, which is rated as C4 according to the classification of the US Salinity Laboratory Staff diagram of irrigation waters (1954). The sodium adsorption ratio (SAR), EC and concentrations of cations such as Na^+ and Ca^{2+} were higher in the treated WW than in the FW (Table 1). The values of EC and SAR, plotted on a US Salinity Laboratory diagram, show that the treated WW is dominantly of the C4–S2 class. The heavy metal contents of the treated WW used were below the limits imposed by the Spanish legislation for irrigation purposes in agricultural soils (B.O.E., 2007). The values of suspended solids of the treated WW were lower than the recommended values by the former legislation (<35 mg l^{-1}). Such WW showed values of biological and chemical oxygen demand rather low compared with those used by other authors (Hentati et al., 2014). Also the microbiological parameters analyzed (total coliforms, salmonella and helminths eggs) were below of the limits established by the Spanish regulation (B.O.E., 2007). The WW used in this study had similar quality parameters than waste waters currently reused for irrigation in agricultural Mediterranean areas of Spain.

3.2. Soil physical–chemical and biochemical parameters

The soil irrigated with treated wastewater (WW) had higher values of electrical conductivity (EC) than the soil irrigated with fresh water (FW), whereas the soil pH decreased with WW irrigation (Table 3). There were no significant differences between the two soils regarding water holding capacity. However, irrigation with WW resulted in significantly-greater aggregate stability in comparison to the soil irrigated with FW.

After 45 years of irrigation with treated WW, the concentrations of total organic C and available P had increased significantly,

Table 3
Soil physical–chemical, biological and biochemical properties of soil irrigated with fresh water and wastewater (mean ± standard deviation, $n = 3$).

	FW	WW
Texture (% sand, silt, clay)	Silty loam (7,76,17)	Silty loam (8,75,16)
pH (H_2O)	8.2 ± 0.1a	7.9 ± 0.1b
EC ($\mu S\,cm^{-1}$)	411 ± 27b	499 ± 43a
AS (%)	43 ± 4b	64 ± 5a
WHC (%)	52.2 ± 1.1a	52.5 ± 1.2a
TOC ($g\,kg^{-1}$)	16.1 ± 7.3b	24.0 ± 7.5a
WSC ($\mu g\,C\,g^{-1}$)	125 ± 15a	159 ± 11a
Available P ($\mu g\,g^{-1}$)	188 ± 1b	258 ± 0a
Cmic ($\mu g\,C\,g^{-1}$)	427 ± 50a	383 ± 50a
Dehydrogenase ($\mu g\,INTF\,g^{-1}$)	89.9 ± 9.0b	106.9 ± 12.6a
Urease ($\mu mol\,NH_3\,g^{-1}\,h^{-1}$)	1.92 ± 0.31b	2.73 ± 0.15a
β-Glucosidase ($\mu mol\,PNP\,g^{-1}\,h^{-1}$)	1.64 ± 0.18b	2.31 ± 0.20a
Phosphatase ($\mu mol\,PNP\,g^{-1}\,h^{-1}$)	1.49 ± 0.12b	1.86 ± 0.17a

EC: electrical conductivity; AS: aggregate stability; WHC: water holding capacity; TOC: soil organic carbon; WSC: water soluble carbon; Cmic: microbial biomass carbon. Values in rows sharing the same letter do not differ significantly ($P < 0.05$) as determined by Student's t test.

by about 49% and 37%, respectively, compared to the soil irrigated with FW (Table 3). However, the type of irrigation had no effect on water soluble C and microbial biomass C (Table 3). Long-term application of wastewater resulted in a significant increase in the levels of urease, β-glucosidase and alkaline phosphatase activities (Table 3). The highest increases were recorded in the urease and β-glucosidase activities, in both about 41% with respect to the soil irrigated with FW.

Principal components analysis (PCA) was used to evaluate the effect of the type of irrigation on the soil physicochemical, biological and biochemical properties (Fig. 1). The first two components explained 81.2% of the total variation. The PC1 explained 53.2% of the variation and separated the samples of plots irrigated with FW from those of plots irrigated with treated WW. The loadings of individual soil properties showed that the samples of treated WW plots were characterised mainly by high values of EC, total organic C and all the enzymatic activities, whereas the samples of FW plots had higher pH values. A negative, significant correlation was found between pH and the relative abundance of bacterial biomass ($R = -0.812$, $P = 0.05$).

