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Chemical and biological properties of a sandy loam soil amended with olive mill waste, solid or liquid form, *in vitro*

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Abstract

Background: The profitable use of the large amounts of olive oil mill wastes produced in Greece, as source of soil organic matter, might be probably beneficial to soil microorganism activity.

Results: A high rate of organic matter biodegradation was observed in soil samples amended with the liquid form of olive mill wastewater, whereas the vice versa results were obtained with the solid form. When the soil was amended with a mixture of both olive mill waste forms, liquid (L) and solid (S), the organic matter of the solid waste (S) showed a well-improved biodegradation; the available forms of P, K, Zn, Mn, and Cu were increased, especially in treatments where the olive mill wastewater, liquid form, was threefold in comparison to the solid form. Moreover, the soil amended with the solid (S) form of olive mill waste reduced bacterial growth significantly, and both waste forms act negative impacts to soil-borne fungi belonging to the genus *Rhizopus*.

Conclusions: The results of this work demonstrated the high potential of olive mill waste, solid or liquid form, added to sandy loam soil in an incubation experiment *in vitro*. The better results for soil quality were obtained when a combining mixture of these materials was added in the ratio 1:3 (solid/liquid).

Keywords: Olive mill waste; Soil organic matter biodegradation; Soil microflora; Soil chemical properties

Introduction

The organic matter as a soil fraction is regulating the biological activity of soils, so a satisfactory content in organic material dominates the soil fertility (Economou et al. 1980; Chouliaras et al. 1998; Gougoulias et al. 2010).

Very important amounts of olive mill waste are produced in olive cultivation areas, ranging between 1.75×10^6 and 2.25×10^6 tons/year of olive mill wastewater for Greece (Kyriazopoulos 2005); then, the profitable use of these organic materials as soil amendments are beneficial both to soil improvement and environmental protection.

Numerous methods are used for the treatments of olive mill waste; then, the respective products added to the soil act various effects on soil properties and plant growth (Ouzounidou et al. 2010). The waste could be applicable as composted material, while the raw waste could have beneficial effects concerning time and cost (Lopez-Pineiro et al. 2010).

The beneficial effects of these amendments are related to soil organic matter increase (Ordonez-Fernadez et al. Ordonez-Fernadez and Carbonnell-Bojollo 2010) then, consequently, to soil chemical and physical property improvement (Nikolaos et al. 2011). According to Lopez-Pineiro et al. (2011), successive applications of the de-oiled two-phase olive mill waste on soil as amendment may be an effective management practice for controlling their ability to increase P availability. The two-phase olive mill waste application to olive grove soil increased organic carbon, total N, available P and K, and in general, increased olive production (López-Piñeiro et al. 2008).

The soil content in organic matter, as well as that added to soils, affects significantly the physical properties of soil; it is useful to quote that the collapse potentials of soils inundated by oil is always less than when inundated by

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water (Labib et al. 2009), and according to López-Piñeiro et al. (2008), the olive mill waste application to olive grove soil increased soil aggregation stability.

The effect of this olive mill waste material on soil microbial activity was also assessed (Kotsou Maria et al. 2004; Hammed et al. 2008; Saadi et al. 2007). Moreover, the antimicrobial effect, probably due to polyphenolic compounds, occurring in olive mill wastewater against fungi was demonstrated by Vagelas et al. (2009a, b). Furthermore, according to Abid et al. (2007), water thermophilic bacteria as well as actinomycetes dominated over eumycetes during composting of the olive mill waste.

Soil amendment using compost derived from olive mill by-products can be an important agricultural practice for supporting and stimulating soil microorganisms and, at the same time, for re-using these by-products, so avoiding their negative environmental impact (Casacchia et al. 2012); furthermore, according to Barjea et al. (2013), liquid and solid olive oil mill waste treatment by composting in a mixture with the organic part of municipal solid waste and rock phosphate opens the way to agricultural spreading. The aim of this work was to examine the effects of biological and chemical properties on soil caused by the rate and the nature of the solid waste or olive mill wastewater *in vitro*.

