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Impact of airborne zinc pollution on the antimicrobial activity of olive oil and the microbial metabolic profiles of Zn-contaminated soils in an Italian olive orchard☆

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ABSTRACT

The growing of microbial resistance leads to a great interest about some natural alternatives to synthetic compounds. This study was carried out in two olive orchards (*Olea europaea* L., cv. Coratina) South Italy (Basilicata region), one located in a polluted area near a fertilizers factory releasing Zn and the other in a control unpolluted site, both managed with similar cultivation techniques. Olive oil samples were studied from both areas during 2014 and 2015. The soil microbiological status of the polluted and unpolluted orchards has been characterized and the antimicrobial effects of olive oils extracted from polluted plants (PP) and control plants (CP) against some phytopathogens have been explored. Results showed that the antibacterial activity of PP oil was significantly higher than CP and this could be due to the high content of some phenolic compounds elicited by air and soil Zn pollution (especially in the layer 0–20 cm). There is no detectable antifungal activity of the studied oils. The metabolic activity (both total and for each carbon substrate group), diversity and evenness of PP soil bacterial communities were significantly different from CP soil, while the effects of soil depth was negligible. The same parameters measured on soil fungal communities are lower in PP soil at 0–20 cm soil depth. The current research clarified the impact of atmospheric Zn pollution on the antimicrobial activity of olive oil and the soil microbial metabolic profiles. The bioactive substances extracted from olive oils growing in Zn-polluted area might be used as antibiotics.

1. Introduction

Olive oil (*Olea europaea* L.) is well known and accepted as the main component of the Mediterranean diet [1,2]. It is well known that the fruits, oil and leaves of olive trees have a nutritional and medicinal uses [3]. Olive oil dietary consumption is often associated with the lower incidences of atherosclerosis, cardiovascular and neurodegenerative diseases, and certain types of cancer [4–6]. These positive effects are mainly due to various phenolic compounds and polyunsaturated fatty acids, which play an essential role in the taste and flavour of olive oil [7].

The phenolic compounds of olive oil have a promising antimicrobial activity against a wide range of pathogens [7–10], and oleuropein [11,12] and hydroxytyrosol [13] are the more effective among them. Romero et al. [14] reported that the dialdehydic form of decarboxymethyl oleuropein aglycon and the dialdehydic form of decarboxymethyl ligstroside aid in inhibiting *Helicobacter pylori*. Bubonja-Sonje et al. [15] indicated that the polyphenols in olive oil can be used as a natural alternative for the prevention of food spoilage by *Listeria monocytogenes*.

In the case olive trees grown in polluted environments, it should be important to define the physico-chemical and microbiological soil status, to ensure the absence of any contaminants in soils and oil. For this purpose, the metabolic diversity of soil microbial communities can be estimated by different methods [16], such as the determination of community-level physiological profiles (CLPPs), having a high discriminating power between microbial communities from different soil environments or subjected to various sources of pollution [17–19].

The aims of the current study was i) to explore the antimicrobial effects of olive against 3 G+ and 3 G- bacteria and 7 phytopathogenic

Abbreviations: 1–D, Simpon's diversity index; AWCD, average well colour development; CFU, Colony-forming unit; CP, control plot; E, Shannon's evenness index; G+, gram-positive; G−, gram-negative; H', Shannon's diversity index; PGI, percentage of growth inhibition; PP, polluted plot; S, richness; U, McIntosh's diversity index; Z, McIntosh's evenness index; OMW, olive mill wastewaters

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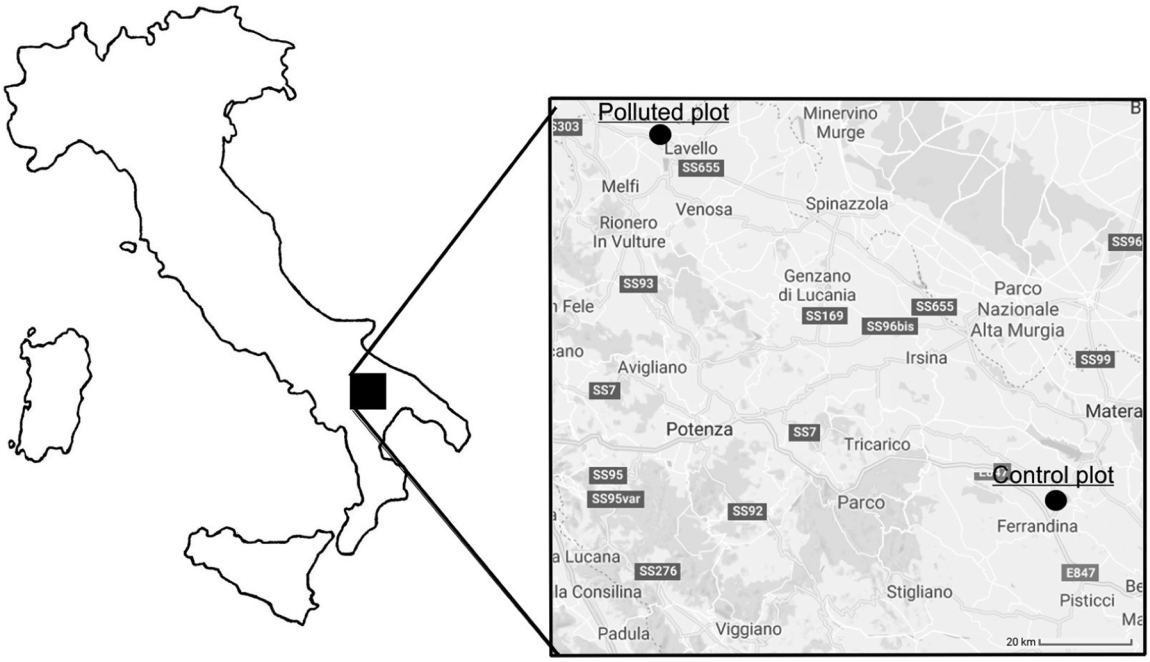


Fig. 1. The studied polluted and control areas.

fungi; and ii) to characterize the soil microbiological status, in terms of microbial metabolic activity and diversity, comparing a zinc-polluted and an unpolluted olive orchard.

2. Materials and Methods

2.1. Plant material and experimental design

The studied 35-year-old olive trees (*Olea europaea* L., cv. Coratina), planted in 1992 at distances of 6 × 3 m, were located in a polluted plot in Lavello (Southern Italy, Basilicata region, N 40°38′, E 15°48′) (Fig. 1). The region is characterized by an arid Mediterranean climate largely influenced by its mild and gentle topography. Polluted plants (PP) were located at 0.5 km from a phosphate fertilizer producing factory located in the industrial area of San Nicola, converting crude phosphate into a granule phosphate fertilizer. During the phosphate attack by sulphuric and phosphoric acids, Zn is released from the industry chimney in the form of inorganic particulate, with an emission at source of 450 kg h^{-1}. The factory emission of Zn in the particulate was found at concentrations of 36.8 Zn g t^{-1} of raw material (mean 2000-2012). Generally, wet deposition dominates during winter, in coincidence with high precipitation. It can occur as washout from smoke below clouds or rainout of particulate taken up by clouds. By contrary, dry deposition dominates in summer period, in association with low precipitation. Therefore, vegetation developed around the factory is continuously exposed to air Zn pollution.

