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One Step Bioremediation of Olive-Oil-Mill Waste by Organoinorganic Catalyst for Humics-Rich Soil Conditioner Production

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Abstract: A new, simple, and rapid one-step integrated method for the biotechnology treatment of raw olive oil mill waste (OMW) is discussed. The innovations introduced involve primarily the application of microaerobic composting processes for OMW bioremediation plus the addition of both a zeolite and a tailor-made biocatalyst extremely rich in soil microorganisms. The latter operates at a wide pH range and provides, apart from soil microorganisms, nutrients to the compost, enhancement to the bio-oxidative phase and acceleration of biochemical reactions during bioremediation. The basic parameters affecting the bioprocess, i.e., electrical conductivity, pH, C/N ratio, specific weight, ash, organic matter, total organic carbon, total Kjeldahl nitrogen, microorganisms, humic substances, and total polyphenols, were monitored systematically to provide insight into the process and evaluate the product obtained. After a biotreatment of just 60 d, a significant reduction in polyphenols (91.4%) and an increased humic substances content (8%)—both serving as maturation indices—were observed. The OMW compost received is stable, free of toxic compounds and pathogens, affords a richness in cenose and a high humic substances content, both vital for soil fertility. Applications of the OMW product received, both in laboratory-scale and field cultivations, confirm its suitability as a first-class soil conditioner for organic farming.

Keywords: olive oil mill waste (OMW); humic substances; biocatalyst; soil conditioner; compost; biodegradation; microorganisms

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1. Introduction

Over 750 million olive trees are cultivated worldwide, 95% of which are located in the Mediterranean region. The top three main olive oil producers worldwide, Spain, Italy, and Greece, account for the 93% of European olive oil production [1].

During olive oil extraction, a large quantity of solid and aqueous residues known as olive oil mill waste (OMW) arises as by-product, containing (by weight) typically 83–94% water, 4–16% organic compounds, and 0.4–2.5% mineral salts [2]. Olive oil mill wastewater is an acidic matrix (pH ranges between 3 and 6) with high solid matter content, bearing a dark color, a strong olive-oil smell, and high electrical conductivity. COD and BOD vary from 40 to 220 and from 35 to 110 g L^{-1}, respectively, a fact strongly related to organic pollution [3]. However, the most notorious pollutant is the phenolic residue constituting 2–15% of the organic fraction, containing low molecular weight compounds (i.e., caffeic acid, tyrosol, hydroxytirosol, p-cumaric acid, ferulic acid, syringic acid, and protocatecholic acid), high molecular weight compounds (i.e., tannins and anthocyanines), and catechol-melaninic polymers [4,5]. Due to their low oil-per-water partition coefficients, phenols favor concentrating into water instead of oil, a fact explaining the increased concentrations of polyphenols in OMW [6]. Especially for OMW discharge, Greek legislation (K.Υ.A.

government decision 127402/1487/Φ15/2016) determines that the liquid waste from the production process after pre-treatment, which includes fat collection, and sedimentation, or other equivalent treatment, is available for hydro fertigation of olive and other trees, with a maximum annual application volume of 8 m^3 per 1000 m^2 land. The waste, kept in closed watertight storage tanks, is applied within 10 days. Several authors argue that spreading OMW on cultivated soils may improve soil fertility and olive plant performance. In fact, significant increases in shoot growth, fruit set and yield, photosynthesis, and concentrations of K, organic matter, phenolic compounds, and microbial populations have been observed [7]. On the other hand, OMW's putrescible nature and insufficiently stable organic matter can cause negative effects in both soil properties and plant growth, e.g., the release of phytotoxic substances, and microbial immobilization of plant nutrients [8,9]. Additionally, the adverse impacts to the atmosphere, aquatic life, and water resources should also be considered [7]. In any case, the high content of organic matter and mineral salts offers the promising possibility for employing OMW as a raw material [10–12] to produce environmentally compatible soil conditioners suitable for organic cultivation, and this perspective is estimable from both the economic and scientific points of view.