Methods

The study was conducted in two incubation experiments. During the first experiment, the waste was tested either in liquid or solid form, added separately to the soil. During the second experiment, adequate mixtures of both forms were tested.

Incubation

In the incubator, the treatments were prepared in triplicates and kept at 28°C for a period of 15 weeks, for each experiment. During the first 3 weeks for each incubation period, the moisture was maintained at two thirds of field capacity, but for the next 3 weeks, the soils were left to dry. This process was repeated until the end of the incubation period. According to Wu and Brookes (2005), the alternation of drying and rewetting soil samples enhances mineralization of both soil biomass organic matter and non-biomass organic matter.

First experiment - separate application of each waste form to soil

In this study, 13.51, 27.03, and 40.54 g of solid waste material containing 2.5, 5.0, and 7.5 g of organic matter were added to 50 g of air-dried, light-textured soil, respectively, which was poor in organic matter. All these materials - soil and waste - were obtained from the region of Larissa (Greece, Table 1). The amounts 41.7, 83.3, and 125.0 g of olive mill wastewater containing 2.5, 5.0, and 7.5 g of organic matter, respectively, were also added into 50 g of the same soil.

Water-soluble extracts during incubation

In the middle of the incubation period, all treatments of the first experiment were leached with distilled water (1:5 (soil/H_2O)), and all water extracts were collected to be analyzed in order to approach the ecological effect of rainfall in natural leaching conditions.

Table 1 Chemical properties of the soil, solid waste, and olive mill wastewater

	Material		
	Soil (Calcaric Fluvisol, 10 cm in depth)	Solid waste (wet basis)	Olive mill wastewater (wet basis)
Texture	Loamy sand	-	-
Cation exchange capacity (cmol kg^{-1})	19	102	-
pH	8.1	4.90	5.10
Organic matter (g kg^{-1})	5.0	185.0	60
$CaCO_3$ (g kg^{-1})	86	-	-
Electrical conductivity, extract (dS m^{-1})	0.5 (one part soil + five parts H_2O)	0.91 (one part waste + five parts H_2O)	1.84 (raw waste)
Total N (g kg^{-1})	0.4	0.82	0.55
Total P (mg kg^{-1})	272	483	308
Total K (mg kg^{-1})	207	770	3,900
Total Mn (mg kg^{-1})	-	14	68
Total Cu (mg kg^{-1})	-	122	13.7
Total Zn (mg kg^{-1})	-	21	6.2

Wet solid and aqueous olive waste produced through a three-phase decanter process from an olive mill located in Larissa was used in this work. The solid wastes or olive husk is a mixture of olive pulp and olive kernel, while the aqueous olive waste (olive mill wastewater) is the water added during preparation and oil extraction process (olive fruit washing, malaxation, and centrifugation). One three-phase centrifuge-type oil mill produces approximately 500 kg of wet solid waste, and 1,200 kg of olive mill waste water is derived per 1,000 kg of processed olives.

Second experiment - application of solid and liquid waste mixture to soil

This experiment was established based on the data of the first experiment; then, an adequate mixture of the two waste forms was chosen in order to be tested. In this study, 24.39 g of solid waste + 8.13 g of olive mill wastewater (3:1 (S/L)), 20.4 g of solid waste + 20.4 g of olive mill wastewater (1:1 (S/L)), and 13.7 g of solid waste + 41.1 g of olive mill wastewater (1:3 (S/L)) were added to 50 g of the same soil, respectively. Each mixture contained 50 g of organic matter.