3.3. Soil microbial community structure

Fig. 2 shows the ratios of specific PLFAs biomarkers and total amount of PLFAs. The proportions of PLFAs assigned to bacteria and fungi were higher in the soil irrigated with fresh water than in the soil irrigated with wastewater. The relative abundances of G+ Bacteria and actinobacteria were similar in both soils. The fungal/bacterial biomass and G−:G+ bacteria ratios were significantly higher in the soil irrigated with fresh water with respect to the soil irrigated with wastewater. The irrigation type had not effect on the concentration of total biomass estimated as total amount of PLFAs ($P = 0.075$).

Changes in the soil microbial PLFA pattern following the different types of irrigation were also analysed by means of PCA (Fig. 3). The first principal component (PC1) explained 71.2% and the second (PC2) 14% of the total variance in the PLFAs. Two of the samples taken in the FW plots showed high concentrations of the unsaturated PLFAs related to fungal biomass (18:2w6c and 18:3w6c) and of PLFAs mainly associated with bacteria (15:0, i15:0, a15:0, 16:1w7c, 17:0, cy17:0, i17:0, a17:0 and cy19:0). The soils irrigated with treated WW only showed high concentrations of PLFA 10Me18:0, that is mainly representative of actinobacteria, the non-specific PLFA 16:1 2OH and PLFA 16:1w7t OH.

4. Discussion

4.1. Effect of treated WW on soil physical–chemical quality parameters

In the current study, the long-term use of treated WW triggered an increment in soil salinity. The increase of EC can be explained by the accumulation of salts in the soil caused by the irrigation with treated WW, although the level of salinity reached in this soil did not provoke deleterious effects on soil structural stability or crop yield. The sodicity hazard is of less magnitude because the treated WW corresponds to medium sodium water (S2). Levy et al. (2003) showed that the sodicity and quality of the irrigation water seem to play a minor role in determining the aggregate stability of soils with a low content of clay—such as the soil used in our study.

Research published on the use of municipal wastewater shows that such wastewaters can improve soil productivity, increasing the soil nutrient status for several potentially limiting nutrients such as N, P and K (Hentati et al., 2014). This reflects the presence of these nutrients in the WW, although the organic C and available P contents of the WW used are relatively low. However, the type of irrigation had no effect on the water soluble C, that is considered as biodegradable organic matter (Caravaca et al., 2002) and can be decomposed easily by microorganisms. This could have been because the labile organic matter deposited on the soil surface was rapidly mineralised, since the high values of soil moisture and the high temperatures of the zone favour this process (Bedford, 2005).

4.2. Effect of treated WW on soil microbial function and soil microbial community structure

The type of irrigation had no effect on the concentration of the total microbial biomass, estimated as the total amount of PLFAs ($P = 0.075$), coinciding with the data of the microbial biomass C determined by the fumigation-extraction method. Kayikcioglu (2012) also did not find shifts in the soil microbial biomass, in a short-term experiment with WW irrigation under Mediterranean conditions. In contrast, other reports recorded an increase in the biomass C in soil irrigated with treated WW, depending on the duration of the irrigation period. For instance, Friedel et al. (2000) and Ramirez-Fuentes et al. (2002) found that microbial biomass C was only increased when the period of irrigation exceeded 80 years. The WW could incorporate exogenous microorganisms into the soil, which may affect native microbial populations (Frenk et al., 2014). In fact, we recorded an abundance of coliform bacteria in the treated WW used for irrigation, including some potential pathogenic genus. However previous studies showed that total coliforms from sewage sludge when applied to soil are short-living, and disappear in several weeks after the application (García-Orenes et al., 2007). In our study, it is possible that the nutrient and organic loads provided with the treated WW originated a favourable environment for the promotion of a specific population of native microflora resulting in a microbial community of similar size to that of the soil irrigated with FW but with a different composition. The relative abundances of fungi, G− bacteria and actinobacteria were similar in the two soils. However, the proportions of bacterial biomass and G+ bacteria were higher in the soil irrigated with treated WW, with respect to the soil irrigated with FW. This finding indicates that the microbiota developed in the soil which was irrigated with treated WW, dominated by G+ bacteria, is able to cycle soil organic matter to a greater extent than that of the soil receiving FW (Mechri et al., 2007). Likewise, it can be pointed out that these populations of G+ bacteria –with characteristic methyl-branched fatty acids (FAs) – were faster growing and more competitive for the available substrates released by the treated WW than the populations of G− bacteria with characteristic monounsaturated (16:1ω7) and cyclic

