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Co-composting of cotton residues with olive mill wastewater: process monitoring and evaluation of the diversity of culturable microbial populations

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Abstract With the aim to recommend an integrated alternative for the combined treatment of olive mill wastewater (OMW) and cotton residues (CR), and the production of high value and environmentally friendly products, two compost piles were set up. The first pile (control, pile 1) consisted of ginned CR, whereas the second (pile 2) was made of CR with the addition of OMW. A series of physicochemical parameters and the culturable microbial diversity in both piles were assessed. Co-composting (pile 2) displayed higher temperatures during the whole process, a prolonged second thermophilic phase and temperature values higher than 40 °C even after the thermophilic stage. Comparing the physicochemical parameters of the pile 2 with those of the pile 1, it was deduced that pH in the former was more acidic during the onset of the process; the EC values were higher throughout the process, while the levels of ammonium and nitrate nitrogen, as well as the NH_4^+/NO_3^- ratios, were lower at most of the sampling dates. By evaluating the abovementioned results, it was estimated that the co-composting process headed sooner toward stability and maturity, Isolated microorganisms from both piles were identified as members of the genera *Brevibacillus*, *Serratia*, *Klebsiella*, and *Aspergillus*, whereas active thermotolerant diazotrophs were detected in both piles at the 2nd thermophilic phase emerging a promising prospect upon further evaluation for enhancing the end-product quality. Our findings indicate that co-composting is an interesting approach for the exploitation of large quantities of agro-industrial residues with a final product suitable for improving soil fertility and health.

Keywords Co-composting parameters · Microbial community dynamics · Phylogenetic analysis · Respiration rates

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Introduction

Current estimates for world olive oil production are about 3,232,000 metric tons, i.e., 2,233,000 metric tons in EU countries and 999,000 metric tons in non-EU countries (European Commission, 2020). However, the extraction of olive oil generates huge quantities of olive mill wastewater (OMW; an aqueous

byproduct of the three-phase decanters) within practically a 3-month winter period. A typical quantity of OMW generated from the olive oil extraction process is 1.2–1.8 m^3 per ton of olives; more than 30 mil m^3 of OMW is produced annually in the Mediterranean basin (Haddad et al., 2017). OMW is a dark and foul-smelling waste, characterized by acidic pH, high electrical conductivity (EC), high C/N ratio, resistance to degradation (mainly due to its significant content of phenolic compounds), and antimicrobial and phytotoxic properties (Paredes et al., 2005). OMW has high potassium content and notable levels of nitrogen, phosphorus, calcium, magnesium, and iron, which constitute key factors for soil fertility (Kavvadias et al., 2011). Unfortunately, the huge quantities of OMW produced annually around the world are mainly disposed of into the environment without treatment, leading to water, air, and land pollution. OMW's discharge in soils has direct detrimental effects not only on plant growth and soil microorganisms' metabolism but also on the physicochemical properties of soil (Ahmed et al., 2019). Co-composting of OMW with other agro-industrial wastes has been suggested to overcome the phytotoxic effects of OMW's direct spreading on soil and at the same time to return nutrients that have been consumed during cultivation back to the croplands (Chowdhury et al., 2013).

Solid agro-industrial wastes, such as ginned cotton residues (CR), constitute an organic material of homogeneous composition and high cellulosic levels, and thus can represent a good substrate for co-composting with OMW (Tejada & Gonzalez, 2003). CR is a byproduct of cotton-ginning processing, composed of bur and stem fragments, immature cottonseed, lint, leaf fragments, and dirt. A huge quantity (3.23 mil tons per year) of CR is produced worldwide, and its disposal is particularly challenging (Sahu & Pramanik, 2015). Several researchers have explored ways to exploit CR, including their use as livestock feed, gardening compost, and raw material for developing products, such as energy pellets, ethanol, or biochar (Hamawand et al., 2016). However, most of the CR is discarded back onto the fields, at the expense of ginning.

Compost is a stabilized and sanitized product that performs a significant role in the soil enrichment process by restoring essential elements assimilated during cultivation. It also helps by minimizing the migration of contaminants into the soil environment through its absorbent action (Chen et al., 2015). The succession of various microbial communities during the composting phases plays a key role in the organic matter transformation and reflects the conditions established in the compost pile (Ryckeboer et al., 2003). Bacteria and fungi have been identified as the two most abundant microbial groups in composting. Bacteria comprise the key factor in the procedure due to their metabolic capabilities and their tolerance at a range of high temperatures. Fungi are present not only in the latter stages, as it was traditionally believed, but also in all phases of the procedure by actively conferring to the degradation of complex organic compounds (Langarica-Fuentes et al., 2014). Many of the microbiological studies conducted so far on OMW composting were based on cultivation-dependent approaches that provide information about culturable microorganisms. Alternatively, molecular methods based on polymerase chain reaction (PCR) amplification of rRNA genes enable the profiling of existing microbial communities, thus avoiding the limitations associated with laboratory culturing.

Greece is one of the main olive oil–producing countries in the Mediterranean region and the first in cotton production among the European Union member states. Therefore, generation of residues from these agro-industrial sectors constitutes a serious economic and environmental puzzle. Accordingly, the goal of the present work was to monitor the process of co-composting of CR with OMW with a view to proposing an environmentally friendly method for the treatment of these major organic agro-industrial byproducts. The physicochemical parameters and the culturable microbial population diversity were assessed and compared to the composting process of CR without the addition of OMW. A significant number of microorganisms originating from different stages of the process, with beneficial properties for the compost, were eventually isolated and identified by phylogenetic analysis.

Materials and methods

Composting processes

CR, originating from a cotton-ginning industry in the Thebes region (Greece), was used as the basic substrate in both piles. OMW was derived from a

three-phase decanter of a mill (processing olives of the Koroneiki variety), located in the Attica region (Greece). The physicochemical characteristics and the chemical composition of raw CR were estimated as follows: total nitrogen 2.01%, total organic carbon 35.81%, C/N ratio = 18/1, pH 6.10, moisture level 10%, EC 22.50 dS/m, water capacity 81%, porosity 93%, and density 1.03 g/cm^3. Additionally, OMW chemical properties and elemental composition were total nitrogen 0.76 g/l, total organic carbon 39.8 g/l, C/N ratio = 52, pH 5.2, EC 12.000 dS/m, BOD 45.000 mg/l, COD 92.000 mg/l, water 89%, and density 1.05 g/cm^3,

The mixtures were composted in a pilot greenhouse plant, in cuboid containers (0.9 m high with a 0.8 × 0.8-m basin). Composting started by adding water (200 l for 70 kg CR) in pile 1, and OMW (210 l for 70 kg CR) in pile 2. Sufficient homogenization of the substrates was applied during watering. Aeration was passive from the bottom to the top. A thick layer of insulating material was used to cover the sides of the containers and minimize temperature losses. Based on the temperature values recorded during the process, the piles were again turned over to improve the homogeneity of the material and help the fermentation process at the 64th day (when temperature started to drop). The moisture of both piles was also controlled during turning by the addition of the necessary amount of water.