Methods of analyses

At the end of the incubation period, soil samples were analyzed using the following methods which are referred by Page et al. (1982):

1. Organic carbon was analyzed by chemical oxidation with 1 mol L^{-1} $K_2Cr_2O_7$ and titration of the remaining reagent with 0.5 mol L^{-1} $FeSO_4$. Total humus compounds (humic + fulvic acids) were extracted at pH 12 with NaOH, and humic acids were precipitated at pH 2 with HCl, according to fractionation of soil organic matter protocol proposed by Chouliaras et al. (1975). The soil organic carbon contents were transformed into organic matter contents by multiplying by 1.724, which is an experimental factor, as reported by Hesse (1972).
2. Both ammonium and nitrate nitrogen were extracted with 0.5 mol L^{-1} $CaCl_2$ and estimated by distillation in the presence of MgO and Dewarda alloy, respectively.
3. Organic phosphorus was measured after mineralization by combustion of the soil sample and subtraction of the mineral phosphorus amounts, which had previously been estimated in the laboratory. The mineral amounts were extracted with 1 mol L^{-1} H_2SO_4, and all forms were measured by spectroscopy.
4. Available P forms (Olsen P) were extracted with 0.5 mol L^{-1} $NaHCO_3$ and measured by spectroscopy.
5. Exchangeable forms of potassium were extracted with 1 mol L^{-1} CH_3COONH_4 and measured by flame photometer.

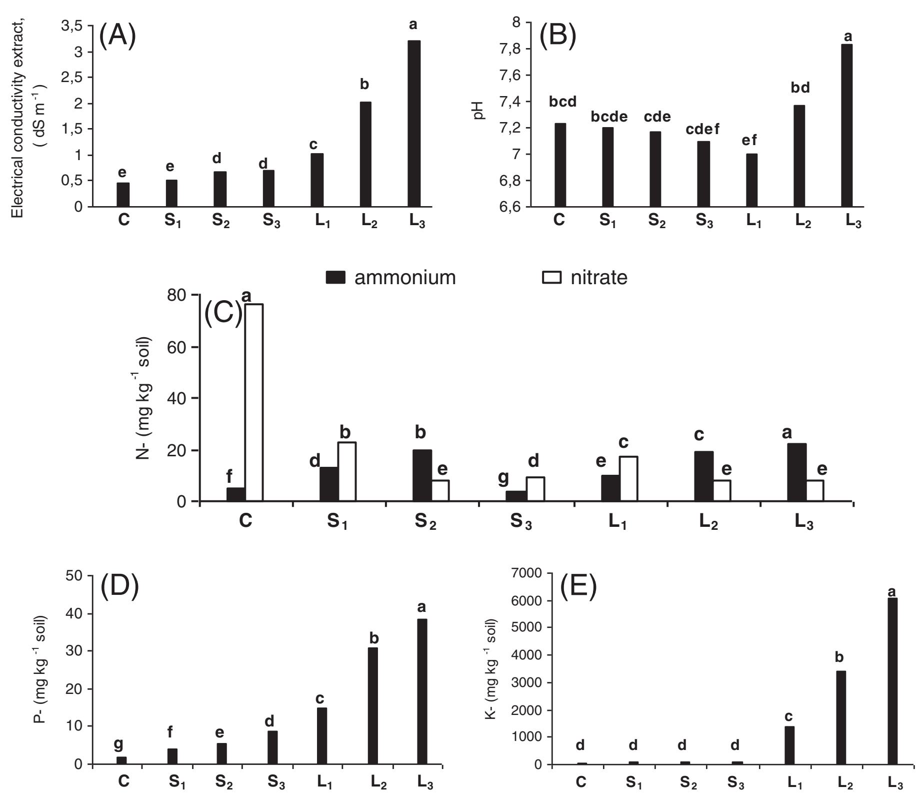

Figure 1 Chemical properties of water extracts. Taken during incubation time after adding solid waste or olive mill wastewater on soil samples. **(A)** Electrical conductivity, **(B)** soil pH, **(C)** ammonium and nitrate contents, **(D)** water-soluble phosphorus, and **(E)** water-soluble potassium. Bars in each characteristic of each graph with the same letter do not differ significantly according to Tukey's test (P = 0.05). C, control (soil); S_1, S_2, or S_3, solid waste 13.51, 27.03, or 40.54 g per 50 g of soil, respectively; L_1, L_2, or L_3, olive mill wastewater 41.7, 83.4, or 125.1 g per 50 g of soil, respectively.