The plot with control plants (CP), was located in Ferrandina (Southern Italy, Basilicata region − N 40°30′, E 16°27′), 90 km south-east of Lavello, in an inland rural area without any industry (Fig. 1). Olive CP were of cv. Coratina and similar in age, plant density and training system to PP. In the control plot, atmospheric Zn was found at concentrations below the instrument detection limits.

2.2. Soil sampling

In October 2015, CP and PP soils were sampled. For each treatment, five composite bulk samples soil were randomly collected from the top soil layers (0-20, 20-40 and 40–60 cm) in the inter-row areas (8-m apart) located at the center of each plot in order to avoid border interferences. Each composite sample was formed by five 7 cm-diameter cores sampled within a 0.50 cm-radius to minimize spatial variability and pooled on site [20,21]. After removal of crop residues, the soil samples were stored immediately at 4 °C in sterilized plastic pots.

2.3. Soil physicochemical parameters

On soil composite samples (soil layers: 0-20, 20-40 and 40–60 cm), soil texture, pH, electric conductivity, organic matter, and contents of Ca, Mg, Na, K and P (Olsen) were determined according to the official methods of the Italian Society of Soil Science [22].

The concentration of metals in the microwave-digestible residual was determined in soil composite samples (soil layers: 0-20, 20-40 and 40–60 cm) after digestion of 1 g in Teflon vessels with 3 ml of HNO_3 70%, 4 ml of HF 40% and 1 ml of $HClO_4$ 70%. Samples were first heated from room temperature to 200 °C for 10 min and kept at this temperature for 15 min in a closed high-pressure microwave system Ethos SEL (Milestone Inc., Schelton, CT, USA). After digestion, vessels were allowed to cool to ambient temperature. Another microwave digestion system ETHOS TC (Milestone, Italy) equipped with VAC-4000 was utilized, and samples were boiled to near dryness in order to evaporate the exceeding acids of HF and $HClO_4$. Operating parameters and power of microwave digestion systems were set according to the procedures reported in the instrumentation manual. The residual solution was subsequently transferred into a 25 mL volumetric flask, and 5 ml of 50% HNO_3 (trace metal grade) were added to preserve the sample for trace metal analyses. Samples were kept at −18 °C until analyses were carried out. Determination of Zn was achieved with inductively coupled plasma mass spectrometer ICP-MS ELAN 6000 DRCe with a cross flow nebulizer (Perkin Elmer, Waltham, MA, USA). ^{103}Rh and ^{187}Re at the concentration 10 µg L^{-1} were used as internal standards. Blank solutions were prepared to correct for contaminants contained in reagents used during sample dissolution. Calibrating standard solutions were used for the calibration of the ICP/MS instrument at different concentrations of metals. The calibration curves were linear in the whole calibrating range ($r \geq 0.9996$). Measures have been replicated thrice and recoveries have been determined using certified soils.

2.4. Soil microbiological characteristics

For the microbiological analysis, three replicates of 10 g-subsamples (dry weight equivalent) of each soil composite sample (0-20 and 20–40 cm) were suspended in 90 ml sterile 0.1% sodium pyrophosphate-one quarter strength Ringer solution (NaCl 2.25 g L^{-1}, KCl 0.105 g L^{-1}, $CaCl_2$ 0.045 g L^{-1}, $NaHCO_3$ 0.05 g L^{-1}, and citric acid 0.034 g L^{-1}) and sonicated for 2 min to disperse microbial cells. Ten-fold serial dilutions of the supernatants were made in sterile one-quarter strength Ringer solution. Sole carbon source utilization patterns of soil microbial communities, also called community-level physiological profiles (CLPPs), were assessed using the Biolog® 96-well Eco-Microplates™ (AES Laboratoire, France), containing 31 different carbon sources for bacteria, and the Biolog® FF Microplates™ (AES Laboratoire, France) and 93 different carbon sources for fungi. For each well of the Biolog® Microplates™ an aliquot of 100 µl of the soil dilution (soil layer: 0–20 cm, the richest in soil microorganisms) at 10^{-3} dilution was used for the assay.

The microplates were incubated at 25 °C in darkness and the colour development was measured every 24 h along 144 h period using a Microplates E-Max Reader (Bio-Rad; Hercules, CA USA) with a E590-nm wavelength filter. The substrate-utilization profiles were analysed on well-absorbance values at the 120 h observation period.

Data were analysed to determine metabolic diversity indices, according to Zak et al. [23]. The indices examined were the following: average well colour development (*AWCD*), that provides a measure of total cultural bacterial activity,

$$AWCD = \sum \frac{(c_i - R)}{W}$$

Shannon's diversity index (*H'*),

$$H' = -\sum p_i (\ln p_i)$$

Shannon's evenness index (*E*), the equitability of activities across all utilized substrates,

$$E = \frac{H'}{\ln S}$$

Simpon's diversity index (1 − *D*), where

$$D = \sum (n_i \times N)^2$$

McIntosh's diversity index (*U*),

$$U = \sqrt{\sum n_i^2}$$

and McIntosh's evenness index (*Z*),

$$Z = \frac{N - U}{N - N/\sqrt{S}}$$

where c_i is the OD_{590nm} in each well; *R* is the OD_{590nm} in the control well; *W* is the number of all the wells; p_i is the OD_{590nm} in each well divided by the mean OD_{590nm} of all the wells; *S* (richness = the number of utilized substrates) is the number of the wells with $OD_{590nm} \geq AWCD$; n_i is the relative OD_{590nm} in each well (c_i − R); *N* is the sum of OD_{590nm} of all the wells.

The carbon substrates of the Biolog® plates were divided into eight and ten main groups of compounds for bacteria and fungi, respectively, and the *AWCD* value for each group was calculated.

2.5. Oil extraction

For both PP and CP plants, oil extraction from olives harvested in November 2014 and November 2015 was carried out using an Abencor system within 24 h after fruit harvesting. Olives (5 kg) were crushed with a hammer mill and were slowly mixed for 30 min at 25 °C. Next, the obtained paste was centrifuged at 3,500 × *g* over 3 min. The oil was then separated by decanting, transferred into dark glass bottles and stored in the dark at 4 °C.

2.6. Oil antibacterial activity and total bacterial counts

Three gram-positive (G +) were tested: *Bacillus megaterium* de Bary, *B. mojavensis* Roberts and *Clavibacter michiganensis* Smith and three gram-negative (G−) *Xanthomonas campestris* Pammel, *X. vesicatoria* Doidge and *Escherichia coli* Migula.

The antibacterial test of studied olive oil was carried out using disc diffusion method of Bhunia et al. [24] and Elshafie et al. [25] as explained here. Bacterial suspension of each tested bacteria was prepared in sterile distilled water adjusted at 10^8 CFU mL^{-1} (OD ≈ 0.2 nm). An aliquot of soft agar (0.7%) and bacterial suspension (9:1; v/v) was prepared and 4 mL was added over King's B media (KB) [26] Petri dish (90 mm, diameter) 10 mL. Blank discs (6 mm)-OXOID were placed after that over KB-plate surfaces and about 15 µL of each oil treatment at concentrations (80, 60 and 40%) (*v/v*) diluted in *n*-hexane mixed with 0.2% Tween 20 were carefully applied over discs. Tetracycline was used as positive control at 1,600 µg mL^{-1}. Tween 20 and *n*-hexane were used as negative control. The antibacterial activity was estimated by measuring the diameter of inhibition hyaline zone (cm) formed around each treated point compared to tetracycline.