Physicochemical parameter estimation

To assess the temperature of the piles, a digital thermometer coupled with a probe was inserted into the piles. Both ambient and compost temperatures were measured daily. The pH and EC were evaluated in a 1:5 (w/v) water-soluble extract. The moisture level was estimated by drying the fresh sample at 60 °C for 2 days. Ammonium (NH_4^+-N) and nitrate (NO_3^--N) nitrogen were extracted with 2 M KCl, and their values were determined by applying the indophenol blue method and the cadmium reduction method, respectively (Keeney & Nelson, 1982). Kjeldahl digestion analysis was used to define total nitrogen (N_T) content (Bremner, 1965).

The samples were taken by mixing subsamples obtained from different sites of the pile, spanning its entire profile (from the bottom to the top).

Microbial analyses and isolation

The population of aerobic culturable microorganisms (bacteria, fungi, and actinobacteria) from each composting phase were determined by decimal serial dilution (10^{-2} to 10^{-8}) and surface plating (0.1 ml) on selective media (Nutrient Agar, Potato Dextrose Agar with 33 μg/ml rose Bengal and 30 μg/ml streptomycin and Glycerol Casein Agar, respectively) in five replicates (Parkinson, 1994; Wellington & Toth, 1994; Zuberer, 1994). To isolate spore-forming bacteria, the first dilution was pre-incubated at 70 °C for 1 day in a water bath and 0.1 ml from the dilutions 10^{-3} to 10^{-7} were surface-plated on Nutrient Agar. To count and isolate cellulolytic bacteria, plates of carboxymethyl cellulose agar were inoculated with 0.1 ml from dilutions 10^{-4} to 10^{-8}. After an incubation period of 3–4 days at 30 °C, plates were flooded with Gram's iodine for 3–5 min and observed for potential zones of clearance around the colonies, as previously described by Kasana et al. (2008). The plates of all the microbial communities were incubated at 30 °C for 4–5 days, and pure cultures were obtained.

The populations of free-living diazotrophs in both piles were estimated by the most probable number of technique (Woomer, 1994). The nitrogen-fixing capacity of diazotrophs was evaluated using the acetylene reduction assay (ARA) (Beauchamp et al., 1991). In brief, serial dilutions of the organic material were performed in vials containing liquid N-free medium (Rennie, 1981). Five vials for each decimal dilution were inoculated and incubated at 30 °C (and at 50 °C for thermophiles). On the 7th day post inoculation, all vials were sealed with rubber stoppers and the gas phase of each vial was modified with acetylene (10% v/v of the air). Cultures were incubated for 1 more day. The amount of ethylene generated from the acetylene reduction was measured by a Perkin-Elmer F-11 gas chromatograph fitted with a flame ionization detector and a Porapak R 80–100 mesh stainless-steel column (2 mm × 2 m). Pure cultures originated from AR-positive vials at 30 °C and cultivated on Rennie's N-free medium solidified with agar (15% w/v).

Statistical analysis

One-way ANOVA at the 0.05 level was used for the analysis of significant difference among the microbial communities' populations between the two piles in each composting phase.

Respiration profiles

The respiration profiles of the microbial communities were assessed by an electrolytic respirometer (Manios & Balis, 1983). Oxygen uptake rates were measured at 30 °C for all the samples and 50 °C only for the samples originating from the thermophilic phases.

Extraction of bacterial/fungal genomic DNA and phylogenetic analysis

Genomic DNA from axenic bacterial cultures of the putative diazotrophic isolates were extracted using the genomic DNA from Tissue Kit according to manufacturer's instructions (Macherey–Nagel). The extraction of DNA from fungi was performed as follows: Potato Dextrose Broth liquid cultures were inoculated with mycelium and incubated for 3 days at 30 °C under agitation. Hyphae were recovered on a 0.45-μm pore filter and washed with distilled water. DNA isolation was performed using a NucleoSpin Plant II kit according to manufacturer's instructions (Macherey–Nagel). DNA concentration and purity were assessed by a Nanodrop ND-1000 spectrophotometer.

To characterize the bacterial isolates (nitrogen-fixing and cellulolytic bacteria), DNA (50 ng) was amplified by PCR using the forward primer fD1 (5′-AGAGTT TGATCCTGGCTCAG-3′) and the reverse primer rD1 (5′-CAAGTCGTAAAAGGTAGCCGT-3′) that amplified the bacterial 16S rRNA gene fragment (Weisburg et al., 1991). For fungal isolates, two regions (ITS-5.8S and 18S rRNA) of rDNA were amplified by PCR. The oligonucleotide primers used for the amplification of the ITS region were the forward primer ITS 1 (5′-TCCGTA GGTGAACCTGCGG-3′) and the reverse ITS 4 (5′-TCC TCCGCTTATTGATATGC-3′). The forward primer for amplification of the 18S rRNA region was nu-SSU-0817 (5′-TTAGCATGGAATAATRRAATAGGA-3′) and the reverse primer was nu-SSU-1563 (5′-ATTGCAATG CRCTATCCCCA-3′). PCR reactions were performed in 50 μl (final volume) mixtures containing 1 × PCR buffer, each deoxynucleotide triphosphate at a concentration of 0.2 mM, each primer at a concentration of 0.2 μM, 2 μl DMSO, and 1 U of DyNAzyme-EXT DNA polymerase (FINNZYMES).

DNA samples were amplified in a BIORAD thermocycler (MJ mini) using the following protocol: initial denaturation at 94 °C for 4 min was followed by 34 cycles of denaturation at 94 °C for 1 min, annealing at 48 °C for 1 min, and extension at 72 °C for 1:30 min. Cycling was completed by a final extension at 72 °C for 10 min. For the characterization of fungi, DNA samples were amplified in a BIORAD thermocycler (MJ mini) to implement the same protocol for both ITS and 18S rRNA genes: initial denaturation at 94 °C for 3 min, followed by 34 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min, and extension at 72 °C for 1:30 min. Cycling was completed by a final extension at 72 °C for 10 min.

To verify the presence of the nifH gene in the putative nitrogen-fixing isolates, genomic DNA (50 ng) was also amplified by PCR using the forward primer PolF (5′-TGCGAYCCSAARGCB-GACTC-3′) and the reverse PolR (5′-ATSGCC ATCATYTCRCCGGA-3′) (Poly et al., 2001). The same PCR conditions were used as those used for the amplification of bacterial 16S rRNA, with the exception that the annealing temperature was set at 55 °C.