6. Available forms of Mn, Zn, and Cu were extracted with DTPA (diethylene triamine pentaacetic acid 0.005 mol L^{-1} + $CaCl_2$ 0.01 mol L^{-1} + triethanolamine 0.1 mol L^{-1}) and measured by atomic absorption.

Soil microflora

To investigate the effect of added material on the soil microflora (bacterial and fungal communities), a small amount of soil was spread onto potato dextrose agar plates and incubated for 2 days at 25°C in darkness. After the incubation period, the number of bacterial colonies formed was counted. The same plates were further incubated as above for another 6 days. After the incubation period, the emerged fungi per plate and per treatment were recognized under the microscope.

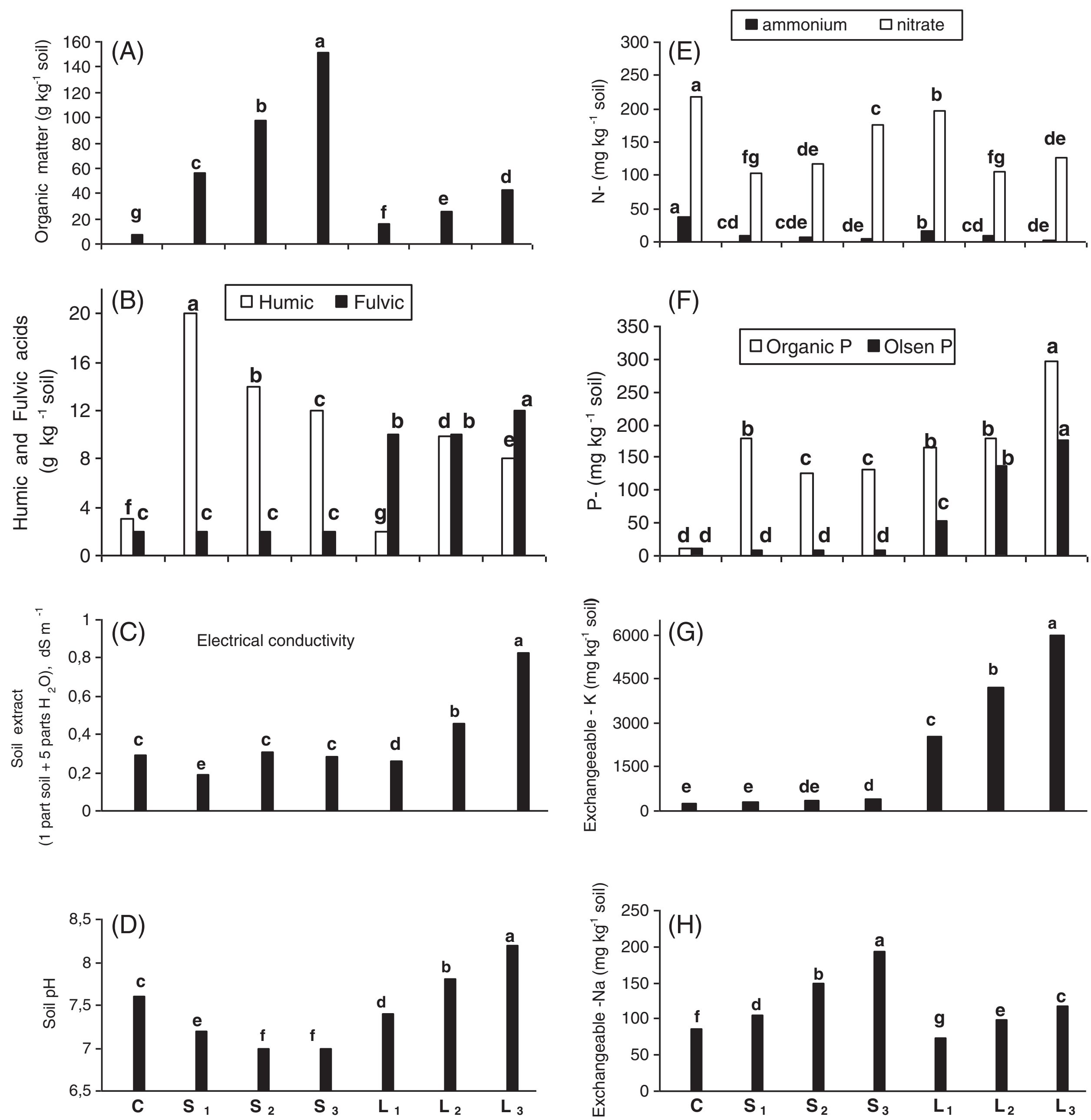