The total counting bacteria test has been carried out using four bacteria (*B. megaterium*, *C. michiganensis*, *X. campestris* and *X. vesicatoria*) to verify eventually the bactericidal effect of tested olive oil against the above-mentioned bacteria. The tested bacteria species were grown overnight in sterile KB and the culture was placed in an incubator (30 °C) until a density of 50 Klett spectrophotometer units was reached at approximately 10^8 CFU mL^{-1}. The cultures were then adjusted at approximately 10^5 CFU mL^{-1} using sterile distilled water. The studied olive oils were sterilized by using 0.22 µm syringe filter. The filtered olive oils were mixed with the appropriate amounts of *n*-hexane as organic solvent to create olive oil concentrations at 1, 10, and 20%. One mL from each concentration was added in sterile eppendorf containing 100 µL from each bacterial suspension; all suspensions were vortexed and incubated at 30 ± 2 °C for 24 h. After that, 100 µl from each suspension was plated over KB media 14 mL petri dishes and the plates were incubated at 30 ± 2 °C for 24 h. The appeared colonies were counted visually and under stereomicroscope Heerbrugg (Switzerland). Each colony was assumed to be arisen from one cell.

2.7. Oil antifungal activity test

Five phytopathogenic fungi were tested for the antifungal activity assay; *Rhizoctonia solani* (Cooke) Wint., *Fusarium oxysporum* von Schlechtendal, *Sclerotinia sclerotiorum* Lib., *Botrytis cinerea* Pers. and *Monilinia laxa* (Aderh. and Ruhland).

The possible fungicidal activity of the studied olive oils was evaluated following the method of Soylu et al. [27] and Elshafie et al. [28] at two different concentrations (1,000 and 10,000 mg kg^{-1}) incorporated with Potato Dextrose Agar (PDA) medium at 45 °C. Fungal disk (0.5 cm) from 96 h fresh culture was inoculated in the center of each Petri dish. All plates were incubated at 22 ± 2 °C for 96 h in darkness conditions and the diameter of fungal mycelium growth was measured in mm. PDA plates without any treatment were inoculated only with fungal disks as negative control. The fungitoxicity was expressed as percentage of growth inhibition (PGI) compared to control plates and calculated according to the formula of Zygadlo et al. [29] as follows:

$$PGI(\%) = \frac{100 \times (GC - GT)}{GC}$$

where PGI is the percentage of growth inhibition, GC is the average diameter of fungal mycelium in PDA (Control), and GT is the average diameter of fungal mycelium on the oil-treated PDA dish.

Table 1
Physicochemical parameters of soils of the two studied sites (PP = polluted plot; CP = control plot). Each value represents the mean (± SE) from composite bulk soil samples (n = 5).

Site	Soil layer	Sand	Silt	Clay	pH	Electric conductivity	Organic matter	Ca	Mg	Na	K	P (Olsen)
		(%)				(mS cm^{-1})	(g kg^{-1})	(mg kg^{-1})				
PP	0–20	56	27	17	6.0	1.98	2.17	6250	3125	186	196	1175
	20–40	56	27	17	5.9	1.95	2.12	5220	2610	114	132	920
	40–60	62	18	20	5.7	1.90	2.10	5000	2500	54	122	500
CP	0–20	72	7	21	7.7	2.42	2.62	1250	1000	42	122	303
	20–40	48	28	24	7.6	2.39	2.33	1040	900	38	112	145
	40–60	44	29	27	7.8	2.36	2.30	520	350	36	103	225

2.8. Statistical analysis

Results obtained from the current research were statistically processed and subjected to analysis of variance by one-way ANOVA, followed by Duncan Post Hoc multiple comparison test with a probability of $P < 0.05$, using *SPSS* statistical software package version 13.0 (2004) to detect the significance between the different oil treatments.

3. Results and discussion

3.1. Soil physicochemical parameters

The physicochemical parameters of soils of the two studied sites in a soil profile of 0–60 cm (Table 1) show that both PP and CP soils were loamy sand soils with similar levels of organic matter. The CP soils have higher values of pH and electric conductivity, while PP soils have higher Ca, Mg, Na, K and P contents.

Regarding Zn, it was mostly concentrated in the superficial layers, thus suggesting Zn depositions due to the factory emissions (Fig. 2). The CP soils contained normal levels of Zn, without showing any contamination, compared to PP soils (Fig. 2).

3.2. Soil microbiological characteristics

Soil bacterial metabolic diversity indices estimated by Biolog® [30,31] refer to the number, variety and variability of microorganisms, including diversity within and between groups, and these parameters were found to be usually lower in metal-contaminated soils [17,19,32,33].

For bacteria, the Biolog® analysis showed that total *AWCD*, a measure of the overall microbial metabolic activity, was significantly greater in CP than in PP, without any significant differences between soil depths (Table 2). Regarding bacterial community diversity, the three diversity indices here used (*H'*, 1–*D*, and *U*) were significantly higher in CP than in PP, while soil depth did not cause significant changes, with the exception of *U* in PP (Table 2). High values of evenness indicate a large number of some groups of microorganisms, without the predominance of few microbial groups [18,23]. The values of both evenness indices (*E* and *Z*) measured for bacteria within the same soil layer were always higher in CP than in PP (Table 2). Finally, richness (*S*), that is the number of utilized substrates, was higher in CP than in PP only at a depth of 20–40 cm (Table 2). From all these results, it appeared clear a metabolic inhibition of bacterial microbial communities in the soils with Zn depositions.

Biolog® CLPP for fungi was different from that observed for bacteria (Table 3). The values of *AWCD* were significantly greater in CP than in PP but only for the soil layer 0–20 cm (Table 3). The diversity indices (*H'*, 1–*D*, and *U*) had trends similar to that of *AWCD* (Table 3). Regarding evenness indices (*E* and *Z*), *E* values were significantly higher in CP for both soil layers, whereas *Z* values in CP were always higher than those in PP (Table 3). Finally, richness (*S*) was always statistically higher in CP than in PP, independently from soil depth (Table 3). The Biolog® analysis on fungal communities revealed a metabolic inhibition in soils interested by Zn depositions but only at soil surface (0–20 cm), where the metal concentration is higher. Fungal communities living in the 20–40 cm soil layer were less affected by the inhibitory effect of Zn.

In our case, Biolog® absorbance values demonstrated that the *AWCD* of almost all principal classes of bacterial carbon substrates were significantly greater in the CP, with the following exceptions: polysaccharides, complex compounds and cellulose at 0–20 cm soil layer and polysaccharides, complex compounds and organic acids at 20–40 cm soil layer (Fig. 3). For fungi, all the functional groups of carbon substrates had a statistically higher *AWCD* in CP for the soil layer 0–20 cm, while only few significant differences (polysaccharides and acid sugars) between CP and PP at a depth of 20–40 cm (Fig. 4).

3.3. Oil antibacterial activity and total bactericidal counts

Both PP (P14, P15) and CP (C14, C15) olive oils were able to significantly inhibit the growth of tested *B. megaterium*, *B. mojavensis*, *X. vesicatoria* and *C. michiganensis* more than the other tested bacteria (Table 4). In addition, all the tested concentrations of all olive oils inhibited completely the growth of *X. campestris* (Table 4).