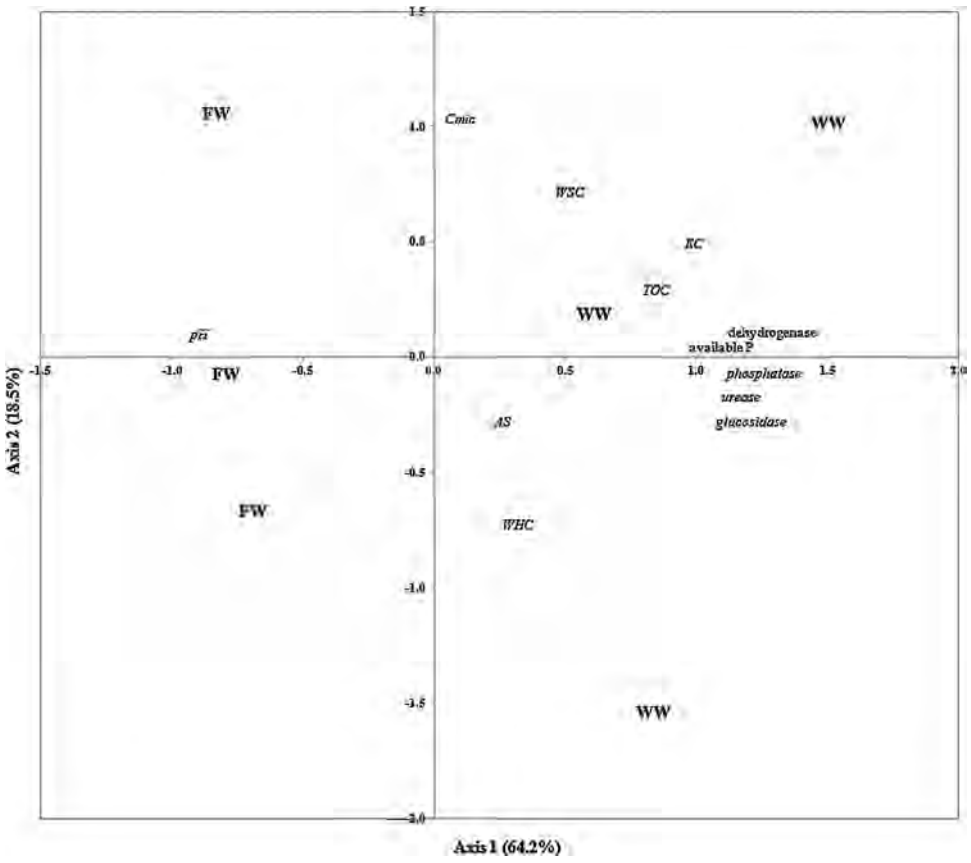

Fig. 1. Score and loading plot from principal component analysis performed on different parameters studied in the soil samples irrigated with fresh water (FW) and wastewater for (WW). TOC: total organic carbon; EC: electrical conductivity; WHC: water holding capacity; WSC: water soluble C; AS: aggregate stability; Cmic: carbon biomass.

(cy17:0 and cy19:0) FAs. Increases in the abundance of the bacterial microflora after irrigation of Mediterranean soils with treated WW were also observed by Frenk et al. (2014) and Hentati et al. (2014), using real-time quantitative polymerase chain reaction and cultivation methods, respectively. In contrast, previous studies using both PLFAs and denaturing gradient gel electrophoresis analyses showed an increase in fungi and a decrease in G+ bacteria after short-term agronomic application of olive wastewaters containing aromatic compounds (Mechri et al., 2007; Rousidou et al., 2010). This suggests that the dominant microbial community is strongly driven by the quality of the organic substrates introduced into the soil by irrigation with treated WW. It is worth noting that spatial heterogeneity in the soil microbial community structure had disappeared after the long-term irrigation with treated WW. It is possible that, in the soil irrigated with the treated WW, there was proliferation of specific microbial communities and more-active functional groups involved in the geochemical cycling of N, P and C. Further, these findings highlight that the effect of treated WW on the community composition is lasting and that the soil bacterial community is not resilient to this disturbance, on our experimental time scale.