Figure 2 Effect of solid waste or olive mill wastewater at the end of the incubation time. (A) Soil organic matter; **(B)** soil humic and fulvic acids; **(C)** water extract electrical conductivity; **(D)** soil pH; **(E)** soil mineral forms of N, ammonium, and nitrate; **(F)** organic and available P; **(G)** available K; and **(H)** exchangeable Na.

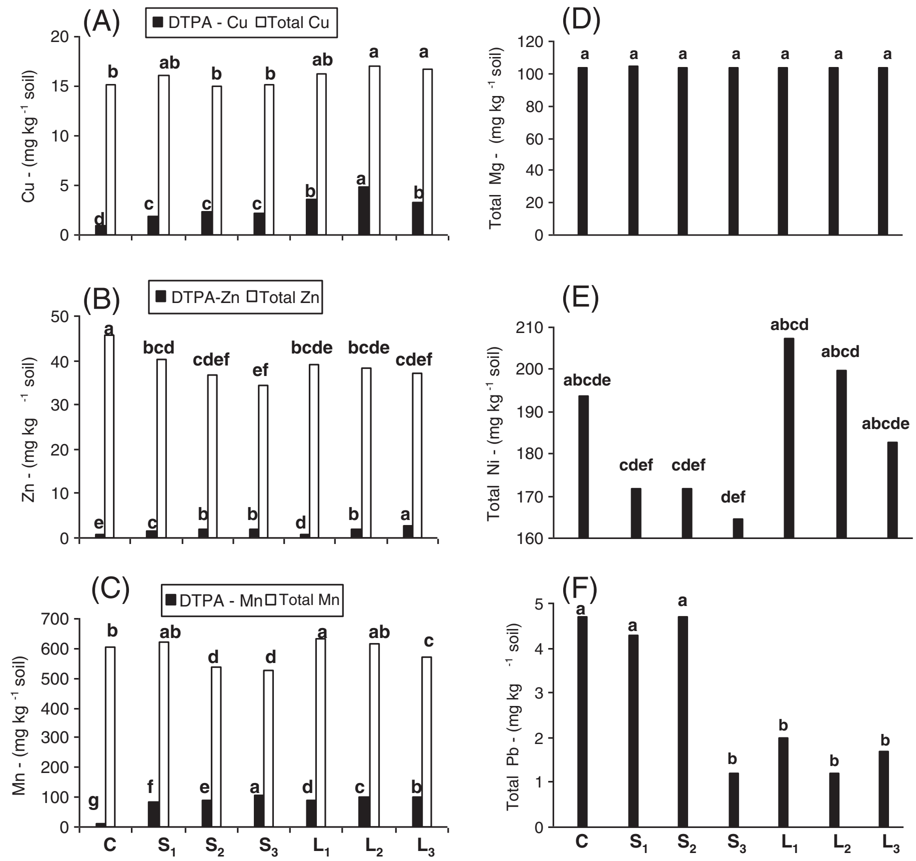

Figure 3 Effect of solid waste or olive mill wastewater added to soil on total and available forms. Cu **(A)**, Zn **(B)**, and Mn **(C)**, or of total Mg **(D)**, Ni **(E)**, and Pb **(F)**.

Statistical analyses

The experiment was repeated, and the completely randomized design with three replications was used. Tukey's procedures were used to detect and separate the mean treatment differences at $P = 0.05$. Statistical analyses were performed by the statistical program Ryan et al. (2005).