In particular, *X. vesicatoria* and *C. michiganensis* were significantly

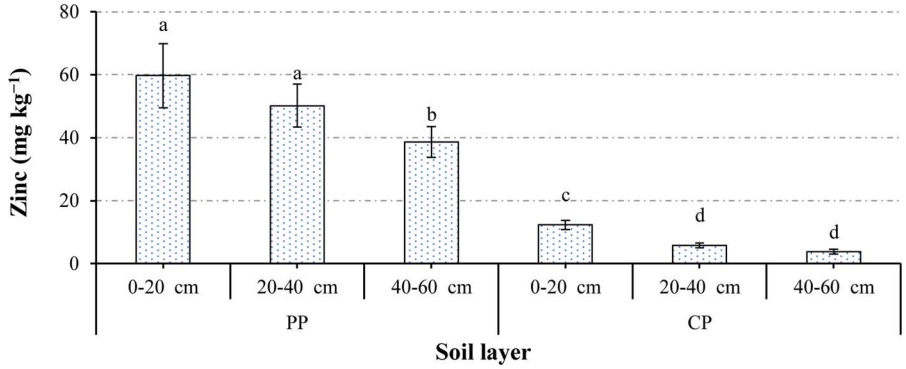


Fig. 2. Zinc levels in soils of the two studied sites (PP = polluted plot; CP = control plot). Each value represents the mean (± SE) from composite bulk soil samples (n = 5). Bars with different letters are statistically different ($P < 0.05$), according to Tukey's B Post Hoc multiple comparison test.

Table 2
Bacterial community indices obtained by Biolog® 96-well Eco-Microplates™ from soils of the two studied sites (PP = polluted plot; CP = control plot). Each value represents the mean (± SE) from composite bulk soil samples (n = 5). Values followed by different letters are statistically different ($P < 0.05$) within columns, according to Tukey's B Post Hoc multiple comparison test. *AWCD* = average well colour development; *H'* = Shannon's diversity index; *E* = Shannon's evenness index; *S* = richness; 1–*D* = Simpon's diversity index; *U* = McIntosh's diversity index; *Z* = McIntosh's evenness index.

Site	Soil layer (cm)	*AWCD*	*H'*	*E*	*S*	*1 − D*	*U*	*Z*
PP	0-20	0.13 ± 0.04 b	2.65 ± 0.04 b	2.47 ± 0.03 b	13.22 ± 1.20 a	0.54 ± 0.06 b	1.80 ± 0.20 b	0.96 ± 0.03 b
	20-40	0.08 ± 0.02 b	2.64 ± 0.06 b	2.24 ± 0.07 c	10.45 ± 1.56 b	0.55 ± 0.09 b	1.09 ± 0.19 c	0.73 ± 0.03 c
CP	0-20	0.26 ± 0.03 a	3.00 ± 0.09 a	2.79 ± 0.05 a	13.12 ± 1.78 a	0.86 ± 0.11 a	2.88 ± 0.23 a	1.13 ± 0.05 a
	20-40	0.26 ± 0.09 a	2.84 ± 0.04 a	2.47 ± 0.13 b	14.85 ± 1.03 a	0.86 ± 0.13 a	3.11 ± 0.14 a	1.10 ± 0.04 a

Table 3
Fungal community indices obtained by Biolog® 96-well Eco-Microplates™ from soils of the two studied sites (PP = polluted plot; CP = control plot). Each value represents the mean (± SE) from composite bulk soil samples (n = 5). Values followed by different letters are statistically different ($P < 0.05$) within columns, according to Tukey's B Post Hoc multiple comparison test. *AWCD* = average well colour development; *H'* = Shannon's diversity index; *E* = Shannon's evenness index; *S* = richness; 1–*D* = Simpon's diversity index; *U* = McIntosh's diversity index; *Z* = McIntosh's evenness index.

Site	Soil layer (cm)	*AWCD*	*H'*	*E*	*S*	*1 − D*	*U*	*Z*
PP	0-20	0.21 ± 0.04 b	2.15 ± 0.30 b	1.71 ± 0.18 c	29.20 ± 3.24 c	0.54 ± 0.10 b	1.80 ± 0.15 b	0.86 ± 0.21 b
	20-40	0.34 ± 0.04 a	3.45 ± 0.23 a	2.04 ± 0.15 b	38.75 ± 5.56 b	0.75 ± 0.08 a	2.16 ± 0.21 a	0.83 ± 0.17 b
CP	0-20	0.35 ± 0.05 a	3.43 ± 0.15 a	2.64 ± 0.43 a	51.60 ± 5.00 a	0.73 ± 0.13 a	2.50 ± 0.13 a	1.13 ± 0.02 a
	20-40	0.38 ± 0.07 a	3.24 ± 0.21 a	2.98 ± 0.19 a	47.20 ± 5.20 a	0.76 ± 0.20 a	2.11 ± 0.17 a	1.10 ± 0.14 a

inhibited by PP oils more than CP oils of both 2014 and 2015. The high bioactivity of PP may be attributed to the presence of some phenolic compounds such as hydroxytyrosol and oleuropein that are considered highly toxic against some pathogenic bacteria, such as *Pseudomonas savastanoi* and *Corynebacterium michiganensis* [34–36]. On the other hand, the most bactericidal polyphenols of olive oil are the dialdehydic form of decarboxymethyl oleuropein and ligstroside aglycons as reported by Brenes et al. [37]. Medina et al. [38] and Zampa et al. [39] reported that olive oil possesses some bioactive components, such as sterols, vitamins, squalene and polyphenols, which play an important role in its antimicrobial activity. Particularly, Medina et al. [40] reported that the antimicrobial activity of olive oil and olive leaf extracts are related to oleuropein and hydroxytyrosol content. Oleuropein was able to inhibit the sporulation of *Bacillus cereus*, while hydroxytyrosol resulted effective against some serious clinical human pathogenic strains such as *Haemophilus influenzae*, *Moraxella catarrhalis* and *Salmonella typhi* [41].

Furthermore, fatty acids and monoglycerides of olive oil have been demonstrated to have a broad spectrum of antibacterial and anti-yeast activity, as reported by Bergsson et al. [42]. The higher amounts of linoleic acid in oil from olive plants exposed to air-pollution than in oils from unpolluted plants may be due to the transformation of oleic acid into linoleic acid, a sequence of enzymatic reactions known as the lipoxygenase pathway. Some abundant volatile compounds such as aldehydes and alcohols are formed from linoleic and α-linolenic acids through a sequence of enzymatic reactions known as the lipoxygenase pathway as discussed by Sanchez and Harwood [43].

In addition, flavan-3-ols has been reported by Daglia [44] that has antibacterial activity against several bacterial species such as *Vibrio cholerae*, *Streptococcus mutans*, *Campilobacter jejuni*, *Clostridium perfringes* and *E. coli*. On the other hand, flavonols are concerned, we can see a remarkable activity against several G+ bacteria such as *Staphylococcus aureus, Lactobacillus acidophilus* and *Actinomyces naeslundii* and G- bacteria such as *Prevotella oralis, Prevotella melaninogenica, Porphyromonas gingivalis,* and *Fusobacterium nucleatum* [44]. Finally, the suggested mechanism of antimicrobial activity of olive oil could be due to the surface activity that damages the plasma membranes of bacterial cells [45].