Amplicons were separated by agarose gel electrophoresis, purified using a PCR clean-up Gel Extraction kit (Nucleospin Extract II—Macherey–Nagel) and ligated with pGEM-Teasy vector (Promega) according to the manufacturer's instructions. *Escherichia coli* XL-Blue competent cells were transformed using the ligation reaction product and blue/white screening was performed to identify transformants containing inserts. Plasmid DNA was obtained from positive clones using the Plasmid DNA Purification kit (Qiagen). Isolated plasmids were again subjected to restriction digestion analysis using the EcoRI restriction enzyme. Purified plasmids were commercially sequenced in an external sequencing provider (CeMIA; Larissa, Greece) in both directions. The nucleotide identity of the sequenced clones was compared to the GenBank database (http://blast.ncbi.nlm.nih.gov/) online to obtain the representative which related most closely to 16S rRNA (for nitrogen-fixing and cellulolytic bacteria) and nifH gene (for nitrogen-fixing bacteria only), and ITS-5.8S and 18S rRNA (for fungi) in the database.

Seed germination indices

After the addition of 250 ml of distilled water in 50 g of fresh compost, the mixture was mechanically

shaken for 2 h, and then filtered. Compost extract (5 ml) was added to culture dishes (9 cm diameter) that contained 5 pieces of filter paper; 25 cress seeds (*Lepidium sativum*) were evenly dispersed on the surface of the filter paper and then incubated in the dark, at 25 °C for 48 h. Water was used as control for the germination index (GI) tests. The procedures were replicated three times for each sample. Root lengths and germination rates were measured, and GI was calculated.

Results and discussion

Evolution of the composting process

In our study, we used CR and OMW that were considered not to be contaminated with pathogenic microorganisms, as usually happens with municipal and sewage wastes, or manures. Temperature increased rapidly at the beginning of the process (Fig. 1). The first thermophilic phase lasted approximately 4 days in both piles. Maximum temperatures of both piles were recorded on the 4th day of the composting process (52 °C in pile 1 and 59 °C in pile 2). The mesophilic phase lasted from the 6th to the 64th day, and after turning and watering, the second thermophilic phase followed and lasted longer in pile 2 compared to pile 1 (7 days and 3 days, respectively). Maximum temperature was observed on the 65th day in pile 1 and on the 67th day in pile 2 (45 °C and 54.5 °C, respectively). The higher temperature measurements and the prolonged second thermophilic phase in pile 2 are probably attributed to OMW addition, which is a substrate rich in carbon compounds and therefore susceptible to intense microbial activity. Prolongation of the thermophilic phase due to the presence of high organic matter content has also been reported by Mari et al. (2003) and Tortosa et al. (2012), whereas Paredes et al. (2002) also observed extended thermophilic phase during the co-composting of CR with OMW Generally, higher temperatures were reported for pile 2 during the whole composting process. The elevated temperature values recorded during the thermophilic phases in both piles in conjunction with their duration were adequate to ensure that both piles were sanitized (Lung et al., 2001; Mehta et al., 2014). The bio-oxidative phase of the composting was considered completed when the temperatures of the piles remained stable and close to that of the atmosphere (Paredes et al., 2005). After the 80th day, the composting process had entered the maturation phase and the temperature in both piles was stabilized (Table 1).

Neither pH nor EC evolution showed sharp changes in either of the two compost piles throughout the experiment (Table 2). The initial pH of pile 2 was

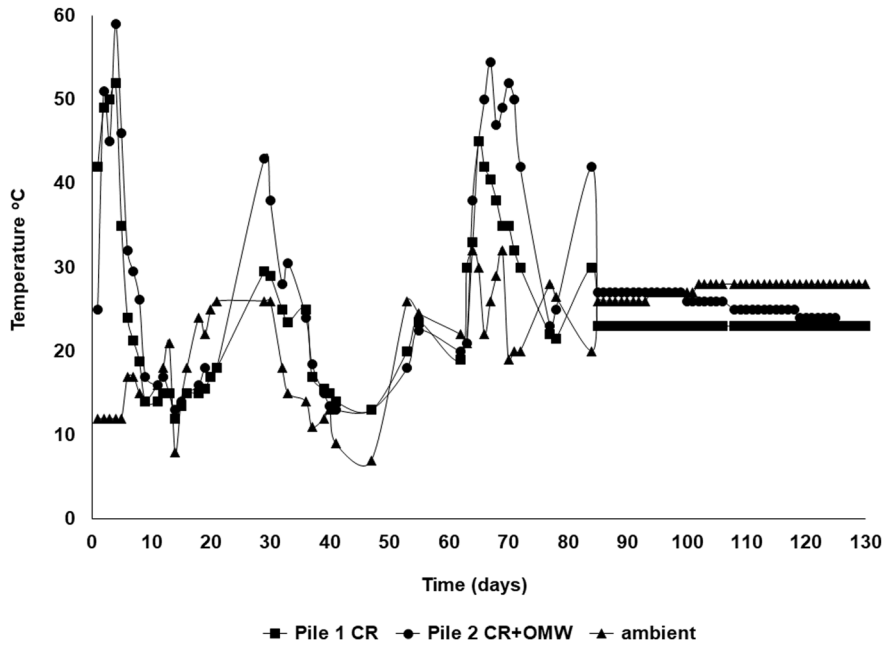

Fig. 1 Temperature profile during composting process in pile 1 (cotton residues—CR, ■) and pile 2 (cotton residues—CR + olive mill wastewater—OMW, ●). The variation in ambient temperature (▲) is also given

Table 1 Sampling dates, compost stages, phase duration, and temperature fluctuations throughout composting of pile 1 (cotton residues—CR) and pile 2 (cotton residues—CR + olive mill wastewater—OMW)

Sampling	Composting time of sampling (days)		Composting phase	Composting phase duration (days)	Temperature fluctuation °C	
a/a	*Pile1 CR*	*Pile2 CR + OMW*			*Pile1 CR*	*Pile2 CR + OMW*
1	0	0	Installation of piles (after turning and watering)	0–1	25–42	25
2	5	6	1st thermophilic phase	2–5	52–35 Max 4th day 52 °C	59–46 Max 4th day 59 °C
3	61	62	Mesophilic phase (64th day turning and watering)	6–64	13–33	13–43
4	67	68	2nd thermophilic phase	65–72	38–45 Max 65th day 45 °C	38–54.5 Max 67th day 54.5 °C
5	130	130	Maturation	73–130	22–23	23–27

slightly acidic (6.45) due to the constituents of OMW; then, pH values increased with time and reached the final values of 7.3 and 7.8 for piles 1 and 2, respectively. These values indicate a classic evolution of a compost under aerobic conditions. The pH values reflect the progressive biodegradation of acids (such as those with carboxylic and phenolic groups) and the mineralization of organic compounds (proteins, amino acids, peptides) into inorganic compounds (Agnolucci et al., 2013). The alkaline pH also suggests the formation of humic substances, which act as pH buffers (Khalil et al., 2001). Final pH values within the range of 6.0–8.5 ensure compatibility with the requirements of most plants (Hogg et al., 2002). Regarding EC values, it is noted that they decreased during the process in both piles, as soluble salts were lost by leaching and were not re-added to the piles. Higher EC values were observed in pile 2, probably due to the soluble salts provided from OMW (Paredes et al., 2005). In any case, EC values were within the optimal EC range of 0.3–12 ms/cm, according to Tomati et al. (2002). This observation is of great importance in the context of an agronomic approach, since it indicates low soluble salt content in the composts and, thus, limited inhibitory

