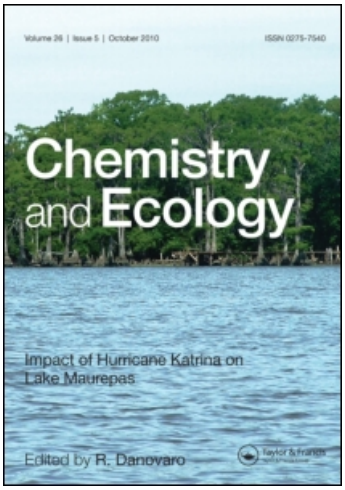

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Effects of amendment with olive mill by-products on soils revealed by nitrifying bacteria

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We investigated the effect of amending soil with olive mill by-products by examining soil characteristics and nitrifying and heterotrophic bacteria content, in comparison with non-amended soils. The effect of the amendment on organic carbon content was also evidenced. No differences were revealed in terms of heterotrophic bacteria, whereas the addition of olive mill by-products increased nitrifying bacteria content in soils with the addition of organic mixtures containing olive mill wastes. Two nitrifying bacterial strains were isolated from amended soils and given the names ISAFOM-B3 and ISAFOM-C2; 16S rDNA gene sequencing assigned them to the genus *Arthrobacter* and to the *α-Proteobacteria* subclass, respectively. A higher nitrate content was revealed in enrichment cultures prepared with amended soils when compared to non-amended ones. Nitrifying bacteria were imaged by fluorescent *in situ* hybridisation. A high total organic carbon content was detected in the amended soils, with an improvement of the humification indexes. This study suggests a positive effect of the addition of olive mill by-products on soils.

Keywords: nitrifying bacteria; 16S rDNA gene; olive mill waste; fluorescent *in situ* hybridisation; humification index

1. Introduction

A pivotal role for environment and soil conditions is carried out by nitrification, the oxidation of reduced forms of nitrogen, which is an essential process in the nitrogen cycle [1]. In its most common form, nitrification involves the oxidation of ammonia to nitrate, via nitrite, by two groups of autotrophic bacteria: ammonia oxidisers and nitrite oxidisers. Chemolithoautotrophic ammonia-oxidising bacteria are responsible for the rate-limiting step of nitrification in a wide variety of environments, making them important in the global cycling of nitrogen. Metabolic diversity within autotrophic nitrifiers is high. Ammonia oxidisers can hydrolyse urea and oxidise carbon monoxide, methane, and a range of recalcitrant organic compounds, while many nitrite oxidisers grow mixotrophically or heterotrophically on organic substrates. Both groups are also

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able to reduce oxidised forms of inorganic nitrogen, leading to the production of NO, N_2O and N_2, particularly at low O_2 concentrations. The traditional, simplistic view of nitrification is further complicated by the existence of heterotrophic nitrification, which is the production of nitrate directly from organic N substrates [2]. In addition, anaerobic nitrification has been demonstrated in wastewater treatment systems [3]. Nitrifying bacteria can also be used to improve anthropogenic damage to the environment by reducing the ammonia content of wastewater in sewage treatment before discharge into aquatic environments [4]. Different aspects of nitrifier ecology have been treated extensively [5].

It is well known that soil amendment changes local microbial activities, depending on the added materials [6,7]. Olive mills produce large amounts of waste (wastewaters and virgin pomace), usually spread on the soil [8]. As olive oil consumption increases worldwide, the olive oil industry is constantly growing, and olive oil producing countries are facing severe environmental contamination problems caused by the lack of feasible or economical solutions to olive mill wastes (OMWs).

Bioremediation and biovalorisation of olive-mill waste practices have been developed [9]. Composted olive mill waste can find application as amendment in agriculture because of its high nitrogen and phosphorous content, although its use must be carefully controlled [10].

The Institute for Mediterranean Agricultural and Forest Systems National Research Council (ISAFoM-CNR) has recently developed a technology that makes it easier to overcome difficulties in recycling raw olive mill effluents for agronomic purposes, producing olive mill waste mixtures (OMWMs) [11]. The organic carbon in OMWMs has a higher degree of humification and lower bio-phenol content [12,13] than raw olive-mill effluents, reducing phytotoxicity and the risk of dissolved organic matter percolation [8].