OMW treatment strategies vary considerably. Neutralization with lime is casually applied prior to OMW disposal in evaporation ponds/lagoons. This treatment does not impose economic burdens, but serious environmental problems may arise, such as overflow and contamination of neighboring systems with polyphenols or other toxic organic compounds, high COD, low dissolved oxygen, the induction of anaerobic conditions, and odor nuisance. Physical processes for OMW treatment, i.e., sedimentation, filtration, flotation centrifugation, and adsorption, are typically applied as a pretreatment step to remove the solids contained. Thermal methods (evaporation, distillation, combustion, and pyrolysis) are mostly used to remove water, but possess high operational costs. The sustainable management of OMW also includes physicochemical methods, i.e., neutralization, precipitation, adsorption, oxidation, and electrocoagulation, which are less expensive but cannot diminish completely the pollution load of OMW. The bioremediation of OMW includes aerobic and anaerobic biological processes, mixing OMW with other agricultural wastes, and enzymatic processing of OMW, e.g., with laccases for phenol oxidation [7]. Combinations of biological methods with oxidation, evaporation/condensation, and composting, are also employed for OMW treatment [13–15]. Bioremediation of OMW via composting is an integrated olive oil waste management technique, and one of the main technologies for recycling OMW [16–21]. This process allows nutrients to return to the cropland, avoiding the drawbacks often observed when applying these wastes directly to soil. OMW absorption onto a solid substrate (lignocellulosic wastes or manures) prior to composting can also be applied. Finally, the co-composting of OMW with other wastes, e.g., poultry manure, has a demonstrated, significant, phenol content decrease [7].

Composts, being a good source of organic matter and fertilizing elements, such as calcium, magnesium, nitrogen, potassium, and phosphorus, ameliorate the structure, chemical and biological fertility, water retention, and cation exchange capacity of soils. They also favor microbial populations of the soil, impairing the development of soil-borne pathogens and reducing heavy metal bioavailability [22]. Thus, composts are of great interest for the rehabilitation of land impoverished by intensive farming and/or facing desertification [21]. However, traditional OMW composting is a time-consuming process that proceeds for months, requiring large land areas, whose end product may sometimes not be as expected, as the entire process is multiparametric. Owing to the complexity and heavy polluting load of OMWs, a single-stage biochemical treatment seems too difficult to achieve complete mineralization at a reasonable cost and can only be successful by menas of well-designed sequential chemical and biological processes with well-defined treatment objectives [15].

In the present work, an integrated solution for actual agro-industrial OMW management is reported based, on a new one-step method for OMW biotreatment. Our innovations primarily involve composting processes under microaerobic conditions for OMW biore-

mediation and the addition of a biocatalyst, i.e., a bacteria consortium from a green-waste compost, in order to achieve almost complete OMW detoxication and humic substances increase in a short biodegradation time. Finally, the bioremediated OMW produced was tested as a soil conditioner in agricultural applications.

2. Materials and Methods

2.1. Materials

Olive mill wastewaters were obtained from an olive oil mill in Rovies of Euboea, Greece, equipped with three-phase centrifugal decanters having an olive processing capacity of 1 ton h^{-1}. Wastes from a two-phase mill containing, in addition, the woody endocarp may also be used equivalently. Plant materials that remain in olive-mill plants and/or other green residues can also be added into the composting pile. Zeolite (90% clinoptilolite) with particle size up to 2 mm in diameter was used (Silver and Baryte Ores S.A., Athens, Greece). Dolomite was purchased from Ionian Kalk S.A., Athens, Greece. Peaty lignite was obtained from Horemi Mines (Megalopolis Basin, Greece) as the particular lignite field material was found rich in humic substances, i.e., over 40 wt.% of lignite on a dry basis [23–25].

2.2. Biocatalyst Preparation

The biocatalyst was prepared as reported previously [26] based on peaty lignite extract enriched with microorganisms. The modifications introduced were necessary in order to increase both the microbial populations and the nutrients, thus enhancing the efficiency of the biocatalyst on the OMW substrate. Specifically, plant residues (min. 1 ton), after being cut to pass a 5-mm sieve, were composted for three months. After the thermophilic stage, when temperature reached 40–45 °C, 10 L of the mixture was collected and mixed with 5 L of water and 0.5 L of powdered milk for extra nutrients. The pH was adjusted to the 7.5–8 range using dolomite (about 200 g). After 24 h, the mixture was stirred to homogenize it, and then, a sample of 1 L was collected every 1 d; ten samples were collected in total. The microbial populations were counted in all fractions received using the process described below. The samples richest in microorganisms proved to be the 3rd and 4th fractions (Table 1).