Table 2 Evolution of the main physicochemical parameters and respiration rates during composting of pile 1 (cotton residues—CR) and pile 2 (cotton residues—CR + olive mill wastewater—OMW) (dry weight basis)

Composting time (days)	Moisture (%)	pH	EC (mS/cm)	N_T (%)	NH_4^+-N (ppm, μg/g d.w.)	NO_3^--N (ppm, μg/g d.w.)	NH_4^+/NO_3^-	ml O_2/100 g d.w./h	
								30 °C	50 °C
Pile 1: CR									
0	73.10	7.20	3.00	1.43	198.03	51.06	3.88	36.64	
5	58.60	7.08	4.55	2.37	117.07	43.52	2.69	34.46	32.07
61	49.13	7.35	4.00	2.55	145.52	67.12	2.17	27.16	
67	73.60	7.70	2.53	3.19	306.18	49.69	6.16	59.95	58.52
130	61.60	7.30	3.96	3.44	193.57	92.69	2.09	12.27	
Pile 2: CR + OMW									
0	73.43	6.45	5.10	1.88	270.13	147.53	1.83	51.56	
6	54.05	7.70	4.19	2.17	46.24	7.47	6.19	29.62	31.38
62	48.53	7.60	5.87	2.86	31.17	19.71	1.58	28.74	
68	73.30	7.80	3.74	3.07	75.40	21.67	3.48	51.13	51.90
130	68.10	7.80	4.87	3.77	47.67	77.16	0.62	12.13	

effect on plant growth and seed germination if applied to soil (Banegas et al., 2007).

The nitrogen content of a compost is an indicator of its fertilizing value. In both piles N_T increased over time. The degradation of labile organic-C compounds reduced the weight of piles and led to increased compost concentrations and therefore increased total nitrogen values. Similar results have also been observed in other experiments (Bernal et al., 1998). The concentration of N usually increases during composting when volatile solid (organic matter) loss is greater than the loss of NH_3 (Bernal et al., 1998). N_T increase can also be attributed to the nitrogen-fixing bacteria active in both piles during the whole composting process.

Nitrogen is continuously transformed during a composting process from insoluble forms (mainly proteins) to soluble (ammonium, NO_2^-, NO_3^-, amino acids, and peptides), due to ammonification and proteolytic reactions. The ammonium-N concentration decreased initially in both piles, due to NH_3 volatilization, leaching losses, and nitrification. Low ammonification rate was expected for lignocellulose-rich substrates as they are less degraded. The changes in ammonium during the bio-oxidative stage strongly depended on temperature. López-Gonzales et al. (2013) noted that ammonium decreased at the beginning of the first and the second thermophilic stages as high temperatures along with alkaline pH led to volatilization. At the late sampling dates of each thermophilic stage, although the same conditions prevailed, ammonia increased instead, perhaps due to pH alteration or to shifts in processes involved in nitrogen dynamics, such as nitrification, leaching, and volatilization. The shift to microbial activities that promote ammonium uptake because of thermal acclimatization may partially explain this behavior. Increased NH_4^+-N concentration on day 67th And 68th in piles 1 and 2, respectively, proved that the mineralization of organic-N compounds was still active, and that the product could not be considered mature at that point of the composting process. After this time, the NH_4^+-N concentration gradually decreased as the compost entered the maturity phase. Compost is considered suitable for use when NH_4^+ concentration decreases and NO_3^- increases in the composting material (Finstein & Miller, 1985). Pile 2 exhibited lower amounts of ammonium-N at most sampling dates of the experiment, perhaps due to a greater loss of volatile N compounds, which is possibly an indication that this pile headed sooner toward maturity.

Nitrate-N was detected in both piles throughout the whole composting process but decreased (especially in pile 1) at the thermophilic phases, due to the low rate of nitrification. OMW is rich in easily biodegradable organic compounds, and this promotes high microbial activity, which leads to increasing demand for mineral nitrogen. Nitrification also hardly occurs under thermophilic conditions (Bernal et al., 1998; Brewer & Sullivan, 2003) since temperatures greater than 40 °C inhibit the activity and growth of nitrifiers. Pile 2 exhibited a lower rate of nitrification, probably due to the antimicrobial activity of OMW. Moreover, pile 2 demonstrated a lower rate of ammonification and ammonium-N loss through volatilization, which led to a lack of substrate for nitrification in pile 2.

In both piles, the NH_4^+/NO_3 ratio displayed an increase at thermophilic stages (the second thermophilic phase for pile 1; both thermophilic phases for pile 2) since high temperatures are inhibitory for nitrifiers' growth and activity. At most sampling dates, the NH_4^+/NO_3^- ratio was lower in pile 2 than that in pile 1, reconfirming that this pile headed sooner toward maturity. The fact that in both piles the NH_4^+/NO_3^- ratio was not low enough by the 68th day was one more indication that both piles had not entered the maturity phase yet, since NH_4^+/NO_3^- ratios of less than 1 are generally considered indicative of a mature compost (Bernal et al., 1998; Brewer & Sullivan, 2003). Bernal et al. (1998) have also pointed out that NH_4^+ to NO_3^- ratio should be in favor of the oxidized form to be considered indicative of the maturity of the compost. In the current research, the final NH_4^+/NO_3^- ratio of pile 2 was lower than 1, while the respective ratio of pile 1 was higher, probably due to the elevated NH_4^+-N concentrations. According to Vuorinen and Saharinen (1997), when NH_4^+/NO_3^- ratio is high, further maturation (curing) is necessary before the agronomic application of the compost.

Microbial community dynamics

The bacterial population in both piles was enhanced at the beginning of the process, decreased during the first thermophilic phase (due to high temperatures), increased significantly during the mesophilic phase, decreased again when temperature reached high values in the second thermophilic phase, and

remained stable until the end of the composting process (Fig. 2). At most sampling dates, total bacteria population was lower in pile 2, probably due to the antimicrobial activity of OMW. The ANOVA results showed that the bacterial communities in pile 1 were significantly increased compared to that in pile 2 at the initial stage of composting and during the first thermophilic phase, but no significant differences were observed during the mesophilic, the second thermophilic, and the maturity phase (Table 3). These results indicate that the mesophiles were the primary active degraders of the fresh organic waste during the initial phase of the process. Mesophilic bacteria were partially killed or inactivated during the thermophilic phases in both piles (at temperatures between 40 and 59 °C).