Nitrifying bacteria were analysed to detect the effect of different kinds of soil amendments, as this bacterial metabolic group is assumed to be a sign of good soil conditions and to contribute to soil fertility [14].

In the present study, the effect of OMWMs spread on soils was investigated using nitrifying bacteria as bio-indicators of soil fertility condition. The wastes were provided from the technology proposed by ISAFoM-CNR.

2. Materials and methods

2.1. *OMWM production and agronomic trials*

The olive mill by-products used in the preparation of OMWMs came from a two-phase decanter olive mill belonging to the Cooperative Nuovo Cilento, located in San Mauro del Cilento (Salerno, Italy). Two mixtures were used, OMWM(b), and OMWM(c), both containing 72% of destoned olive mill residues and the following hygroscopic materials: wool wastes, straw, and sawdust, added at concentrations of 11%, 8.5%, and 8.5%, in the first mixture, and then 0%, 14%, and 14% in the second, respectively. The chemical characteristics of the OMWMs used in the trials are reported in Table 1. The agronomic trial was run in triplicate, in an olive tree cultivation area at the Casteldoglio farm in Perugia, Italy, using a randomised two block design (30 plants per plot). Soils were amended yearly in spring with $9\,t\,ha^{-1}$ of two different kinds of OMWMs during a five year trial (2001–2005). Control plots were fertilised with $100\,kg\,N\,ha^{-1}$ of urea.

2.2. *Soil sampling and characterisation*

Soil samples were axenically collected in triplicate from OMWM-amended and non-amended soils at a depth of 20 cm, at an equal distance from each olive tree, and were maintained in

Table 1. Main chemical characteristics of the olive mill waste mixtures used in the trials (data are the mean of three replicates, cv < 5%).

		OMWB(b)	OMWB(c)
Moisture	%	67.3	63.5
pH		5.66	5.41
Electrical conductivity	$dS(m)^{-1}$	1.56	1.51
TOC	%	42.1	45.2
TEC	%	25.7	20.5
HA+FA	%	17.4	13.1
DH		64.2	63.8
N	%	1.96	1.07
C/N		21.5	42.2

Note: TOC, total organic carbon; TEC, total extractable carbon; HA, humic acid; FA, fulvic acid; DH, degree of humification = (HA+FA)× 100/TEC; HI, humification index = [TEC–(HA+FA)]/(HA+FA); N, nitrogen; C, carbon.

sterile bags (Wirl-pak, Nasco) until their arrival to the laboratory, where they were processed immediately. The soil was a sandy-clay-loam with a pH value of 7.9, specific electrical conductivity of 0.37 dS m^{-1}, total calcium carbonate of 17.4% (d.w.), total organic carbon (TOC) 1.6% (d.w.), and a cation exchange capacity of 15.4 cmol(+) kg^{-1}. These characteristics were determined on a representative soil sample collected in 2001 before the first OMWM soil application. During the agronomic trial, further soil samples (0–20 cm depth) were collected yearly in spring (three sub-samples per plot), before spreading the OMWMs for TOC analyses. TOC was determined by the Springer–Klee method [15]. Total extractable carbon (TEC), humic acids (HA) and fulvic acids (FA), extracted from air-dried samples with 0.1 M NaOH and $Na_4P_2O_7$, were determined as reported by Ciavatta et al. [16]. All other soil chemical parameters reported in this paper were determined according to standard procedures [17].

2.3. *Heterotrophic bacteria in amended and non-amended soils*

Three aliquots of amended and non-amended soils, collected in aseptic conditions, were used for heterotrophic microorganism enumeration. Experiments were carried out both in complex medium Tryptone Soya Agar (TSA) and in Sabouraud medium specific for growth of yeasts and moulds (Oxoid, Milan, Italy). Serial dilutions were prepared in 0.85% NaCl solution and aliquots of 100 μl of the different dilutions spread on the surface of Petri dishes with the two media alternatively, and the Petri dishes were incubated at 28 °C for 48 h, according to standard procedures [1]. Evaluation of microbial populations were carried out according to Student–Newmann–Keuls test for $p = 0.05$.