Results

First experiment - separate application of each waste form to soil

Water extracts

The studied water extracts, taken by leaching the soil treatments in the middle of the incubation period, showed a significant increase of soil salinity and alkalinity in all the

Table 2 Effect of olive oil mill waste on the number of bacterial colonies and fungi

	C	Treatments					
		Solid waste mill			Olive mill wastewater		
		S1	S2	S3	L1	L2	L3
Number of bacterial colonies	121 b	112 a,b	109 a,b	96 a,b	70 a	124 b	301 c
Microorganisms							
Fusarium species	+	–	–	+++	–	–	+++
Aspergillus species	+	–	+	–	–	++	–
Mucor species	++	+++	+++	–	–	–	–
Rhizopus species	+++	–	–	–	–	–	–
Acremonium species	–	+	–	–	–	–	+

Columns with the same letters (a, b, and c) do not differ significantly according to Tukey's test ($P = 0.005$). Fungal mycelia were graded from + (weak growth) to +++ (strong growth), and – (not detected). C, control (soil); S_1, S_2, or S_3, solid waste 13.51, 27.03, or 40.54 g per 50 g of soil, respectively; L_1, L_2, or L_3, olive mill wastewater 41.7, 83.4, or 125.0 g per 50 g of soil, respectively.

samples amended with olive mill wastewater; in these water extract treatments, a significant elevation in P and K water-soluble forms was also attested in comparison with the control (Figure 1).

End of incubation period

The organic contents of soil treated at the end of the incubation period proved a high percentage of organic matter biodegradation, about >60%, for all the samples amended with soluble waste. On the contrary, a strong resistance to biodegradation has been shown by samples treated with solid waste; a significant increase of salinity and alkalinity was also revealed for treatments with the two upper rates of liquid applications, and available forms of P and K were also found significantly increased in these treatments with olive mill wastewater (Figure 2).