In general, most of the tested bacteria were sensitive to the studied olive oils either from PP and CP plants. However, the inhibitory effect was higher against G+ than G- bacteria in agreement with Zampa et al. [39]. A high content of phenolic compounds in the tested olive oils could be the main reason for its antimicrobial activity [37]. In particular, the dialdehydic form of decarboxymethyl oleuropein and ligstroside aglycons possess a strong bactericidal activity as reported by Brenes et al. [37]. In particular, olive oil (2014) at a concentration of 20% from both PP and CP significantly decreased *B. megaterium* growth, followed by olive oil (2014) at 10% and olive oil (2014) at 20% (Fig. 5A). By contrary, olive oil (2015), either at 1% or 10% in PP and at 1% in CP showed the lowest inhibition activity on *B. megaterium* survivorship. Regarding *C. michiganensis* (Fig. 5B), results indicated that all the tested olive oils from both PP and CP significantly decreased its survivorship, excepting the olive oil extracted from CP olive trees at 1% (2015). For *X. campestris* (Fig. 5C), all the tested olive oils from both PP and CP significantly decreased its survivorship, excepting the olive oil extracted from CP plants at 1% (2014 and 2015). Finally, the olive oils extracted from CP (2014 and 2015) at 10% and 20% significantly decreased the growth of *X. vesicatoria* (Fig. 5D). Finally, olive oil (2015), either at 1% or 10% in PP and 1% in CP had the lowest inhibition activity against *X. vesicatoria*.

Other by-products deriving from olive oil production could be used as antibiotics instead of olive oil. Major risks might exist for olive mill wastewaters (OMW) due to the atmospheric deposition of Zn on olive surfaces and the higher solubility of these elements in water rather than in oil, so OMW disposal could be dangerous without any control/regulation. The use of OMW as antimicrobials/antibiotics could be also in competition with their use in agriculture as co-composting for soil organic fertility restoration [46], while using poor-quality oil is economically advantageous.

3.4. Antifungal activity of olive oils

Results on antifungal activity test demonstrated that all tested concentrations of all olive oil treatments did not show any fungicidal effect against all tested phytopathogenic fungi.

4. Conclusions

The obtained results can be considered an indicator of the impact of air pollution on the antimicrobial activity of olive oil. Furthermore, the analysis of Biolog data for soil carbon source utilization pattern demonstrated that Zn pollution had a significant negative impact on microbial community structure and functional diversity. The results demonstrated that the olive oil extracted from Zn-polluted plants, often

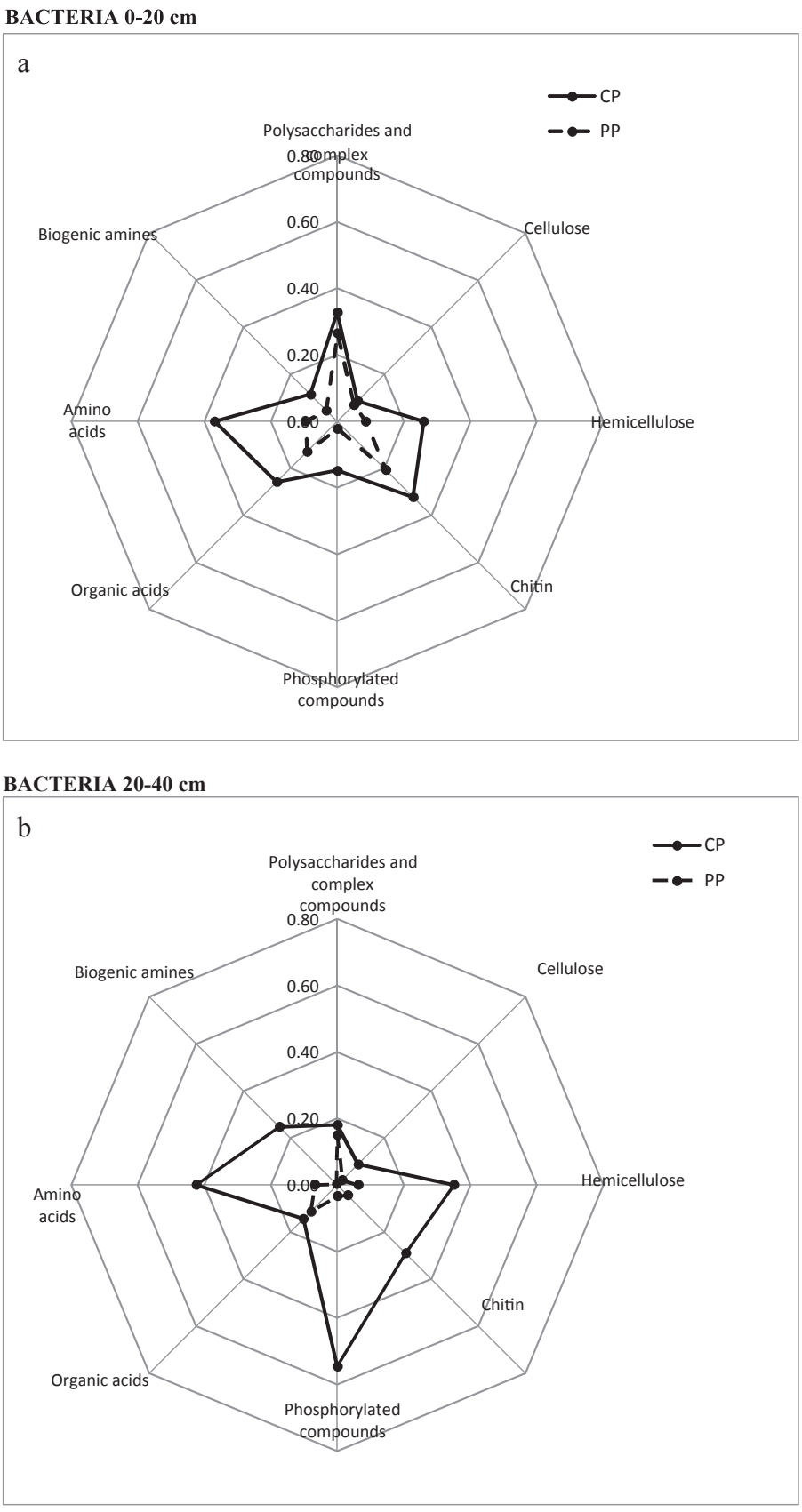
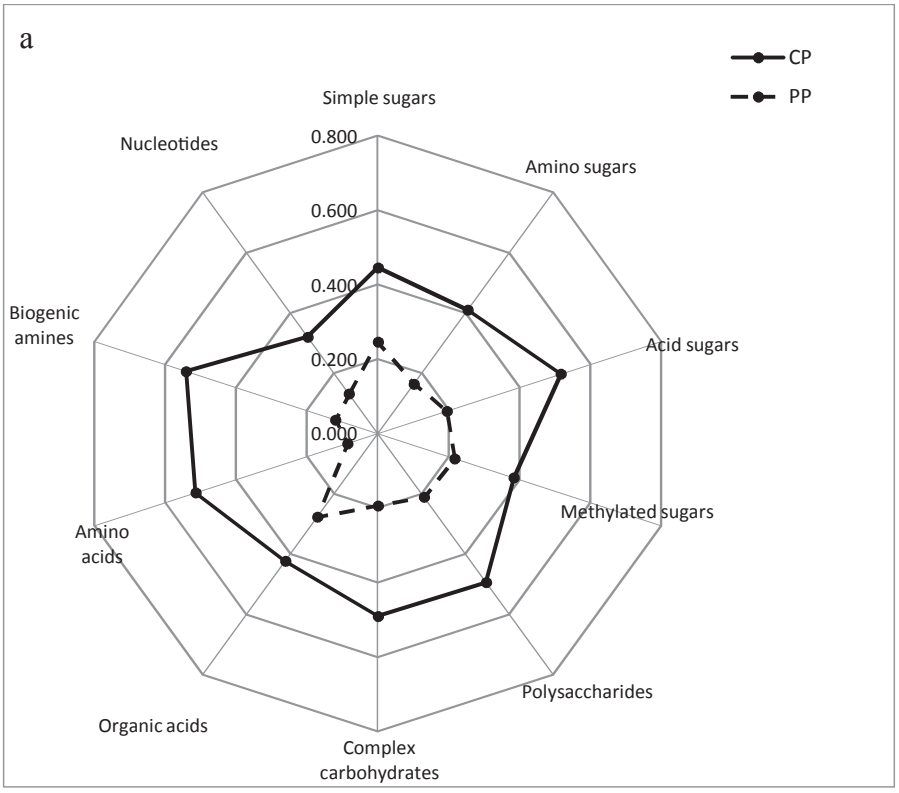