2.4. *Nitrifying bacteria in amended and non-amended soils*

Quantification of nitrifying bacteria were determined in synthetic nutrient medium (SNM) [18] containing: 3.6 mM NH_4Cl (0.192 g l^{-1}), 17.8 mM $NaHCO_3$ (1.49 g l^{-1}), 0.4 mM K_2HPO_4 (0.069 g l^{-1}) 0.41 mM $MgSO_4$ $7H_2O$ (0.100 g l^{-1}), 1.25 mM NaCl (0.074 g l^{-1}), at pH 7.8 ± 0.2, in double distilled water with no detectable dissolved organic carbon. Aliquots of serial dilutions were spread on the surface of SNM Petri dishes and incubated at 28 °C for at least two weeks. Evaluation of microbial populations were carried out according to according to Student–Newmann–Keuls test for $p = 0.05$.

2.5. *Enrichment and isolation of nitrifying bacteria*

Enrichment cultures were carried out in SNM; various 250 ml flasks were filled with 50 ml of SNM and inoculated with 0.5 g of soil samples each. The inoculated flasks were incubated at 28 °C for four weeks and growth was detected by microscopic analyses throughout the incubation time. Then an aliquot of 100 μl from each culture was transferred and spread on solid SNM, and incubated at 28 °C for three-four weeks. After this period, colonies showing different shapes were selected as nitrifying strains. Colonies were streak purified at least three times, and isolated strains were stored in liquid culture containing 30% sterile glycerol (v/v) in liquid nitrogen.

2.6. *Nitrate content detection by ionic chromatography analyses*

Samples collected from nitrifying bacteria enrichment cultures were filtered through filters with 0.45 μm diameter pores and analysed immediately for nitrate (NO_3^-) content by ion chromatography (Dionex DX-120). An AS14 analytical column with an AG14 guard column was used for anion analysis; the eluent was a 3.5 mM Na_2CO_3 + 1.0 mM $NaHCO_3$ solution. The sample loop was of 1 ml, and the eluent flow rate was 1.2 ml min^{-1}. Determinations were carried out against standard solutions (prepared daily) using the method of standard additions. Data quality was checked by analysing blanks (MQ waters used for dilutions) and by simultaneous analysis of standard reference materials (SRM) with certified values. Replicate and duplicate samples were run daily and the relative standard deviation of the duplicates was always = 1%.

2.7. *Nitrifying bacterial strain characterisation and 16S rDNA gene analyses*

After the growth of isolates on solid medium, the colonies were observed under a stereomicroscope and Gram determination was carried out (Gram stain kit, Carlo Erba). For the 16S rDNA sequencing of isolated bacterial strains, a single colony was suspended in 50 μl bi-distillated water and treated for 5 min at 100 °C. Amplification of the 16S rRNA gene was performed using 10 ng of genomic DNA in 20 μl of 1X 'Amplitaq' buffer (10 mM Tris-HCl; 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% gelatin) with 150 ng each of the primers 27f (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1495r (5′-CTACGGCTACCTTGTTACGA-3′), 250 μM each dNTPs and I U of 'Amplitaq' (Perkin-Elmer). The reaction mixtures were incubated at 95 °C for 1 min and 30 s and then cycled 35 times through the following temperature profile: 95 °C for 30 s, annealing temperature (Ta) for 30 s and 72 °C for 4 min. Ta was 60 °C for the first five cycles, 55 °C for the next five cycles and 50 °C for the last 25 cycles. Finally, the mixtures were incubated at 72 °C for 10 min and at 60°C for 10 min; 2 μl of each amplification mixture was analysed by agarose gel (1.2% w/v) electrophoresis in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) containing 0.5 μg ml^{-1} (w/v) ethidium bromide. Sequencing was carried out at the Bact 16S biomolecular research service (BMR) (CRIBI Biotechnology Centre, University of Padua, Italy). Consensus sequences of the isolates were compared with those deposited in GenBank using the BLAST program [19].

2.8. *Sequence accession numbers*

The 16S rDNA nucleotide sequences obtained in this study were deposited in the GenBank database with GenBank accession numbers DQ867308 and DQ867309 for isolated strains ISAFOM-B3, and ISAFOM-C2 respectively.

Table 2. Probes for nitrifying bacteria detection used in this study.