Soil enzymes are considered soil quality indicators due to their quick response to any perturbation in the soil. Increases in urease activity due to WW irrigation over a short period of time have been reported (Brzezinska et al., 2006). Other work found significant enhancement of alkaline phosphatase activity in soils irrigated with WW for a shorter period than in our study (4 or 5 years) (Truu et al., 2009) and Chen et al. (2008) observed an enhancement of various enzymatic activities in soils irrigated with treated WW for 10 years. By contrast, other studies showed a significant decrease of enzymatic activities (β-glucosidase and phosphatase) with WW irrigation, related to the presence of metals such as Cr or Co (Brzezinska et al., 2006; Kayikcioglu, 2012). The WW used in our experiment did not contain detectable levels of heavy metals (data not shown). The fact that enzyme activities were higher in soils irrigated with treated WW may be due to the addition of labile organic matter and/or the existing differences in microbial populations (Friedel et al., 2000; Chen et al., 2008; Alguacil et al., 2012). However, the WW irrigation did not increase the content of hydrosoluble organic C, as mentioned above. Whilst the activity of the soil microbial community was increased, the microbial biomass C remained unaffected, which could indicate the presence of more-active microorganisms in the soil irrigated with WW, compared to the soil irrigated with FW. Positive, significant correlations were found between the percentage of stable aggregates and the urease ($R = 0.931$, $P = 0.007$), β-glucosidase ($R = 0.982$, $P < 0.001$), alkaline phosphatase ($R = 0.971$, $P = 0.007$) and dehydrogenase ($R = 0.899$, $P = 0.015$) activities. These findings suggest that the improvement in soil aggregate stability of the soil irrigated with WW can be attributed to the reactivation of the microbial activity of this soil.

In conclusion, the long-term use of treated WW for the irrigation of a semiarid orange-tree orchard promoted the establishment of a specific and persistent microbial community that was functionally more active, altering the native microbial community composition of the soil. The changes in the soil bacterial community structure may be attributed to the organic substrates, nutrients and microorganisms introduced into the soil with the WW. The WW provoked lasting increases in bacterial abundance, particularly in G+ bacteria, indicating that the composition of the bacterial community is sensitive and not resilient to this anthropic action. Further studies should be performed, with molecular tools, to identify the dominant functional groups and their impact on agroecosystem sustainability.