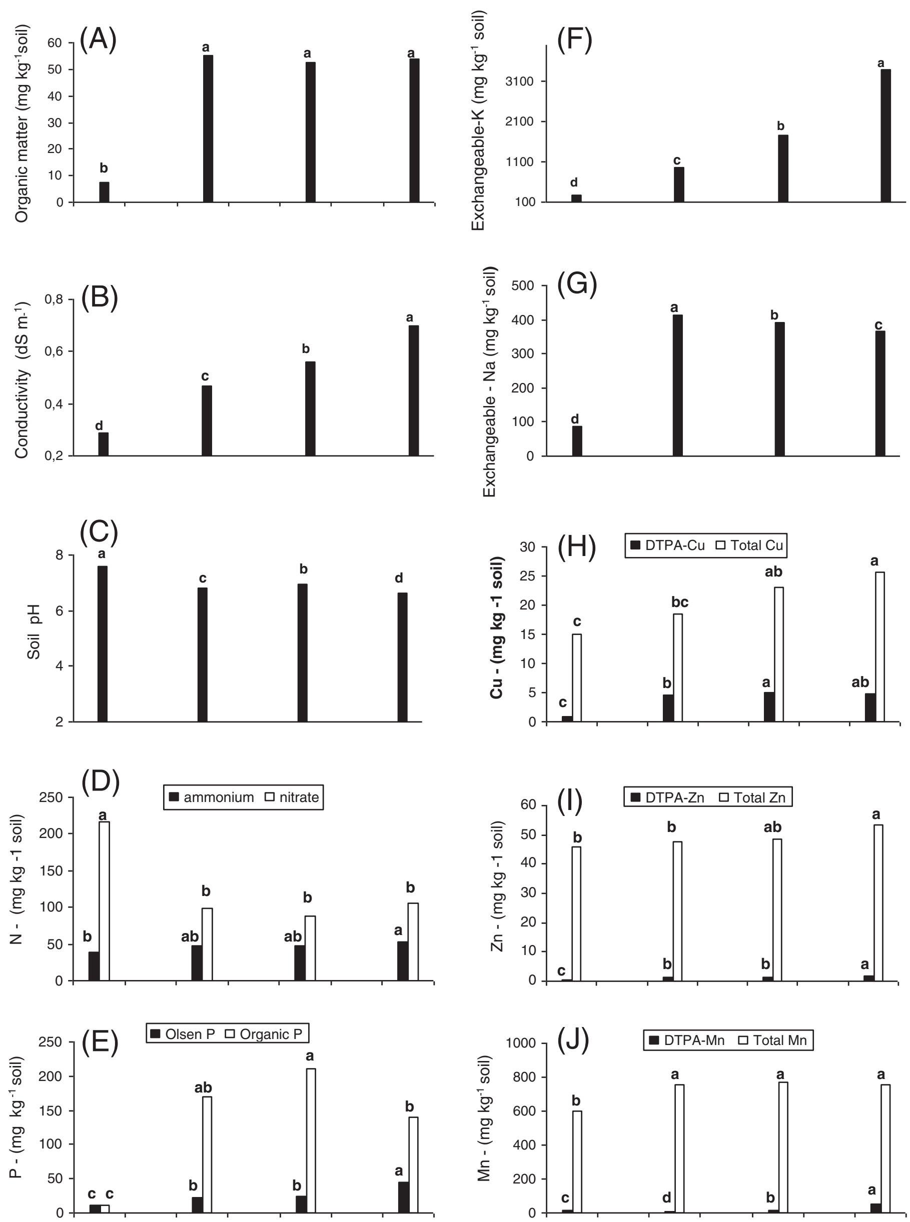

Figure 4 Effect of different mixtures of solid waste and olive mill wastewater added to soil. On organic matter contents **(A)**, on electrical conductivity of soil extract, 1:5 (soil/H_2O) **(B)**, on soil pH **(C)**, on nitrogen contents **(D)**, on P form contents **(E)**, on exchangeable K contents **(F)**, on exchangeable Na contents **(G)**, on Cu forms **(H)**, on Zn forms **(I)**, and on Mn forms **(J)**. C, control (soil); 24.39 g of solid waste + 8.13 g of olive mill wastewater (3:1 (S/L)), 20.4 g of solid waste + 20.4 g of olive mill wastewater (1:1 (S/L)), and 13.7 g of solid waste + 41.1 g of olive mill wastewater (1:3 (S/L)) were added correspondingly to 50 g of the same soil. Bars in each characteristic of each graph with the same letter do not differ significantly according to Tukey's test (P = 0.05).

The NO_3^- content of all treatments at the end of the incubation period is higher in comparison with NH_4^+, but that ($N\text{-}NO_3^-/N\text{-}NH_4^+$) ratio is lower in the case of water leaching extracts, taken earlier than the end of the incubation period; then, nitrification process is unachieved. A significant increase for P organic synthesis was also revealed for all treatments.

A significant elevation in available forms for Cu, Mn, and Zn in comparison to the control for all treatments was attested, and the increase of exchangeable Na did not cause any sodicity risk for soil (Figure 3).

The humic acid contents are always predominating in comparison with fulvic acid contents for treatments with solid waste, which is a very ligneous material (Figure 2). According to our results, soil bacterial numbers increased significantly by L3 (upper rate of olive mill wastewater, Table 2). On the contrary, S1, S2, and S3 treatments (solid waste) reduced total soil bacterial number as well as olive waste of both forms acted a significant negative effect to the genus *Rhizopus.*

Second experiment - application of solid and olive mill wastewater mixture to soil

The mixture of these two sorts of waste materials added to soil produced a well-decomposed organic mixture during incubation, concerning organic matter for both solid or olive mill wastewater; the organic matter of these mixtures is decomposed about >40%; then, the organic matter of solid waste is better decomposed under the effect of the olive mill wastewater, in comparison to the results of the first experiment where solid waste was separately added to soil. The salinity was further increased in the case of the mixture 1:3 (S/L), but soil pH was slightly affected in all treatments. At the end of the incubation period, relatively high amounts of $N\text{-}NH_4^+$ forms yet existed, but $N\text{-}NO_3^-$ forms predominate in comparison to $N\text{-}NH_4^+$ forms.