Probe	*E. coli* 16S rRNA position	Probe sequence	Specificity
Nb 1000	1000–1012	5′-TGCGACCGGTCATGG-3′	*Nitrobacter* spp.
NIT3	1035–1048	5′-CCTGTGCTCCATGCTCCG-3′	*Nitrobacter* spp.
Nso190	190–208	5′-CGATCCCCTGCTTTTCTCC-3′	Ammonia-oxidising bacteria
Nso1225	1225–1244	5′-CGCGATTGTATTACGTGTGA-3′	Ammonia-oxidising bacteria

2.9. *DAPI staining and observation*

Aliquots (100 μl) of samples were fixed in 4% paraformaldehyde and stained with 10 μl of 4',6-diphenylindole (DAPI, Sigma, Milan) solution, at 10 μg ml^{-1}, and incubated in the dark at room temperature for 5 min, then 5.0 μl were added to a slide and observed under fluorescent light using a Diaplan microscope (Leitz) with a 100× objective.

2.10. *Fluorescent* in situ *hybridisation (FISH) experiments*

Each sample was fixed in paraformaldehyde 4% and then maintained at −20 °C in 50% ethanol. Three μl of fixed cell suspension were then immobilised on a pre-cleaned glass slide with black rims, air-dried, and dehydrated with three different ethanol solutions (50%, 80%, and 98%) [14]. Each well was then filled with 8 μl of hybridisation buffer containing 360 μl of 5 M NaCl, 40 μl of 1 M Tris-HCl, 2 μl of 10% SDS, and formamide added at a concentration of 20% and 35% for NIT3 and Nso190 probes respectively, and brought up to 2 ml with demineralised water. All probe sequences are listed in Table 2. Probes (1μl of a solution 25 ng μl^{-1}) were added to each well, and slides incubated for 2 h at 46 °C. Slides were then washed with a buffer containing 2.15 ml of 5 M NaCl, 1 ml of 1 M Tris-HCl, 50 μl of 10% SDS, filled with demineralised water to a final volume of 50 ml, at 48°C, and maintained at this temperature for 15 min. The slides were then washed with demineralised water to stop any reaction, dried under air flux, and observed under a microscope, adding citifluor (agar) to enhance the fluorescence [20]. Cells hybridised with a molecular probe conjugated with FITC or with TRITC were observed under the appropriate fluorescent light, using a Diaplan (Leitz) microscope with a 100× objective. Pictures were taken with a Photometrics camera (Snap c.f.).

3. Results

3.1. *Soil characterisation*

Soils at the Casteldoglio farm, amended with OMWMs over a period of five years, from 2001 to 2005, showed modification of organic carbon parameters with respect to the control soils, where urea was added (Table 3). In particular, percentages of TOC in amended soils showed an increase compared to the control, by adding both OMWM(b) and OMWM(c), with a lower effect when the former mixture was added. A similar result was also observed in the same samples with regard to the percentage of TEC and HA+FA. Considering humification indexes, an increase in the degree of humification (DH) and a reduction of the humification index (HI) were recorded in amended soils as a signal of an improvement in soil quality.

Table 3. Carbon content and humification parameters in soil amended with OMWMs for five years. Data are referred to the mean value determined over the 2001–2005 period.

		OMWB(b) soil	OMWB(c) soil	Control soil
TOC	%	1.87 b	1.73 b	1.40 a
TEC	%	1.07 b	0.93 b	0.78 a
HA+FA	%	0.69 b	0.61 b	0.46 a
DH	%	64.5	65.6	59.0
HI		0.36	0.34	0.41
TEC/TOC	%	57.2	53.8	55.7

Note: Data flagged by the same letter are not significantly different according to Student–Newmann–Keuls test for $p = 0.05$. TOC, total organic carbon; TEC, total extractable carbon; HA, humic acid; FA, fulvic acid; DH, degree of humification = (HA+FA) × 100/TEC; HI, humification index = [TEC – (HA+FA)]/(HA+FA).