Fig. 3. Radar diagrams of (a) bacterial *AWCD* of all the principal classes of carbon substrates, identified by Biolog® 96-well Eco-Microplates™, from soils (a = 0–20 cm; b = 20–40 cm) of the two studied sites (PP = polluted plot, continuous line; CP = control plot, dashed line). Means (n = 5) with the * are statistically different ($P < 0.05$), according to Tukey's B Post Hoc multiple comparison test.

FUNGI 0-20 cm



FUNGI 20-40 cm

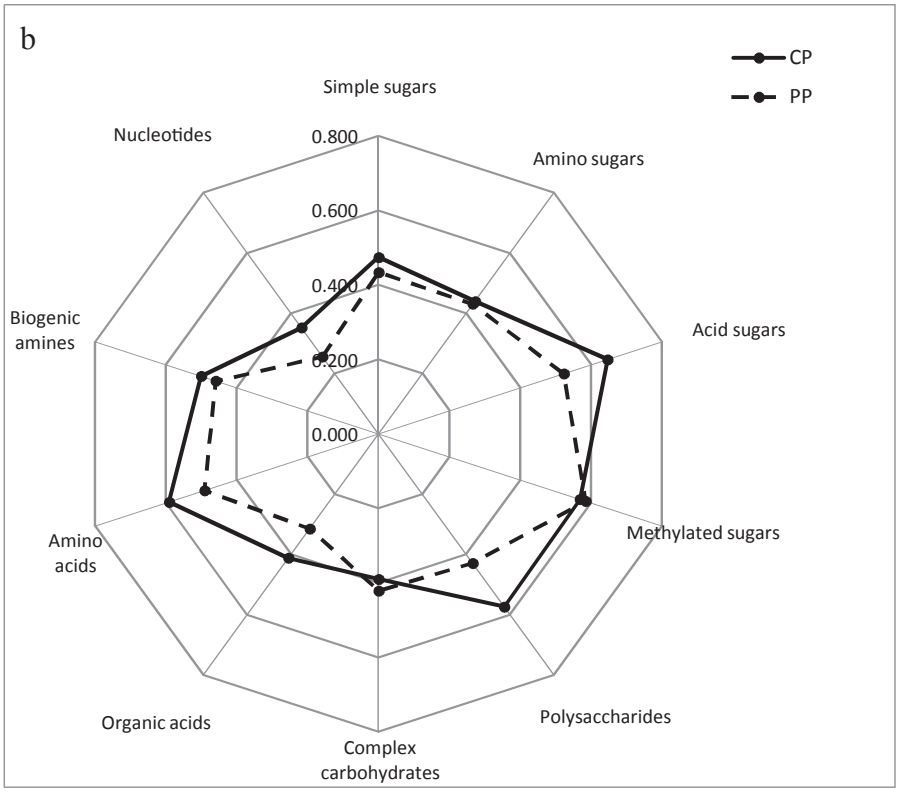


Fig. 4. Radar diagrams of fungal *AWCD* of all the principal classes of carbon substrates, identified by Biolog® 96-well FF Microplates™, from soils (a = 0–20 cm; b = 20–40 cm) of the two studied sites (PP = polluted plot, continuous line; CP = control plot, dashed line). Means ($n = 5$) with the * are statistically different ($P < 0.05$), according to Tukey's B Post Hoc multiple comparison test.

Table 4
Antibacterial activity of olive oil against some phytopathogenic bacteria.

Treatments		Diameter of inhibition zones (cm) G+			Diameter of inhibition zones (cm) G-		
Olive oil	Conc. %	*B. megaterium*	*B. mojavensis*	*C. michiganensis*	*X. campestris*	*X. vesicatoria*	*E. coli*
C14	80	2.10 ± 0.14ab	1.10 ± 0.14b	2.15 ± 0.21a	4.50 ± 0.0a	1.15 ± 0.21b	0.00 ± 0.0b
	50	1.40 ± 0.14b	0.75 ± 0.21c	1.60 ± 0.14ab	4.50 ± 0.0a	0.70 ± 0.28c	0.00 ± 0.0b
	20	0.80 ± 0.14c	0.40 ± 0.14 cd	1.00 ± 0.14b	4.50 ± 0.0a	0.40 ± 0.14c	0.00 ± 0.0b
P14	80	1.35 ± 0.21b	1.15 ± 0.21b	2.60 ± 0.28a	4.50 ± 0.0a	2.50 ± 0.21a	0.00 ± 0.0b
	50	0.85 ± 0.07c	0.40 ± 0.14 cd	1.95 ± 0.21ab	4.50 ± 0.0a	1.80 ± 0.14ab	0.00 ± 0.0b
	20	0.45 ± 0.07 cd	0.25 ± 0.07 cd	1.50 ± 0.21ab	4.50 ± 0.0a	1.00 ± 0.14b	0.00 ± 0.0b
C15	80	1.35 ± 0.07b	0.75 ± 0.07c	1.80 ± 0.14ab	4.50 ± 0.0a	1.40 ± 0.14ab	0.00 ± 0.0b
	50	0.80 ± 0.14c	0.35 ± 0.07 cd	1.45 ± 0.21ab	4.50 ± 0.0a	1.00 ± 0.14b	0.00 ± 0.0b
	20	0.55 ± 0.07 cd	0.15 ± 0.07 cd	1.10 ± 0.14b	4.50 ± 0.0a	0.60 ± 0.14c	0.00 ± 0.0b
P15	80	1.85 ± 0.21ab	0.75 ± 0.07c	2.30 ± 0.28a	4.50 ± 0.0a	2.40 ± 0.14a	0.00 ± 0.0b
	50	1.20 ± 0.14b	0.35 ± 0.07 cd	1.85 ± 0.21ab	4.50 ± 0.0a	1.75 ± 0.21ab	0.00 ± 0.0b
	20	0.80 ± 0.14c	0.15 ± 0.07 cd	1.45 ± 0.21ab	4.50 ± 0.0a	1.20 ± 0.14b	0.00 ± 0.0b
Tetracycline	1600 μg ml^{-1}	3.35 ± 0.21a	2.90 ± 0.14a	2.70 ± 0.14a	4.50 ± 0.0a	2.90 ± 0.28a	2.500.21a
n-hexane		0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00c	0.00 ± 0.0b	0.00 ± 0.00d	0.00 ± 0.0b
Control		0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00b	0.00 ± 0.00d	0.00 ± 0.00b

Where: C14: olive oil extracted from olive trees in unpolluted area during 2014; P14: olive oil extracted from olive trees in polluted area during 2014; C15: olive oil extracted from olive trees in unpolluted area during 2015; P15: olive oil extracted from olive trees in polluted area during 2015. Values are recorded as the mean diameter of inhibition zone (cm) ± standard deviations. Values followed by the different letter in each vertical column are significantly different at $P < 0.05$ according to Duncan test. Data were obtained from three replicates.