Table 1. Microbial populations during biocatalyst preparation.

Fraction No.	Microbial Populations (c.f.u. g^{-1})		
	Bacteria	**Fungi and Yeasts**	**Actinomycets**
1	2×10^3	5×10^2	2×10^2
2	6×10^4	2×10^4	4×10^3
3	6×10^6	3×10^6	2×10^4
4	8×10^6	5×10^6	3×10^6
5	2×10^6	4×10^6	3×10^6
6	2×10^6	4×10^6	3×10^6
7	2×10^6	4×10^6	3×10^6
8	2×10^6	2×10^6	2×10^6
9	2×10^6	2×10^6	2×10^6
10	2×10^6	2×10^6	2×10^6

Afterwards, the mixture was stirred for 48 h to further increase the microbial populations. If necessary, sodium pyrophosphate ($Na_4P_2O_7$) was used to adjust the pH to 7.5. Finally, 1 ton of raw peaty lignite (particle diameter less than 2 mm) was added to every 20 L of mixture and the whole was left for one week. The biocatalyst prepared as described above contains humic substances and minerals (30 and 38%, dry basis, respectively), and can be bagged and stored at 5 °C for more than one year. The microbial population contained therein consists of 10^7 colony-forming units/1 g (c.f.u. g^{-1}) of bacteria (total

mesophilic aerobic and spore-forming), 10^5 c.f.u. g^{-1} of fungi and yeasts and 10^7 c.f.u. g^{-1} of actinomycetes, on wet basis.

2.3. Bioremediation Process

In order to prepare the compost piles, the OMW was first mixed with green wastes from the oil mill plant (1:1 *w*/*w*). The green wastes were cut using a Vermeer, BC 1000 XL brush chipper, to 1–3 cm pieces. A biocatalyst (25 kg m^{-3}) and zeolite (10 kg m^{-3}) were then added, and the mixture was stacked to piles of about 20 m^3 (Scheme 1). Zeolite facilitates the growth of the microbial population of the biocatalyst and improves the soil quality thanks to its high cation exchange capacity and its enhanced surface area and porosity. Furthermore, natural zeolite (clinoptilolite) in a sewage sludge composting product was found to take up heavy metals, reducing their bioavailability [27].

Scheme 1. Formation of piles for olive oil mill waste (OMW) biotreatment.

The stacks were covered with a waterproof, gas-permeable fleece fabric of continuous polypropylene filaments. The main parameters characterizing both the OMW and the green wastes are summarized in Table 2. The addition of the biocatalyst plays a vital role in microaerobic biotreatment, i.e., vermicomposting, via the release of the microbial load contained, thus simplifying the process and allowing us to skip both analyses and conditioning of the microbial populations. The microaerobic environment was not accurately defined [28,29]; however, a value of 5% O_2 was reported [30]. The turning of the windrows was carried out with a loading machine whenever a temperature drop was observed to ensure proper aeration during the thermophilic stage. The wetting of the mixture was also carried out to keep the moisture levels within the range of 50–60%. This was important to keep the moisture levels high enough for composting and also prevent the nutrients from washing off. Sampling was carried out by collecting about 100 g of the composting mixture from five different spots of the pile, ten times in total. Prior to analysis, the sample particles were further crushed to pass a 12.5-mm sieve and then stored at 5 °C to inhibit any biological activity.

It should be stressed that the OMW did not undergo bioremediation by themselves; the basic physicochemical parameters remained practically constant during the first 20 d. Untreated OMW (control sample) decays with time and rot, having a high solid matter content, a dark color, an annoying odor, and physicochemical characteristics strongly related to organic pollution.

Table 2. Basic physicochemical parameters of olive oil mill waste before and after composting.

Parameter	OMW	Green Wastes (GW)	Compost	
			Initial	60-d Treatment
Moisture (%)	90.3	58.1	68.1	48.9
Electrical conductivity (dS m^{-1})	11	0.99	1.92	2.8
pH	5.48	6.85	5.7	7.3
Bulk density (kg L^{-1})	0.98	0.12	0.33	0.4
Ash (% *w*/*w*)	19.8	7.3	14.0	21.9
Organic matter (% *w*/*w*)	80.2	92.7	86.0	78.1
Total organic carbon (% *w*/*w*)	45.7	53.8	49.9	40.3
Total Kjeldahl nitrogen (% *w*/*w*)	1.7	2.0	1.9	1.3
Humic substances (% *w*/*w*)	-	1.8	2.8	8.5
Total phenols (mg kg^{-1})	374.3	93.2	280.3	32.3

2.4. Physicochemical Analysis

Moisture was estimated by drying the samples in a laboratory furnace at 110 ± 5 °C until constant weight [31].