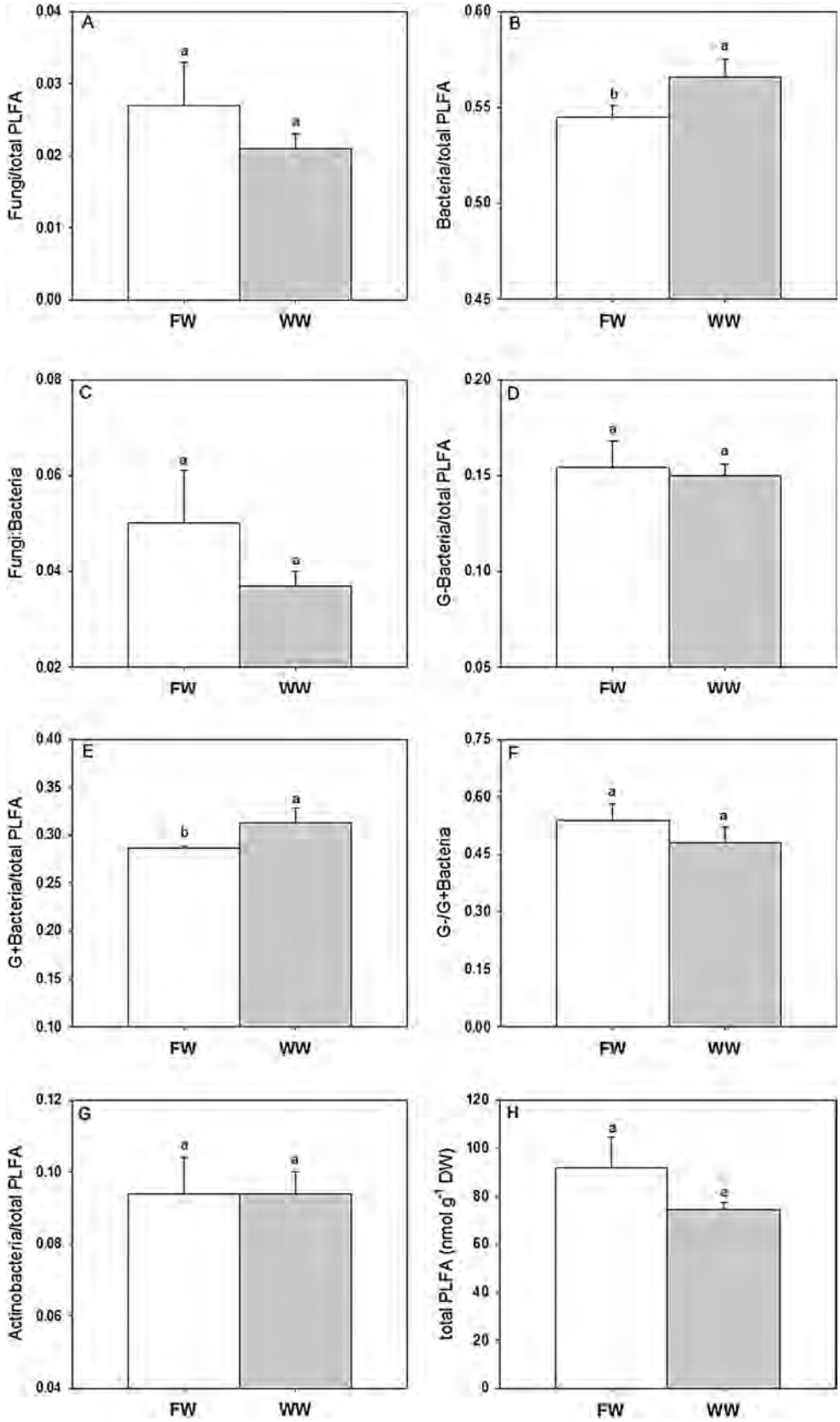

Fig. 2. Ratios of PLFA biomarkers and total amount of PLFAs (mean ± standard deviation) of the soil samples irrigated with fresh water (FW) and wastewater (WW). Bars sharing the same letter do not differ significantly ($P < 0.05$) as determined by Student's *t* test.