3.2. *Microbiological studies*

Growth of heterotrophic bacteria from amended and non-amended soils was carried out in order to detect the effects of olive mill by-products on both prokaryotic and eukaryotic soil microorganisms, at least with regard to the cultivable portion. Both amended and non-amended soils showed the presence of heterotrophic bacteria with values ranging from 10^5 to 10^7 colony forming units (CFU) g^{-1} fresh weight (f.w.) (Figure 1(A) & (B)). Higher values of heterotrophic bacteria, corresponding to 3.0×10^7 and 1.6×10^7 CFUg^{-1} (f.w.), were detected in the OMWM(b) amended soils and in the non-amended ones, respectively (Figure 1(A)). These

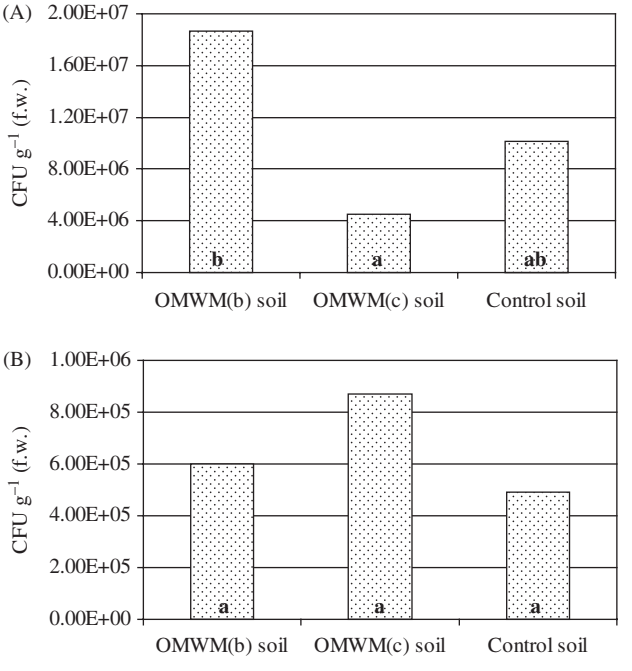

Figure 1. Heterotrophic microorganisms quantification, expressed as CFU g^{-1} (f.w.) in amended (OMWM(b) and OMWM(c)) and non-amended (control) soils investigated in TSA medium adapted for growth of heterotrophic bacteria (A); and in Sabouraud medium specific for growth of yeasts and moulds (B). Cultures were incubated at 28 °C for 48 h (columns marked with the same letter are not significantly different according to Student–Newmann–Keuls test for $p = 0.05$).

results suggest a low effect of the olive mill by-product amendment with respect to heterotrophic bacteria.

Concerning growth of yeasts and moulds on specific medium in the amended areas OMWM(b) and OMWM(c), values of 9.0×10^5 CFUg^{-1} (f.w.), and 1.2×10^6, CFUg^{-1} (f.w.), respectively, and values of 8.5×10^5 CFUg^{-1} (f.w.), in the control of non-amended soil, were detected, with low differences seen in the soils treated in the two different ways (Figure 1(B)).

The presence of nitrifying bacteria was investigated in olive oil mill waste amended and non-amended soils as one of the fundamental bioindicators of soil fertility. Higher levels of nitrifying bacteria were detected in the amended soils, where values of 10^5 CFU g^{-1} (f.w.) were reached compared to values of 10^3 and 10^4 CFU g^{-1} (f.w.) registered in the non-amended ones. Higher values were found in the amended soils (by adding OMWM(b) and OMWM(c)), with concentrations of 5.4×10^5 and 2.3×10^5 CFUg^{-1} (f.w.), respectively (Figure 2). Although only slight differences were noted between the two, a positive effect on soils in terms of an increase in nitrifying bacteria was suggested.

Enrichment cultures aimed at isolating nitrifying bacteria were carried out in synthetic nutrient medium and in the presence of NH_4Cl. Low turbidity was detected in the different enrichment cultures after 3–4 weeks of incubation both in amended and non-amended soils. Chemical analyses by ion chromatography showed a higher nitrate concentration in the amended soils, and in particular in the OMWM(b) amended ones (Figure 3). Nitrate content in these samples was the result of the microbial activity in transforming ammonia of the culture medium. This activity was carried out by bacteria present in the original soils, amended and non-amended, used as inocula for enrichment. Nitrate contents in the different samples were an indirect measure of the presence