Organic P content increased significantly in all treatments, and available forms of P, K, and Mn were increased especially at the mixture 1:3 (S/L); also, the available forms of Cu and Zn increased for all treatments. The increase of exchangeable Na did not cause any sodicity risk for soil by these mixtures either (Figure 4).

Moreover, it seems that olive mill waste (both phases) acts a significant impact on soil-borne fungi belonging to the genus *Rhizopus* (Tables 2 and 3), but it might have allowed the growth of some fungi species of *Aspergillus* and *Fusarium* (Tables 2 and 3).

Discussion

When the two kinds of waste were added separately, a strong resistance to biodegradation has been proved by solid waste; the organic matter of solid waste is better decomposed under the effect of the olive mill wastewater, when the two waste sorts are added together. A probable explanation for that effect could be the higher content in fulvic acids of olive mill wastewater, and these more labile compounds are estimated as more biodegradable by soil microflora, (Jacquin and Chouliaras 1976). The salinity elevation is more affected by olive mill wastewater application, and it is important to remark the increased presence of available forms of P, K, Zn, Mn, and Cu in the soils amended with the mixture of solid + olive mill wastewater, in the ratio 1:3 (S/L).

Table 3 Effect of solid waste and olive mill wastewater on the number of bacterial colonies and fungi

	Control	Treatments		
		3:1 (S/L)	1:1 (S/L)	1:3 (S/L)
Number of bacterial colonies	121 c	50 a	74 b	38 a
Microorganisms				
Fusarium species	+	−	++	++
Aspergillus species	+	+++	−	−
Mucor species	++	+	++	++
Rhizopus species	+++	−	−	−

Columns with the same letters (a, b, and c) do not differ significantly according to Tukey's test ($P = 0.005$). Fungal mycelia were graded from + (weak growth) to +++ (strong growth), and − (not detected). C, control (soil), 24.39 g of solid waste + 8.13 g of olive mill wastewater (3:1 (S/L), 20.4 g of solid waste + 20.4 g of olive mill wastewater (1:1 (S/L)), and 13.7 g of solid waste + 41.1 g of olive mill wastewater (1:3 (S/L)) were added correspondingly to 50 g of the same soil.

Waste addition might be attributed to the increase of availability of heavy metals, such as Zn and Cu, which reduced bacterial growth. Toxicity of these heavy metals (Zn and Cu) to the organism (soil bacterial community) is well known (Frostegard et al. 1993; Giller et al. 1998; Novak et al. 2005). Moreover, this study showed that the soil amended with 1:3 (S/L) mixture increased the growth of fungi belonging to the subdivision *Deuteromicotina*, class *Deuteromycetes* (*Fusarium*) and the fungus belonging to the genus *Mucor* (class *Zygomycetes*); these soil fungi are resistant to toxic metals, such as Zn and Cu (Kao et al. 2006), and it seems that in the present study, these fungi species increased the exchangeable K in soil. According to the literature, microorganisms like fungi species *Aspergillus* and *Fusarium* are known for their resistance to heavy metals (Kapoor et al. 1999; Ahmad et al. 2005; Bishnoi and Garima 2005). Based on this, we can conclude that soil amended with 1:3 (S/L) olive mill waste mixture could be an alternative way to chemical control.

Conclusions

The organic matter of solid waste is better decomposed under the effect of the olive mill wastewater, when the two waste sorts are added together; when the soil is amended with the mixture of solid + olive mill wastewater on soil, in the ratio 1:3 (S/L), an important increase of available forms of P, K, Zn, Mn, and Cu was remarked. As far as it concerns soil microflora, solid waste acted a limitation effect to total soil bacterial number as well as olive waste of both forms acted a significant negative effect to the genus *Rhizopus*.

Abbreviations

S: Solid material of olive mill waste; L: liquid material of olive mill waste.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NG, first author, principal contribution to the work. All authors read and approved the final manuscript.

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