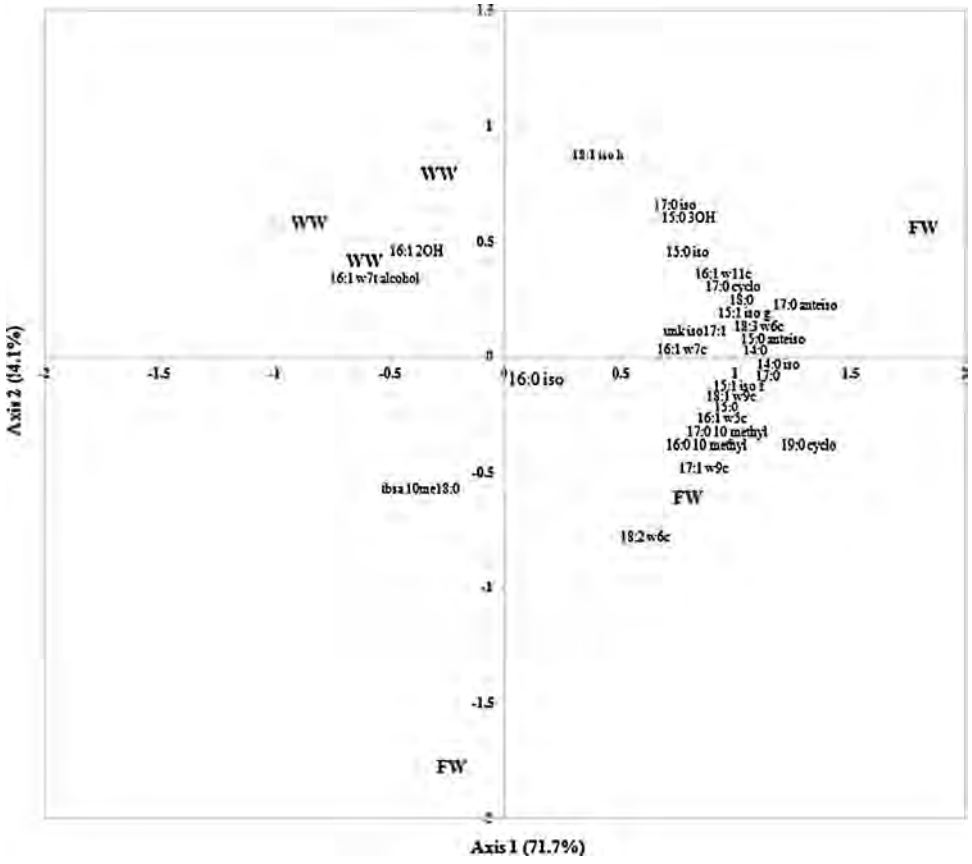

Fig. 3. Score and loading plot from principal component analysis performed on the phospholipid fatty acids (PLFAs) of soil samples irrigated with fresh water (FW) and wastewater (WW).

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References

Adrover, M., Farrús, E., Moyà, G., Vadell, J., 2012. Chemical properties and biological activity in soils of Mallorca following twenty years of treated wastewater irrigation. J. Environ. Manage. 95, S188–S192.

Alguacil, M.M., Torrecillas, E., Torres, P., García-Orenes, F., Roldán, A., 2012. Long-term effects of irrigation with waste water on soil AM fungi diversity and microbial activities: the implications for agro-ecosystem resilience. PLoS One 7, e47680.

Angin, I., Yaganoglu, A.V., Turan, M., 2005. Effects of long-term wastewater irrigation on soil properties. J. Sustainable Agric. 26, 31–42.

American Public Health Association (APHA), 2005. Standard methods for the examination of water and wastewater. In: Eaton, A.D., Clesceri, L.S., Rice, E.W., Greenberg, A.E. (Eds.), Water Pollution Control Federation. , 21st ed. American Water Works Association, Washington, DC, p. 1368.

Barbera, A.C., Maucieri, C., Cavallaro, V., Ioppolo, A., Spagna, G., 2013. Effects of spreading olive mill wastewater on soil properties and crops, a review. Agric. Water Manage. 19, 43–53.

Bedford, A.P., 2005. Decomposition of *Phragmites australis* litter in seasonally flooded and exposed areas of a managed reedbed. Wetlands 25, 713–720.

Benito, E., Gomez-Ulla, A., Díaz-Fierros, F., 1986. Descripción de un simulador de lluvia para estudios de erodibilidad y estabilidad de agregados al agua. An. Edafol. Agrobiol. 45, 1115–1126.

B.O.E., 2007. Real Decreto 1620/2007, de 7 de diciembre, sobre la reutilización de las aguas depuradas. B.O.E., Madrid (Spain).

Bossio, D.A., Scow, K.M., Gunapala, N., Graham, K.J., 1998. Determinants of soil microbial communities: effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. Microb. Ecol. 36, 1–12.

Brzezinska, M., Tiwari, S.C., Stepniwska, Z., Nosalewicz, M., Bennicelli, R.P., Samborska, A., 2006. Variation of enzyme activities, CO_2 evolution and redox potential in an eutric histosol irrigated with wastewater and tap water. Biol. Fertil. Soils 43, 131–135.

Caravaca, F., Masciandaro, G., Ceccanti, B., 2002. Land use in relation to soil chemical and biochemical properties in a semiarid Mediterranean environment. Soil Tillage Res. 68, 23–30.

Chen, W., Wu, L., Frankenberger, W.T., Chang, A.C., 2008. Soil enzyme activities of long-term reclaimed wastewater-irrigated soils. J. Environ. Qual. 37, S36–S42.

DeGrood, S.H., Claassen, V.P., Scow, K.M., 2005. Microbial community composition on native and drastically disturbed serpentine soils. Soil Biol. Biochem. 37, 1427–1435.

Díez, J.A., 1982. Consideraciones sobre la utilización de la técnica extractiva de Burriel–Hernando para la evaluación de fósforo asimilable en suelos. An. Edafol. Agrobiol. 41, 1345–1353.

El Hassani, F.Z., Zinedine, A., Mdaghri Alaui, S., Aissam, H., Errachidi, F., Merzouki, M., Benlemlih, M., 2007. Effect of olive mill vegetable water spreading on soil microbial communities and soil properties. World J. Agric. Sci. 3, 663–669.