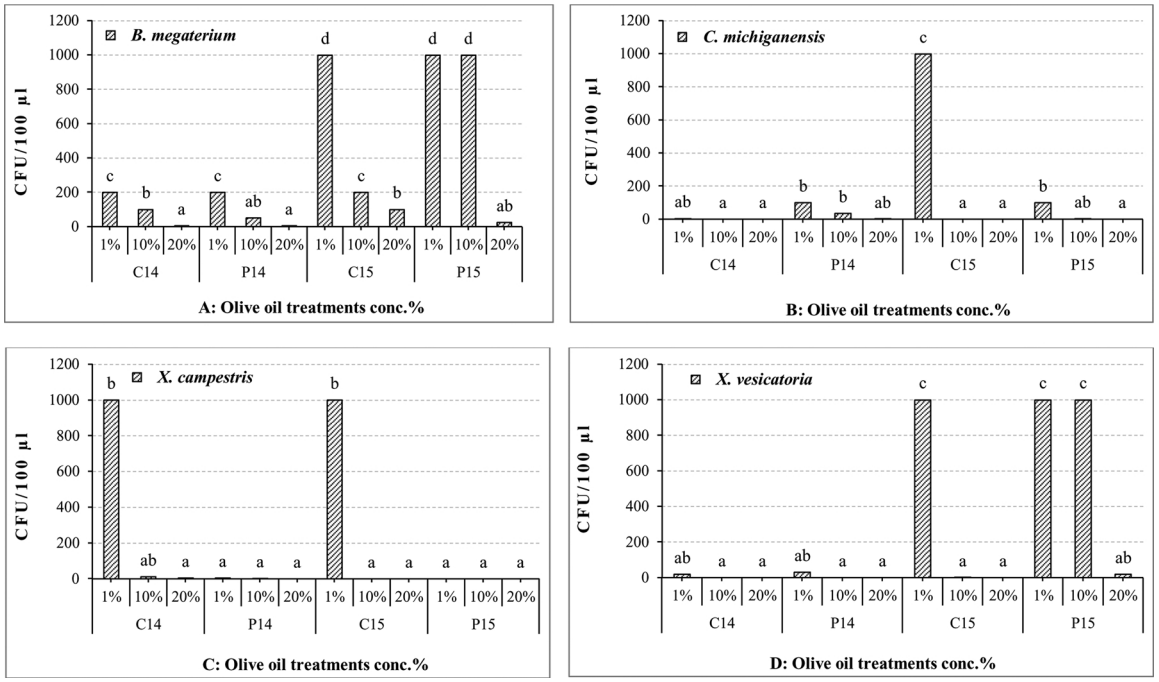


Fig. 5. Total counting bacteria assay. Where: C13: olive oil extracted from olive trees in the control area during 2014; P13: olive oil extracted from olive trees in the polluted area during 2014; C14: olive oil extracted from olive trees in the control area during 2015; P14: olive oil extracted from olive trees in the polluted area during 2015. A: *B. megaterium*; B: *C. michiganensis*; C: *X. campestris*; D: *X. vesicatoria*. Bars with different letters indicate mean values significantly different at $P < 0.05$ according to *Tukey* (B) test. Data are expressed as mean of three replicates.

having a low organoleptic quality and is not suitable for dietary consumption, might be eventually used as antibiotics. Other future studies should explain better the possible antifungal activity of olive oil and whether or not there are differentiable factors that could affect negatively the bioactivity of olive oil according to the air pollution conditions.

Conflict of interest

The authors declare no conflict of interest

Author disclosure statement

No competing financial interests exist.

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References

[1] C. Cioffi, M.S. Pesca, P. De Caprariis, A. Braca, L. Severino, N. De Tommasi, Phenolic compounds in olive oil and olive pomace from Cilento (Campania, Italy) and their antioxidant activity, Food Chem. 121 (2010) 105–111.

[2] A. Sofo, V. Nuzzo, A.M. Palese, C. Xiloyannis, G. Celano, P. Zukowskyj, B. Dichio, Net CO_2 storage in Mediterranean olive and peach orchards, Scientia Horticulturae 107 (2005) 17–24.

[3] M.G. Soni, G.A. Burdock, M.S. Christian, C.M. Bitler, R. Crea, Safety assessment of aqueous olive pulp extract as an antioxidant or antimicrobial agent in foods, Food Chem. Toxicol. 44 (2006) 903–915.

[4] A.E. Binkoski, P.M. Kris-Etherton, T.A. Wilson, M.L. Mountain, R.J. Nicolosi, Balance of unsaturated fatty acids is important to a cholesterol-lowering diet: comparison of midoleic sunflower oil and olive oil on cardiovascular disease risk factors, J. Am. Diet Assoc. 105 (2005) 1080–1086.

[5] S. Cicerale, L. Lucas, R. Keast, Biological activities of phenolic compounds present in virgin olive oil, Int. J. Mol. Sci. 11 (2010) 458–479.

[6] S. Cicerale, L.J. Lucas, R.S. Keast, Antimicrobial, antioxidant and anti-inflammatory phenolic activities in extra virgin olive oil, Curr. Opin. Biotechnol. 23 (2012) 129–135.

[7] H. Karaosmanoglu, F. Soyer, B. Ozen, F. Tokatli, Antimicrobial and antioxidant activities of Turkish extra virgin olive oils, J. Agr. Food Chem. 58 (2010) 8238–8245.

[8] E. Medina, C. Romero, M. Brenes, A. De Castro, Antimicrobial activity of olive oil, vinegar, and various beverages against foodborne pathogens, J. Food Protec. 70 (2007) 1194–1199.

[9] E. Mancini, I. Camele, H.S. Elshafie, L. De Martino, C. Pellegrino, D. Grulova, V. De Feo, Chemical Composition and Biological Activity of the Essential Oil of *Origanum vulgare* ssp. *hirtum* from Different Areas in the Southern Apennines (Italy), Chem. Biodiver. 11 (2014) 639–651.

[10] H.S. Elshafie, E. Mancini, I. Camele, L.D. Martino, V. De Feo, *In vivo* antifungal activity of two essential oils from Mediterranean plants against postharvest brown rot disease of peach fruit, Indus. Crops Produc. 66 (2015) 11–15.

[11] P.M. Furneri, A. Piperno, A. Saija, G. Bisignano, Antimicrobial activity of hydroxytyrosol, Antimicrob. Agents Ch. 48 (2004) 4892–4894.

[12] G. Yildiz, V. Uylaser, Natural an antioxidants: oleuropein, J. Agricul. Fac. Uludag. Uni. 25 (2011) 131–142.

[13] G. Bisignano, A. Tomaino, R.L. Cascio, G. Crisafi, N. Uccella, A. Saija, On the in-vitro antimicrobial activity of oleuropein and hydroxytyrosol, J. Pharm. Pharmacol. 51 (1999) 971–974.

[14] C. Romero, E. Medina, J. Vargas, M. Brenes, A. De Castro, *In vitro* activity of olive oil polyphenols against Helicobacter pylori, J. Agric. Food Chem. 55 (2007) 680–686.