Temperature is considered to be an important factor affecting fungal growth. In both piles, fungal populations were higher in mesophilic temperatures, since most fungi are mesophilic (5–37 °C), with an optimum temperature of 25–30 °C (Ryckeboer et al., 2003). The optimal temperature for thermophilic fungi is 40–55 °C, the maximum being 60–62 °C. At temperatures above 60 °C, fungi are killed or transiently present in the form of spores (Beffa et al., 1996b; Ryckeboer et al., 2003). Statistical processing showed that viable fungal numbers were significantly lower in pile 2 in all stages of the process compared to pile 1, probably due to the constituents of OMW (Fig. 2). Fungi were still active in both piles at the second thermophilic stage, since they are the main degraders of cellulose and chitin, an observation that is in agreement with previous reports (Ryckeboer et al., 2003). The higher fungal population numbers were observed at the mesophilic phase in both piles (1.11×10^8 and 1.84×10^7 cfu/g d.w. for piles 1 and 2, respectively) (Table 3).

Actinomycetes' population was significantly increased in pile 1 compared to pile 2 during the whole composting process, with the exception of mesophilic actinomycetes (Fig. 2). Enhanced population numbers in pile 1 at the last sampling dates probably indicated that this pile still contained degradable compounds, such as cellulose, hemicellulose, and lignin at the respective composting phase (Table 3). Moreover, actinomycetes are known to develop slower than most bacteria and fungi do and seem to be rather ineffective competitors when nutrient levels are high. Generally, actinomycetes play an important role in composting by degrading natural polymers and colonizing organic materials after bacteria and fungi have consumed the easily degradable fractions (Beffa et al., 1996b; Ryckeboer et al., 2003).

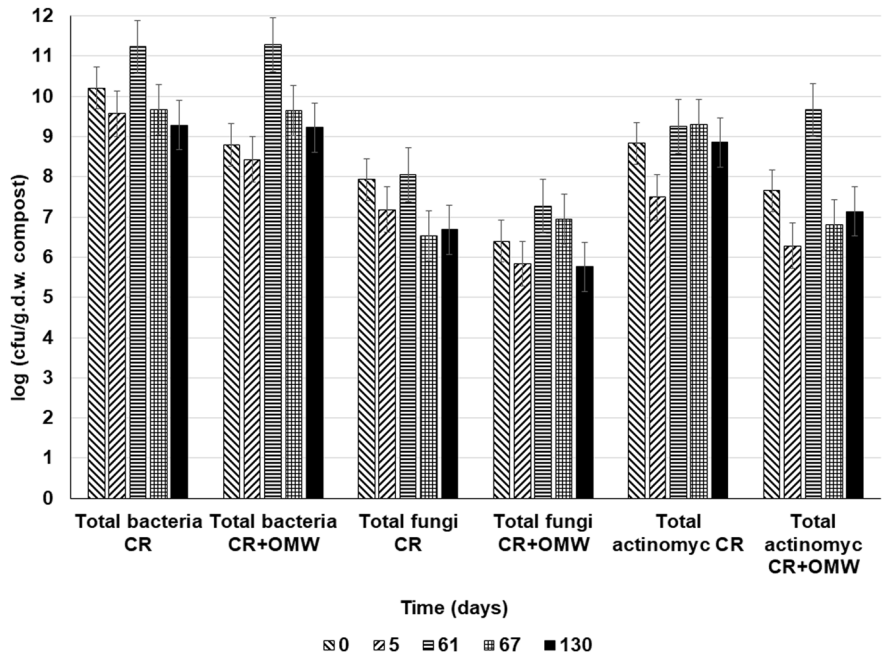

Fig. 2 Temporal variation of total bacteria, total fungi, and total actinomycete populations during the composting process of piles 1 (cotton residues—CR) and 2 (cotton residues—CR + olive mill wastewater—OMW). Population estimates are expressed as log10 (cfu/g d.w.)

Table 3 Dynamics of the major microbial group populations during composting of pile 1 (cotton residues—CR) and pile 2 (cotton residues—CR + olive mill wastewater—OMW), expressed in colony forming units per dry weight basis (cfu/g d.w. compost)

Composting time (days)	Total bacteria	Fungi	Actinomycetes	Spore-forming bacteria	Cellulolytic bacteria	Nitrogen-fixing bacteria 30 °C	Nitrogen-fixing bacteria 50 °C
Pile 1: CR							
0	$1.64\times10^{10}\pm3.1\times10^{9}a$	$8.46\times10^{7}\pm2.6\times$	$6.80\times10^{8}\pm2.8\times10^{8}e$	$1.27\times10^{9}\pm2.9\times10^{8}a$		$1.02\times10^{7}\pm2\times10^{5}e$	
5	$3.76\times10^{9}\pm5.3\times10^{8}b$	$1.50\times10^{7}\pm5.2\times10^{6}b$	$3.10\times10^{7}\pm6.6\times10^{6}c$	$8.28\times10^{8}\pm9.2\times10^{7}b$		$4.33\times10^{6}\pm1.1\times10^{6}d$	0
61	$1.71\times10^{11}\pm5.5\times10^{10}c$	$1.11\times10^{8}\pm1.7\times10^{7}c$	$1.77\times10^{9}\pm7.2\times10^{8}b$	$1.26\times10^{9}\pm3.83\times10^{8}e$	$5.95\times10^{10}\pm1.8\times10^{10}a$	$5.50\times10^{6}\pm1.6\times10^{6}a$	
67	$4.58\times10^{9}\pm2.2\times10^{9}d$	$3.50\times10^{6}\pm5\times10^{5}d$	$2.04\times10^{9}\pm5.2\times10^{8}d$	$1.40\times10^{10}\pm2.65\times10^{9}c$	$1.20\times10^{11}\pm2\times10^{10}c$	$3\times10^{7}\pm8.7\times10^{6}b$	$1.66\times10^{4}\pm4.9\times10^{3}a$
130	$1.92\times10^{9}\pm6.27\times10^{8}e$	$4.83\times10^{6}\pm3.8\times10^{5}e$	$7.27\times10^{8}\pm2.2\times10^{8}a$	$8.08\times10^{8}\pm1.49\times10^{8}d$	$8.38\times10^{10}\pm1.2\times10^{10}b$	$2.33\times10^{7}\pm4.5\times10^{6}c$	
Pile 2: CR + OMW							
0	$6.13\times10^{8}\pm1.91\times10^{8}b$	$2.50\times10^{6}\pm6.9\times10^{5}b$	$4.41\times10^{7}\pm9\times10^{6}a$	$1.17\times10^{8}\pm1.6\times10^{7}c$		$1.43\times10^{6}\pm5.1\times10^{5}a$	
6	$2.66\times10^{8}\pm6.3\times10^{7}a$	$6.93\times10^{5}\pm1.5\times10^{5}c$	$1.91\times10^{6}\pm3.2\times10^{5}b$	$8.38\times10^{7}\pm8.5\times10^{6}d$		$1.4\times10^{4}\pm3.6\times10^{3}c$	0
62	$1.91\times10^{11}\pm7.4\times10^{10}c$	$1.84\times10^{7}\pm1.1\times10^{6}d$	$4.53\times10^{9}\pm9.4\times10^{8}d$	$1.35\times10^{9}\pm4.8\times10^{8}e$	$4.65\times10^{9}\pm9.5\times10^{8}c$	$2.57\times10^{5}\pm1.4\times10^{5}b$	
68	$4.28\times10^{9}\pm1.5\times10^{9}d$	$8.72\times10^{6}\pm1.3\times10^{6}e$	$6.34\times10^{6}\pm2.4\times10^{6}c$	$2.58\times10^{9}\pm5.3\times10^{8}a$	$1.71\times10^{10}\pm4.46\times10^{9}b$	$1.02\times10^{5}\pm2\times10^{3}d$	$7\times10^{2}\pm1\times10^{2}b$
130	$1.7\times10^{9}\pm4.4\times10^{8}e$	$5.81\times10^{5}\pm1.8\times10^{5}a$	$1.39\times10^{7}\pm2\times10^{6}e$	$7.62\times10^{9}\pm1.4\times10^{9}b$	$1.57\times10^{10}\pm2.34\times10^{9}a$	$4.8\times10^{7}\pm1.2\times10^{7}e$	