Federle, T.W., 1986. Microbial distribution in soil-new techniques. In: Megusar, F., Gantar, M. (Eds.), Perspectives in Microbial Ecology. Slovene Society for Microbiology, Ljubljana, pp. 493–498.

Filip, Z., Kanazawa, S., Berthelin, J., 1999. Characterization of effects of a long-term wastewater irrigation on soil quality by microbiological and biochemical parameters. J. Plant Nutr. Soil Sci. 162, 409–413.

Forster, J.C., 1995. Soil physical analysis. In: Alef, K., Nannipieri, P. (Eds.), Methods in Applied Soil Microbiology and Biochemistry. Academic Press Inc., San Diego, CA, pp. 106–111.

Frenk, S., Hadar, Y., Minz, D., 2014. Resilience of soil bacterial community to irrigation with water of different qualities under Mediterranean climate. Environ. Microbiol. 16, 559–569.

Frostegård, Å., Bååth, E., Tunlid, A., 1993. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biol. Biochem. 25, 723–730.

Friedel, J.K., Langer, T., Siebe, C., Stahr, K., 2000. Effects of long-term waste water irrigation on soil organic matter, soil microbial biomass and its activities in central Mexico. Biol. Fertil. Soils 31, 414–421.

García, C., Hernández, M.T., Costa, F., 1997. Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun. Numer. Methods Eng. 28, 123–134.

García-Orenes, F., Roldán, A., Guerrero, C., Mataix-Solera, J., Navarro-Pedreño, J., Gómez, I., Mataix-Beneyto, J., 2007. Effect of irrigation on the survival of total coliforms in three semiarid soils after amendment with sewage sludge. Waste Manage. 27, 1815–1819.