For the pH determination [32], 5 g of the sample was mixed with 50 mL of distilled water, stirred for 1 h, and left to settle for 10 min. The pH value was calculated using a PHS-3D pH-meter (Beijing Jia Hua Zhong Xin Technology Co., Ltd., Beijing, China) device. Electrical conductivity (EC) measurements [33] were carried out similarly in 1:5 (*w*/*v*) sample:water mixtures with the use of a Konduktoskop E365B (Metrohm, Herisau, Switzerland) appliance.

The bulk density (BD) of the samples was estimated according to the pycnometer method [34] by weighing 100 mL of compost. For the calculation of the bulk density on a dry basis, BD_{db}, the following correction was applied,

$$BD_{db} = BD\frac{100 - \%\ \text{moisture}}{100} \quad (1)$$

Ash was determined by the combustion of dry samples at 825 °C K for 4 h [35]. Total organic matter (TOM) was calculated (a) as follows [36],

$$\%\ \text{organic matter} = 100\ -\ \%\ \text{ash} \quad (2)$$

and (b) following [37]; 1 g sample was first powdered and dried, as described previously, and then placed in a Thermolyne by Sybron 1500 Furnace at 100 °C. The temperature of the oven was increased to 330 °C K and kept constant for 24 h. TOM was determined as the percentage of the weight loss. Both methods applied ended with similar results and the mean value was used.

Total organic carbon (TOC) was determined after the digestion of the sample with an acidified dichromate solution [38].

$$2\ Cr_2O_7^{2-} + 3\ C + 16\ H^+ \rightarrow 4\ Cr^{3+} + 8\ H_2O + 3\ CO_2 \quad (3)$$

The TOC results obtained were about 55–58% of the TOM values [39].

The total nitrogen (TN) was determined using a Kjeldhal system according to the Wieninger method [40].

TOC and TN results were further used to define the C/N ratio, which is a biovailability indicator for the nutrients.

The determination of the total phenolic compounds was carried out by the Folin–Ciocalteu spectrophotometric method [41]. The isolation of soluble phenolic compounds was carried out by extraction into a methanol containing HCl solution (twice). The supernatant containing the phenols was collected in a Falcon test tube. Then saturated Na_2CO_3 and Folin-Ciocalteu reagent were added and the mixture afforded a blue color. The quantification of phenols was based on UV-vis absorbance readings (Cary 3E UV–

vis spectrophotometer) at 750 nm against a linear calibration curve of gallic acid. The concentration of phenolic compounds was then reported as a gallic acid equivalent.

For the extraction and determination of the humic substances contained in the compost samples, the following process was applied [23]: 2 g of the sample was placed in an Erlenmeyer flask, together with 40 mL of a 0.1 M $Na_4P_2O_7$/NaOH solution. The mixture was agitated with the use of an IKAKS 130 Basic device for 0.5 h and left for another 12 h. The humic substances were then separated in a T54 centrifuge, MLW, Leipzig, Germany (20 min at 3600 min^{-1}). For the quantification of humic substances, 1 mL of the solution obtained was diluted in 250 mL water and UV-vis absorbance readings at λ_{max} = 550 nm were acquired. All humic matter contents were calculated from a linear calibration curve established by plotting absorbance at λ_{max} against the humic substances concentration using standard humic substances derived from lignite. Dilute solutions were prepared as humic substances appear mostly colloidal at high concentrations.

For the heavy metal determinations, 0.200 g of the compost samples were added to a 5 mL HNO_3/HCl solution 3:1 *v/v* and the mixture was digested in a MARS EXPRESS microwave oven (CEM, NC, USA) at 800 W. The solution obtained was then diluted to a final volume of 50 mL. Pb, Cd, Cu, Cr, and Ni concentration measurements were carried out in a GFAAS-PERKIN ELMER 6000 (Shelton, CT, USA) graphite furnace, and Zn was processed via flame atomic absorption spectroscopy (FAAS-PERKIN ELMER 2380, Shelton, CT, USA).