Data are shown as the mean value of three replicates ± standard deviation. Data analyzed by one-way ANOVA, $p<0.05$. Different lowercase letters within a column denote significant differences among the microbial populations of pile 1 and pile 2, at the same composting time. Data within a column followed by the same lowercase letter are not significantly different at the $p<0.05$ level

Increased spore-forming bacteria population numbers were observed at the 2nd thermophilic phase in both piles (Fig. 3). ANOVA results indicated that endospore-forming bacterial populations were significantly enhanced in pile 1 (in comparison to pile 2) at both thermophilic phases (Table 3). This was attributed to spores' tolerance to high temperatures since thermophilic spore-forming bacilli are usually active during the composting process at temperatures between 50 and 65 °C (Beffa et al., 1996a).

Bacteria able to degrade cellulose were not detected before the mesophilic phase in any of the piles (Table 3). In both piles, higher populations of cellulolytic bacteria were recorded in the 2nd thermophilic

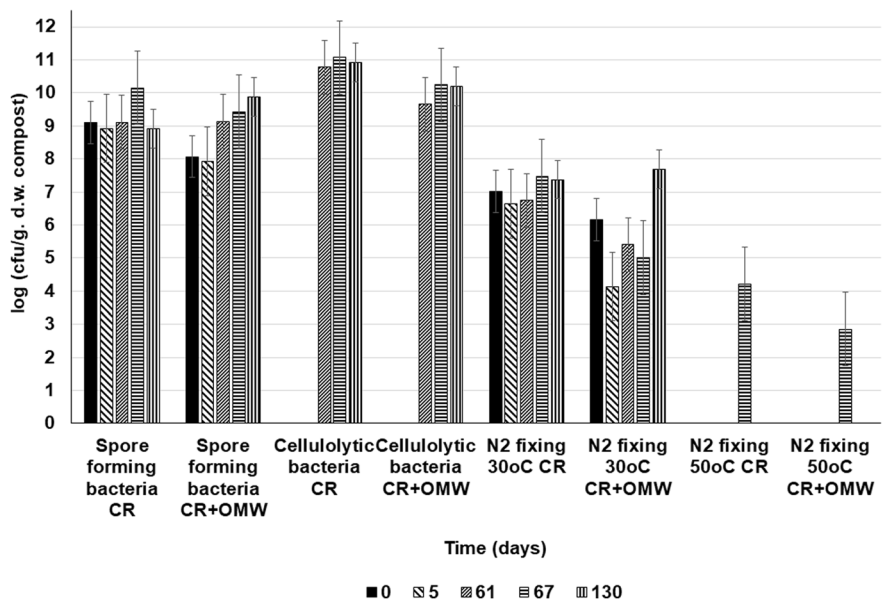

Fig. 3 Temporal variation of spore-forming bacteria, viable cellulolytic bacteria, viable mesophilic, and viable thermophilic diazotroph populations during the composting process of piles 1 (cotton residues, CR) and 2 (cotton residues—CR + olive mill wastewater—OMW). Population estimates are expressed as log10(cfu/g d.w.)

phase since the optimal temperature for cellulose degradation is around 65 °C, indicating that thermostable enzymes are mainly involved in this process (Ryckeboer et al., 2003; Upreti & Joshi, 1984). Cellulolytic bacterial populations developed the same pattern in both piles throughout the composting process, although pile 1 sustained higher populations compared to pile 2. Cellulolytic populations were detected even during the maturation phase (Fig. 3). The general growth pattern demonstrated by cellulolytic bacteria in the present study seems to be attributable to the fact that these microorganisms decompose cellulose, hemicellulose, and lignin after the readily degradable organic fractions have been assimilated (Tiquia, 2002; Tuomela et al., 2000).

The population of diazotrophs incubated at 30 °C was high during the whole composting process in both piles (Table 3). Pile 2 supported lower numbers of nitrogen-fixing bacteria at most of the sampling dates, indicating that the antimicrobial properties of OMW had a negative effect on diazotrophs. On the contrary, Balis et al. (1996) contented that OMW, defined by a low content in nitrogenous organic components and richness in carbon sources, offers a highly favorable environment for the growth of free-living nitrogen-fixing microorganisms. The presence of active thermotolerant nitrogen-fixing microbiota in both piles at the 2nd thermophilic phase was a significant finding (Fig. 3). Perhaps, part of the initial population of the diazotrophic bacteria adapted well at high temperatures and stayed active during the thermophilic phase, although the population in pile 1 was significantly higher than that observed in pile 2. The population of nitrogen-fixing bacteria reached the maximum value in pile 2 during maturation (4.8×10^7 cfu/g d.w). The final product was enriched in nitrogen-fixing microorganisms as population remained high in both piles at the 130th day. Galli et al. (1997) observed appreciable nitrogenase activity at the end of the thermophilic phase. Our results upon the free-living nitrogen-fixing bacterial population are of great importance, since there are not enough reports on this subject. The potential use of both cellulose degradation and nitrogen fixation is significant with respect to rapid composting, enhancing the compost quality and the sustainable management of the lignocellulosic agricultural waste. As mentioned in the literature, the inoculation of two cellulolytic nitrogen-fixing *Bacillus* strains in the composting of rice straw has been shown to reduce the composting time by 40–43% and increase the total nitrogen, phosphorus, and potassium level in the compost (Abdel-Rahman et al., 2016). In the present study, it was proved that free-living diazotrophs, remarkably enhanced during the composting process, are responsible for the fertilizing value of the end-product.