García-Orenes, F., Morugán-Coronado, A., Zornoza, R., Scow, K., 2013. Changes in soil microbial community structure influenced by agricultural management practices in a Mediterranean agro-ecosystem. PLoS One 8, e80522.
Guisande, C., Barreiro, A., Maneiro, I., Riveiro, I., 2006. Tratamiento de datos. Díaz de Santos, Spain, pp. 356p.
Hedlund, K., 2002. Soil microbial community structure in relation to vegetation managements on former agricultural land. Soil Biol. Biochem. 34, 1299–1307.
Hentati, O., Chaker, S., Wali, A., Ayoub, T., Ksibi, M., 2014. Effects of long-term irrigation with treated wastewater on soil quality, soil-borne pathogens, and living organisms: case study of the vicinity of El Hajeb (Tunisia). Environ. Monit. Assess. 186, 2671–2683.
Kayikcioglu, J.H., 2012. Short-term effects of irrigation with treated domestic wastewater on microbiology activity of a Vertic xerofluvent soil under Mediterranean conditions. J. Environ. Manage. 102, 108–114.
Levy, G.J., Mamedov, A.I., Goldstein, D., 2003. Sodicity and water quality effects on slaking of aggregates from semiarid soils. Soil Sci. 168, 552–562.
Li, X., Liu, F., Li, G., Lin, Q., Jensen, C.R., 2010. Soil microbial response, water and nitrogen use by tomato under different irrigation regimes. Agric. Water Manage. 98, 414–418.
Mechri, B., Echbili, A., Issaoui, M., Braham, M., Elhadj, S.B., Hammami, M., 2007. Short-term effects in soil microbial community following agronomic application of olive mill wastewaters in a field of olive trees. Appl. Soil Ecol. 36, 216–223.
Mechri, B., Attia, F., Tekaya, M., Cheheb, H., Hammani, M., 2014. Agronomic application of olive mill wastewaters with rock phosphate increase the 10Me18:0 fatty acid marker of actinomycetes and change rhizosphere microbial functional groups under long-term field. Soil Biol. Biochem. 70, 62–65.
Morugán-Coronado, A., García-Orenes, F., Mataix-Solera, J., Arcenegui, V., Mataix-Beneyto, J., 2011. Short-term effects of treated wastewater irrigation on Mediterranean calcareous soil. Soil Tillage Res. 112, 18–26.
Nelson, D.V., Sommers, L.E., 1982. Total carbon, organic carbon, and organic matter. In: Page, A.L., Miller, R.H., Keeney, D.R. (Eds.), Methods of Soil Analysis. Part 2. Chemical and Biological Methods. American Society of Agronomy and Soil Science Society of America, Madison, WI, pp. 539–579.
Olsson, P.A., Bååth, E., Jakobsen, I., Söderström, B., 1995. The use of phospholipid and neutral lipid fatty acids to estimate biomass of arbuscular mycorrhizal fungi in soil. Mycol. Res. 99, 623–629.
Ortega-Larrocea, M.P., Siebe, C., Estrada, A., Webster, R., 2007. Mycorrhizal inoculum potential of arbuscular mycorrhizal fungi in soils irrigated with wastewater for various lengths of time, as affected by heavy metals and available P. Appl. Soil Ecol. 37, 129–138.
Oved, T., Shaviv, A., Goldrath, T., Mandelbaum, R.T., Minz, D., 2001. Influence of effluent irrigation on community composition and function of ammonia-oxidizing bacteria in soil. Appl. Environ. Microbiol. 67, 3426–3433.
Pereira, L.S., Oweis, T., Zairi, A., 2002. Irrigation management under water scarcity. Agric. Water Manage. 57, 175–206.
Ramirez-Fuentes, E., Lucho-Constantino, C., Escamilla-Silva, E., Dendooven, L., 2002. Characteristics, and carbon and nitrogen dynamics in soil irrigated with wastewater for different lengths of time. Bioresour. Technol. 85, 179–187.
US Salinity Laboratory Staff, 1954. Diagnosis and Improvement of Saline and Alkali Soils. Agricultural Handbook No. 60. US Department of Agriculture, Washington, DC, pp. 160p.
Roldán, A., García-Orenes, F., Lax, A., 1994. An incubation experiment to determinate factors involving aggregation changes in an arid soil receiving urban refuse. Soil Biol. Biochem. 26, 1699–1707.
Rousidou, C., Papadopoulou, K., Zervakis, G., Singh, B.K., Ehaliotis, C., Karpouzas, D.G., 2010. Repeated application of diluted olive mill wastewater induces changes in the structure of the soil microbial community. Eur. J. Soil Biol. 46, 34–40.
Tabatabai, M.A., 1994. Soil enzymes. In: Weaver, R.W., Angle, J.S., Bottomley, P.S. (Eds.), Methods of Soil Analysis. Part 2. Microbiological and biochemical properties. SSSA Book Series No. 5. Soil Science Society of America, Madison, WI, pp. 775–833.
Toze, S., 2006. Reuse of effluent water e benefits and risks. Agric. Water Manage. 80, 147–159.
Saadi, I., Laora, Y., Ravivb, M., Medinab, S., 2007. Land spreading of olive mill wastewater: effects on soil microbial activity and potential phytotoxicity. Chemosphere 66, 75–83.
Truu, M., Truu, J., Heinsoo, K., 2009. Changes in soil microbial community under willow coppice: the effect of irrigation with secondary-treated municipal wastewater. Ecol. Eng. 35, 1011–1020.
Van der Wal, A., van Veen, J.A., Smant, W., Boschker, H.T.S., Bloem, J., Kardol, P., van der Putten, W.H., de Boer, W., 2006. Fungal biomass development in a chronosequence of land abandonment. Soil Biol. Biochem. 38, 51–60.
Frostegård, Å., Tunlid, A., Bååth, E., 2011. Use and misuse of PLFA measurement in soils. Soil Biol. Biochem. 43, 1621–1625.
Vance, E.D., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil microbial biomass C. Soil Biol. Biochem. 19, 703–707.
Zelles, L., Bai, Q.Y., Ma, R.X., Rackwitz, R., Winter, K., Beese, F., 1994. Microbial biomass, metabolic quotient and nutritional status determined from fatty acid patterns and poly-hydroxybutyrate in agriculturally-managed soils. Soil Biol. Biochem. 26, 439–446.
Zelles, L., 1997. Phospholipid fatty acid profiles in selected members of soil microbial communities. Chemosphere 35, 275–294.
Zelles, L., 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterization of microbial communities in soil: a review. Biol. Fertil. Soils 29, 111–129.
Zornoza, R., Guerrero, C., Mataix-Solera, J., Scow, K.M., Arcenegui, V., Mataix-Beneyto, J., 2009. Changes in soil microbial community structure following the abandonment of agricultural terraces in mountainous areas of eastern Spain. Appl. Soil Ecol. 42, 315–323.