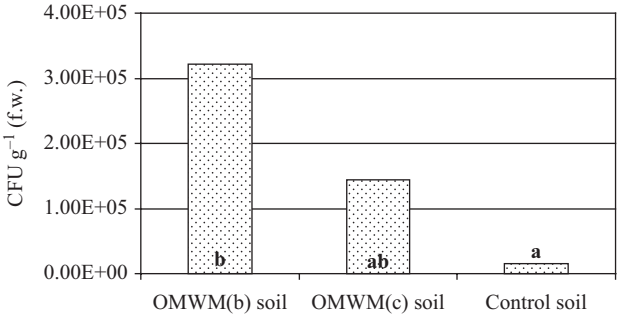

Figure 2. Nitrifying bacteria quantification, expressed as CFU g^{-1} (f.w.) in amended (OMWM(b) and OMWM(c)) and non-amended (control) soils investigated in SNM medium in the presence of NH_4Cl. Cultures were incubated at 28 °C for 3 weeks (columns marked by the same letter are not significantly different according to Student–Newmann–Keuls test for $p = 0.05$).

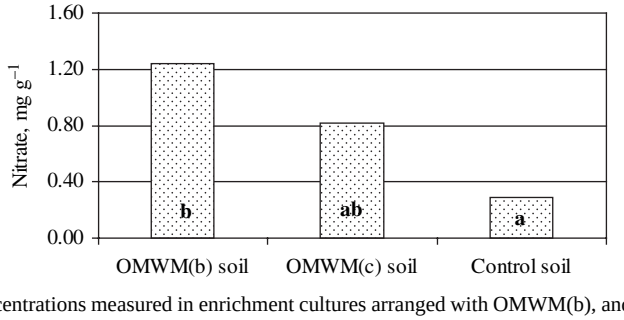

Figure 3. Nitrate concentrations measured in enrichment cultures arranged with OMWM(b), and OMWM(c) soil samples in comparison to control soil; data are referred on dry matter base (columns marked by the same letter are not significantly different according to Student–Newmann–Keuls test for $p = 0.05$).

of nitrifying bacteria, producing low biomass and transforming high quantities of substrate to obtain energy.

Nitrifying bacteria were isolated from the same enrichment cultures after 3 weeks of incubation on solid medium, which developed small colonies on its surface. Two strains were isolated from enrichment cultures OMWB(b) and OMWB(c), and were named ISAFOM-B3 and ISAFOM-C2 respectively. The former were round colonies, 0.5 mm in diameter, moist, with regular margins, cream coloured, and resulted positive to Gram-staining. The latter were also round colonies, 0.3 mm in diameter, smooth, mucoid, cream coloured, Gram-negative. Isolated strains were then identified by 16S rDNA gene sequencing, and BLAST analyses assigned them to the genus *Arthrobacter* and to the subclass *α-Proteobacteria* respectively. The closest genus of strain ISAFOM-B3 resulted *Arthrobacter* sp. strain CU19 (DQ643143), with a sequence similarity of 99%. Isolated strain ISAFOM-C2 showed sequence similarity of 100% with the α-proteobacterium strain A7-2 (AY017049).

3.3. *Microscopic study*

Microscopic analyses were carried out using the DAPI probe to detect all the microorganisms present in the specimens, in all the different shapes, and the same images were also observed after treatment with molecular probes specific for different groups included in the nitrifying bacteria, selecting the subject of analyses. FISH analyses gave positive signals with probes NIT3 and Nb1000 for *Nitrobacter* spp. and with probe Nso190 for ammonia-oxidising bacterial strains. No signals were detected with probe Nso1225. Results of the DAPI staining in an enrichment culture obtained with OMWB(b) amended soil used as inoculum showed different cell shapes (rod-and coccoid-shaped), since this probe locates all microbial communities. A selection for nitrifying bacteria was evidenced in the same image treated with the NIT3 probe conjugated with FITC, where rod-shaped cells were no longer evident but round cells instead appeared. An enrichment culture from OMWB(c) amended soil sample stained with DAPI and Nso190 probes respectively, showing an aggregate of rod-shaped bacterial cells, pointed out also in the presence of the probe specific for bacteria *Nitrobacter* sp. A signal in amended soils stained with Nb1000 probe conjugated with TRITC was also detected.

4. Discussion

Soil organic carbon parameters analysed in this study showed an improvement in soil fertility due to OMWM amendments. This result agrees with the findings of Alburquerque et al. [6], which detected an overall positive effect and an improvement of soil conditions by adding olive mill by-product compost.