Respiration rates

In the present research, respiration rates at 30 °C followed the same pattern in both piles (Table 2, Fig. 4). The respiration rates decreased at the beginning of the composting process, until the mesophilic phase, approaching a value of 28 ml $O_2 \times 100$ g d.w./h and afterwards increased during the 2nd thermophilic phase, approaching a value of 51 to 60 ml $O_2 \times 100$ g d.w./h. During the maturation phase, the respiration rates decreased and remained stable in both piles, thus indicating the stability of the final product (Fig. 4). Notably, the respiration rate during the onset of the composting process was higher in pile 2, although the microbial population was less compared to that of pile 1. Even though most microbial communities may have been affected by the antimicrobial activity of OMW, the endogenous microbiota of the two nutrient-rich substrates in pile 2 was more active initially. Afterwards, the respiratory activity in each pile was proportional to the level of the microbial populations installed. The potential of the microbial communities to metabolize carbon sources at different environmental temperatures in the compost piles can be described by the respiratory profiles obtained (Mari et al., 2003).

Respiration rates at 50 °C also followed the same pattern in both piles (Table 2). An increase was observed at the 2nd thermophilic phase (Fig. 4). This is also an indication that the microbiota of the two composts was still active and well adapted not only at mesophilic but at thermophilic temperatures as well. Our results confirmed that respirometric tests were a useful indicator of the microbial activity which can describe the progress and stabilization of the composting process.

At the end of the composting process, the high microbial biomass and the low respiration rates observed could be related to the depletion of easily degraded organic compounds and/or to the changes in the composition of the microbial community. During

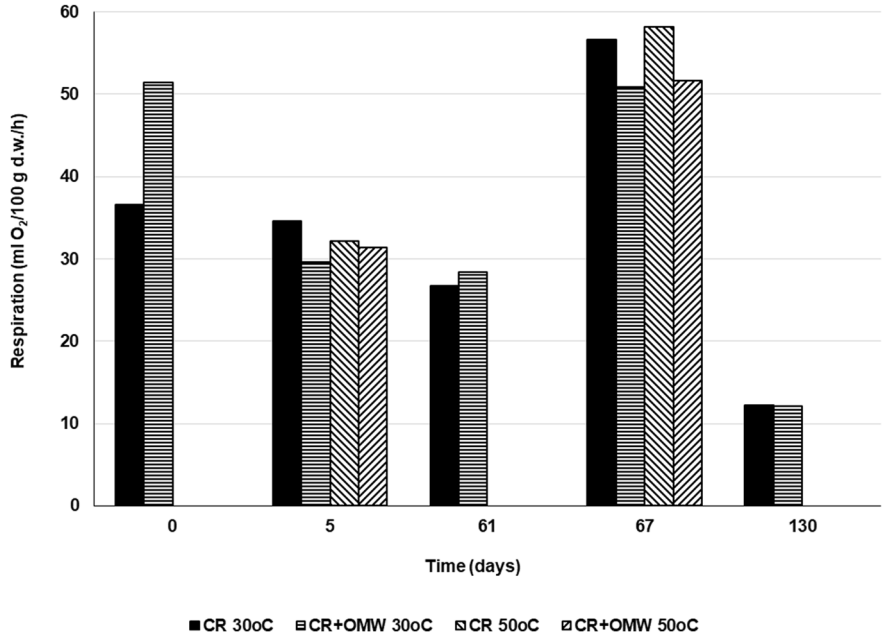

Fig. 4 Temporal variation of the respiratory activity of the samples originating from pile 1 (cotton residues—CR) and pile 2 (cotton residues—CR + olive mill wastewater) after incubation at 30 °C and 50 °C. Respiration rates are expressed as ml O_2/100 g d.w./h

the maturity phase, both piles exhibited low respiration rates but enhanced microbial population numbers revealing a rather inactive microbial community. It has been documented that although the bacterial population decreases during the cooling phase compared to the thermophilic phases, its taxonomic and metabolic diversity increases markedly (Beffa et al., 1996b). Suarez-Estrella et al. (2013) proved the importance of mature compost as a source of effective biological control agents, since more than 60% of the microorganisms selected for their broad antagonistic spectrum were isolated during the maturation phase.

Identification of compost microbial isolates

Eighteen nitrogen-fixing bacteria were isolated into axenic cultures, originating from different sampling dates from both piles. All isolates gave positive results in ARA. Of particular interest were the isolates Co1, Co2, and Co5 exhibiting higher nitrogen fixation activity and a high percentage of identity with *Klebsiella variicola*, *Klebsiella pneumoniae*, and *K. variicola*, respectively, according to 16S rRNA gene sequences. Comparative analyses of nucleotide sequences of amplified nifH gene fragments revealed that isolate Co1 exhibited identity with *K. variicola*, Co2 with *K. grimontii*, and Co5 with *K. variicola* (Table 4). Based on nifH and 16S rRNA gene sequences, the identification of N_2-fixing bacteria was entirely consistent at the genus level. Therefore, the diversity of the nifH gene can be used to study the diversity of the bacterial community able to fix N_2 (Poly et al., 2001; Venieraki et al., 2011).

Published scientific literature concerning the genus *Klebsiella* provides information that certain species possess plant growth-promoting traits and many of them are diazotrophs; hence, Chen et al. (2016) reported that twenty-one strains of *K. variicola*, one strain of *K. pneumoniae*, and five out of eight strains of *K. quasipneumoniae* contained nitrogen-fixing genes. *K variicola* strain DX120E, a colonizer of sugarcane roots and shoots, was able to fix N_2 in association with sugarcane plants, and promoted sugarcane growth; thus, it showed potential as a biofertilizer (Lin et al., 2015). *K. pneumoniae* has been reported to protect rice from sheath and seedling blight while it keeps nematode populations under control, and thus could be considered a biological control agent (Liu et al., 2018). *K. grimontii* is a newly identified species closely related to *K. oxytoca*, certain strains of which are diazotrophs (Lutfu Cakmakci et al., 1981). Mukherjee et al. (2020) isolated, characterized, and assessed a facultative thermophilic, nitrogen-fixing *Klebsiella* sp., which had multiple plant growth-promoting traits.

Ten cellulolytic bacteria originating at different sampling dates were isolated into axenic cultures.