[15] M. Bubonja-Sonje, J. Giacometti, M. Abram, Antioxidant and antilisterial activity of olive oil, cocoa and rosemary extract polyphenols, Food Chem. 127 (2011) 1821–1827.

[16] T. Gomiero, D. Pimentel, M.G. Paoletti, Environmental impact of different agricultural management practices: conventional versus organic agriculture, Crit. Rev. Plant Sci. 30 (2011) 95–124.

[17] M. Boshoff, M. De Jonge, F. Dardenne, R. Blust, L. Bervoets, The impact of metal pollution on soil faunal and microbial activity in two grassland ecosystems, Enviro. Res. 134 (2014) 169–180.

[18] A. Sofo, A. Ciarfaglia, A. Scopa, I. Camele, M. Curci, C. Crecchio, C. Xiloyannis, A.M. Palese, Soil microbial diversity and activity in a Mediterranean olive orchard using sustainable agricultural practices, Soil Use Manage. 30 (2014) 160–167.

[19] Y. Xie, J. Fan, W. Zhu, E. Amombo, Y. Lou, L. Chen, J. Fu, Effect of heavy metals pollution on soil microbial diversity and bermudagrass genetic variation, Front Plant Sci. 7 (2016) 12pp.

[20] J.R. Bacon, G. Hudson, A flexible methodology for the characterisation of soils: a case study of the heavy metal status of a site at Dornach, Sci. Total Environ. 264 (2001) 153–162.

[21] G. Tian, J.M. Vose, D.C. Coleman, C.D. Geron, J.T. Walker, Evaluation of the effectiveness of riparian zone restoration in the southern Appalachians by assessing soil microbial populations, Appl. Soil Ecol. 26 (2004) 63–68.

[22] SISS, Italian Society of Soil Science, Metodi di Analisi Chimica del Suolo, Franco Angeli Editore, Milano, Italy, 2000.

[23] J.C. Zak, M.R. Willig, D.L. Moorhead, H.G. Wildman, Functional diversity of microbial communities: a quantitative approach, Soil Biol. Biochem. 26 (1994) 1101–1108.

[24] M.C. Bhunia, B. Johnson, Ray Purification, characterization and antimicrobial spectrum of a bacteriocin produced by Pediococcus acidolactici, J. Appl. Bacteriol. 65 (1988) 261–268.

[25] H.S. Elshafie, S. Sakr, S.M. Mang, V. De Feo, I. Camele, Antimicrobial activity and chemical composition of three essential oils extracted from Mediterranean aromatic plants, J. Med. Food 19 (2016) 1096–1103.

[26] E.O. King, M.K. Ward, D.E. Raney, Two simple media for demonstration of pyocyanin and fluorescin, J. Lab. Clin. Med. 44 (1954) 301–307.

[27] E.M. Soylu, S. Kurt, S. Soylu, *In vitro* and *in vivo* antifungal activities of the essential oils of various plants against tomato grey mould disease agent Botrytis cinerea, Int. J. Food Microbiol. 143 (2010) 183–189.

[28] H.S. Elshafie, E. Mancini, S. Sakr, L. De Martino, C.A. Mattia, V. De Feo, I. Camele, Antifungal activity of some constituents of *Origanum vulgare* L. essential oil against postharvest disease of peach fruit, J. Med. Food 18 (2015) 929–934.

[29] J.A. Zygadlo, C.A. Guzman, N.R. Grosso, Antifungal properties of the leaf 410 oils of *Tagetes minuta* L. and *Tagetes filifolia* Lag, J. Essen. Oil Res. 6 (1994) 617–621.

[30] E.A. Hobbie, L.S. Watrud, S. Maggard, T. Shiroyama, P.T. Rygiewicz, Carbohydrate use and assimilation by litter and soil fungi assessed by carbon isotopes and BIOLOG® assays, Soil Biol. Biochem. 35 (2003) 303–311.

[31] M.P. Singh, Application of Biolog FF MicroPlate for substrate utilization and metabolite profiling of closely related fungi, J. Microbiol. Meth. 77 (2009) 102–108.

[32] X. Zhang, X. Zhao, M. Zhang, Functional diversity changes of microbial communities along a soil aquifer for reclaimed water recharge, FEMS Microbiol. Ecol. 80 (2012) 9–18.

[33] M. Liao, X.M. Xie, Effect of heavy metals on substrate utilization pattern biomass, and activity of microbial communities in a reclaimed mining wasteland of red soil area, Ecotoxicol. Environ. Safety 66 (2007) 217–223.

[34] P. Venkatasubbaiah, W.S. Chilton, Phytotoxins of *Botryosphaeria obtuse*, J. Nat. Prod. 53 (1990) 1628–1630.

[35] R. Capasso, A. Evidente, L. Schivo, G. Orru, M.A. Marcialis, G. Cristinzio, Antibacterial polyphenols from olive oil mill waste waters, J. Appl. Bacteriol. 79 (1995) 393–398.

[36] E. Tripoli, M. Giammanco, G. Tabacchi, D. Di Majo, S. Giammanco, M. La Guardia, The phenolic compounds of olive oil: structure, biological activity and beneficial effects on human health, Nut. Res. Rev. 18 (2005) 98–112.

[37] M. Brenes, E. Medina, C. Romero, A. De Castro, Antimicrobial activity of olive oil, Agro. Food Indus. Hi.Tech. 18 (2007) 6–8.

[38] E. Medina, A. De Castro, C. Romero, A. Brenes, Comparison of the Concentrations of Phenolic Compounds in Olive Oils and Other Plant Oils: Correlation with Antimicrobial Activity, J. Agric. Food Chem. 54 (2006) 4954–4961.

[39] A. Zampa, S. Silvi, M. Servili, G. Montedoro, C. Orpianesi, A. Crecí, In vitro modulatory effects of colonic microflora by olive iridoids, Microb. Ecol. Health Dis. 18 (2006) 147–153.

[40] E. Medina, A. de Castro, C. Romero, E. Ramírez, M. Brenes, Effect of antimicrobial compounds from olive products on microorganisms related to health, food and agriculture. Microbial pathogens and strategies for combating them, Science, Technology and Education (A. Míndez-Vilas, Ed.) (2013) 1087–1094.

[41] S. Paudel, T. Magrati, J.R. Lamichhane, Antimicrobial activity of wild olive crude extracts in vitro, Int. J. Pharma. Sci. Res. 2 (2011) 110–113.

[42] G. Bergsson, J. Arnfinnsson, O. Steingrimsson, H. Thomàr, *In vitro* killing of Candida albicans by fatty acids and monoglyc-erides, Antimicrob. Agents Chemother. 45 (2001) 3209–3212.

[43] J. Sanchez, J.L. Harwood, Biosynthesis of triacylglycerols and volatiles in olives, Eur. J. Lipid Sci. Technol. 104 (2002) 564–573.

[44] M. Daglia, Polyphenols as antimicrobial agents, Curr. Opin. Biotechnol. 23 (2012) 174–181.

[45] B. Juven, Y. Henis, B. Jacoby, Studies on the mechanism of the antimicrobial action of oleuropein, J. Appl. Bacteriol. 35 (1972) 559–567.

[46] T. Casacchia, A. Sofo, S. Zelasco, E. Perri, P. Toscano, In Situ Olive Mill Residual Co-Composting for Soil Organic Fertility Restoration and By-Product Sustainable Reuse, It. J. Agr. 7 (2012) 35–38.