The nitrate content detected in enrichment cultures was relatively high, resulting from growth and metabolism of nitrifying bacteria. The main source of nitrogen was probably constituted by wool wastes, rich in nitrogen, present in the OMWMs as one of the hygroscopic materials. Nitrate detection could represent a way of measuring the activity of nitrifying bacteria producing low biomass [1].

The effect of the OMWM amendment on soils was not evident with respect to heterotrophic microorganisms, including bacteria yeasts and moulds, although in the case of the eukaryotic microorganisms pathways of growth in the amended soils seemed better than the non- amended ones. Nitrifying bacteria cultures led to the isolation of two bacterial strains, one belonging to the genus *Arthrobacter,* including ammonia-oxidising bacteria able to use organic carbon sources [21]; and another to the subclass *α-Proteobacteria,* where ammonia-oxidising bacterial strains

were also found [22]. Culture media may reveal bacterial strains that do not necessarily represent the most abundant population present in native samples [23–26]. Furthermore, as with other microorganisms, portions of viable cells in an environmental sample that is actually amenable to laboratory culture conditions may be quite small [25,27]. Nitrogen sources and a high carbon content in the amended soils used as inocula for enrichment cultures could have favoured ammonia-oxidising bacteria able to use organic carbon sources as well.

The high nitrogen content in soils, probably induced by OMWM amendment, is related to the high presence of nitrifying bacteria. Mixture OMWM(b) showed higher nitrogen content and higher amounts of nitrifying bacteria. OMWM amendment therefore had a positive effect on soils at the Casteldoglio farm analysed in this study, from both the chemical and microbiological points of view. Other bio-indicators were used to detect the effects of the addition to soils of olive mill by-product compost, such as enhanced plant growth, in particular of *Lolium perenne* L. [6]. The germination index was used as an indicator of the good quality of soils after the addition of a compost of olive oil mill wastewater containing other agroindustrial and urban wastes as well [28]. Olive mill compost addition to soil yielded greater potato plant growth [29]. The benefit of olive mill wastewater and agricultural wastes compost demonstrated the potential sustainable agronomic production of potatoes [30]. Compost originating from sludge also issued from the OMW evaporation ponds with poultry manure [31]. Composting of olive mill wastes plus straw is another alternative, where a substantial decrease in both the organic matter and C/N ratio and an increase in the nitrogen content were observed [32,33]. Composting of solid olive mill by-product added to soils showed good potential for cultivating peppers under commercial conditions [34]. A study conducted on soils amended with OMWMs, where monitoring of microbial communities was chosen as a parameter of soil conditions, showed an improvement in microbial activity [35]. Italian agricultural plots were tested for the presence of nitrifying bacteria [36,37], and in this context both *Nitrosospira*- and *Nitrosomonas*-like sequences were found in plots fertilised with swine manure, while only *Nitrosospira*-like sequences were found in non-fertilised plots. These results seem to support the more traditional idea that high-ammonia conditions stimulate nitrosomonad populations [38]. In this study nitrifying bacteria were tentatively used as an index of soil conditions after olive oil mill by-product amendment.

Microscopic analyses pointed out the presence of ammonia- and nitrite-oxidising bacteria, both in the enrichment cultures and in the original soils. This confirms the presence of nitrifying bacteria in the OMWM amended soils, suggesting their good conditions to be the result of OMWM amendment. *Nitrobacter* spp. are also globular, and similar structures were stained with probe NIT3. Different reports showed the efficiency of FISH techniques in detecting nitrifying bacteria in various environmental specimens [39,40]. Ammonia and nitrite oxidising bacteria were found by microscopic analyses in OMWM amended soils, although some interference of FISH probes with soils was detected where the images were less clear. Ammonia-oxidising strains were isolated by culturing. These bacterial strains are most likely quick-growing and well adapted to soil characteristics, with high carbon content, although other bacteria are present and active in the same soils. A combination of cultural and microscopic approaches, the latter supported by molecular probes, could give useful insights for studies on olive oil mill by-product amendment to soils.

5. Conclusion

This study suggests that the addition of olive oil mill by-products to soils increases the relative nitrogen content and that of nitrifying bacteria. The latter were considered as bioindicators of the fertility of soils. Use of olive mill by-products as an amendment to soils is suggested as a positive process for agronomic purposes and for valorisation of these substrates.

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