Table 4 Characterization of the N_2-fixing and cellulolytic bacterial isolates through 16S rRNA and nifH gene sequences, and the fungal isolates through ITS and 18S rRNA sequences. The isolates originated from different sampling stages of composting pile 1 (cotton residues—CR) and pile 2 (cotton residues—CR + olive mill wastewater—OMW)

Sampling stage	Isolate/sequence	Description	Query cover	Percent. identity
N_2-fixing bacteria				
Mesophilic pile 2	Co1 (16S rRNA)	*Klebsiella variicola*	98%	99.80%
	Co1 (nifH)	*Klebsiella variicola*	100%	99.70%
1st thermophilic pile 2	Co2 (16S rRNA)	*Klebsiella pneumoniae*	98%	99.73%
	Co2 (nifH)	*Klebsiella grimontii*	100%	99.09%
1st thermophilic pile 1	Co5 (16S rRNA)	*Klebsiella variicola*	98%	99.73%
	Co5 (nifH)	*Klebsiella variicola*	100%	99.39%
Cellulolytic bacteria				
2nd thermophilic pile 1	Cell2 (16S rRNA)	*Serratia marcescens*	95%	99.24%
Mesophilic pile 1	Cell8 (16S rRNA)	*Brevibacillus agri*	100%	99.35%
Mesophilic pile 1	Cell9 (16S rRNA)	*Brevibacillus agri*	100%	99.75%
Fungi				
Installation pile 2	K1As1 (ITS)	*Aspergillus fumigatus*	100%	100%
	K1As1 (18S rRNA)	*Aspergillus fumigatus* *Aspergillus terreus*	99% 99%	99.61% 99.61%
Mesophilic pile 2	K3As4 (ITS)	*Aspergillus flavus* *Aspergillus oryzae*	100% 100%	99.83% 99.83%
	K3As4 (18S rRNA)	*Aspergillus flavus*	99%	99.74%
		Aspergillus oryzae	99%	99.74%
2nd thermophilic pile 2	K4As8 (ITS)	*Aspergillus niger* *Aspergillus tubigensis*	100% 100%	99.83% 99.83%
	K4As8 (18S rRNA)	*Aspergillus flavus*	99%	99.61%
		Aspergillus oryzae	99%	99.61%

Three specific isolates (Cell2, Cell8, and Cell9) displayed higher cellulolytic activity. Comparative analyses of nucleotide sequences of amplified 16S rRNA fragments revealed that Cell2 was identical to *Serratia marcescens*. The isolates Cell8 and Cell9 exhibited great similarities with *Brevibacillus agri* (Table 4). Bacteria belonging to the genus *Serratia* are commonly found in raw OMW (Venieri et al., 2010). *Serratia* species produce bioactive products and possess biocontrol capabilities; Ahmed et al. (2018) reported that rhizobacterial strains belonging to *B. brevis*, *B. agri*, and *B. formosus* expressed suppression on *Salvia officinalis* wilt and root rot diseases, probably due to the production of several inhibitory metabolites (such as HCN, chitinase, and siderophores). *Brevibacillus* spp. have been described as biological control and soil bioremediation agents (Panda et al., 2014).

The three fungal isolates that were identified originated from both piles, at different composting stages. Comparative analyses of nucleotide sequences of amplified 18S rRNA fragments showed that the isolates exhibited identity with species of the genus *Aspergillus*. Particularly, the isolate K1As1 showed great similarity with *A. fumigatus* and *A. terreus*, while isolates K3As4 and K4As8 were identified as *A. flavus* and *A. oryzae* (Table 4). The ITS1 domain sequences were compared for similar nucleotide sequences with the BLAST search of NCBI and demonstrated that K1As1 had great similarity with the species *A. fumigatus*, K3As4 was identical to *A. flavus* and *A. oryzae*, whereas K4As8 was identical to *A. niger* and *A. tubigensis* (Table 4).

Fungi have an important role in the composting process, especially during thermophilic and maturation phases, as degraders of recalcitrant materials such as cellulose and lignin (Tortosa et al., 2020). Ascomycota have been described as the predominant phylum in OMW composting (Ntougias et al., 2013). Genera such as *Aspergillus* have been broadly

described in many composting processes during all phases and have been characterized as thermophilic microorganisms (Langarica-Fuentes et al., 2014). Ribeiro et al. (2019) identified *A. fumigatus* and yeasts distributed at all phases of the process, whereas *A. candidus* emerged only in a single phase (early at the mesophilic stage).

Compost products have been reported to contribute to plant growth and to the suppression of plant pathogens, improving the overall environmental quality. Some important enzymes of the compost's microbiota such as protease; chitinase; lipase; and b-1,3 glucanase are responsible for their suppressive effects on phytopathogenic fungi (EL-Masry et al., 2002). Composts have been used as sources for isolating potential biocontrol agents, such as *Bacillus subtilis*, *Bacillus licheniformis*, and *Penicillium chrysogenum* strains, with significant antagonistic effects on plant pathogens (Milinković et al., 2019; Suarez-Estrella et al., 2013). The microorganisms isolated in the present study and identified as members of the genera *Brevibacillus*, *Serratia*, *Klebsiella*, and *Aspergillus* could be further studied for their potential capacity as plant growth promoters or biological control agents against phytopathogens.

Phytotoxicity (GI index)

GI in pile 1 exceeded 80% for the 100% compost extract, and it was over 90% for the 50% compost extract. According to the criteria for the in vitro evaluation of compost stability, the material generated from pile 1 was stable and could be used in plant production. GI in pile 2 was lower (59.10% for 100% compost extract and 86.75% for 50% compost extract), but still within limits to be considered nontoxic for plants (Milinković et al., 2019). This result indicates that GI depends on the initial composition of the basic substrate used and is in agreement with previous reports (Nafez et al., 2015). GI represents a measure of how ready the final product is to support seed germination at a level that is not harmful to plant growth. Loss of phytotoxicity is a measure of the maturity level of the compost.

Conclusions

In the current study, co-composting of OMW with cellulose-rich CR was recommended, for an effective treatment of both wastes and the production of a high-value product suitable for biofertilizer. On the basis of the results obtained, although initially the addition of OMW was slightly toxic to the microbial populations, co-composting accelerated the treatment process and reduced the time needed to reach maturity leading to a final product with improved quality. The GI value that was between the proper limits and the appropriate pH and EC indicated that the mature compost can be used for agronomic purposes. It was also confirmed that respirometric tests are useful indicators for a reliable description of the composting process progress, the dynamics of the microbial population, and the maturity of the compost. The results upon the thermotolerant, free-living, nitrogen-fixing bacterial population are of importance, since few pertinent reports are available. It was also demonstrated that the free-living diazotrophs, which were significantly enhanced during the composting process, were responsible for the fertilizing value of the end-product. Several culturable microorganisms were isolated (identified as members of the genera *Brevibacillus*, *Serratia*, *Klebsiella*, and *Aspergillus*), which could be further evaluated for their properties as biological control agents, plant growth promoters, and composting accelerators for the production of a final product with useful traits. In countries like Greece that have large quantities of problematic agricultural residues to manage, co-composting is an easy and effective method leading to a valuable final product, i.e., an organic fertilizer and soil amendment with high nitrogen content, improved porosity, enhanced water and nutrient retention and carbon sequestration in the form of organic matter. The addition of such products in soils is also safer than that of composts originating from municipal wastes or manures, due to the absence of heavy metals or pathogenic microorganisms dangerous to humans and animals.

Availability of data and material The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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