All analyses are reported on dry basis and involve an error of ±8%.

2.5. Microbial Populations

The progressive dilution technique was employed for the determination of the microbial populations. For the extraction, 1 g of the sample was added to 10 mL of Ringer solution. The mixture was agitated for 30 min and then left to settle for another 15 min. Total mesophilic aerobic and spore-forming bacteria were cultivated on nutrient agar at 306 K and the colonies were counted after 2 d of incubation [26]. Bacteria accomplish the biotransformation of organic substrates; during composting they participate in the degradation of proteins, lipids, cellulose, and lignin. They are fast-growing microorganisms and their abundance increases during the mesophilic phase where the availability of easily useable organic substances exists, hence dominating initial decomposition. They play a key role in the degradation of nitrogen in composting, are linked with C/N variation and compost stabilization, and regulate humic substances formation [42]. Spore-forming bacteria, thanks to their ability to break down complex organic molecules and their tolerance to unfavorable conditions and environmental stresses, play a significant role in organic matter recycling. In addition, controlled OMW spreading was found to increase the spore-forming bacteria population in soil microbiota [43]. Filamentous fungi and yeast populations were determined on Sabouraud dextrose agar supplemented with streptomycine (0.03 g L^{-1}) [26,44,45], incubated for 7 d at 25 °C K. Actinomycetes were cultivated on a Rose Bengal Agar supplemented with Chloramphenicol (2 mL for each 500 mL of medium) to suppress bacteria and enumerated after 10 d of incubation at 28 °C. Colonies were stained intensely and uniformly pink pinpoint actinomycetes were observed [46].

Colonies of *E. coli* cultivated on MacConkey Agar with salt were counted after 24 h of incubation at 37 °C K. Thanks to their pink-to-red color, *E. coli* colonies can be easily identified and enumerated among other organisms also cultivated on the same medium, e.g., *Proteus* spp., *Salmonella* spp. and *S. Aureus*. In the case of *Salmonella*, the samples were counted after 24 h of incubation at 37 °C K. XLT 4 Agar [44] was used as the cultivation medium. *Salmonella* spp. colonies, after 18-24 hours incubation, appear as black or black-centered with a yellow periphery. If the plates are incubated further, the colonies will become entirely black, or pink to red with black centers.

All samples were run in duplicate in a sterile environment. Control cultures were also run in the absence of microbes studied. Each microbial population was counted in c.f.u. g^{-1}.

2.6. Plant Germination/Cultivation Experiments

For the germination experiments, lettuce (*Lactuca sativa* L.) was selected. The choice of seedlings was considered unnecessary, due to the high growth rate of lettuce and also because the initial stages of development were skipped. Thus, lettuce seeds were employed to obtain a meaningful estimation of the germination capacity of the various substrates (Laboratory of Ecophysiology, Department of Biology, National and Kapodistrian University, Athens, Greece).

Four different substrates were produced by mixing OMW compost and perlite; the amount of OMW compost in the substrate was 0, 33, 50, 100% (v/v), respectively. Before placing the samples into the containers, perlite and OMW compost were thoroughly mixed to homogenize. The room temperature was set at 18 °C, and the photoperiod was 16 h light and 8 h dark. Ten lettuce seeds were placed in each container in order to obtain statistically acceptable results. Based on the substrate saturation tests, it was decided to water each container with 70 mL of tap water. This process was repeated in triplicate. Blank samples were also prepared, consisting of 200-mL containers having exclusively perlite as the substrate. In order to calculate the growth rate, stems above 2 cm were cut, then dried at 105 °C for 24 h and weighed (dry basis). This process was repeated several times during the first 20 d from the day the first seed germinated.

For the chlorophyll determination, 5 g of leaves were weighed after the large nerves had been removed. Then the leaves were cut into small pieces (dimensions of a few mm) using a stainless-steel knife and placed into a porcelain mortar. A small quantity of $CaCO_3$ and 1–2 mL of acetone were added and the mixture was ground until the tissue became completely crushed and homogenized. Then the remaining quantity of acetone (out of 40 mL acetone in total) was poured gradually into the mortar, followed by light stirring. Thus, the photosynthetic dyes were extracted into the acetonic extract, which afforded a dark green color. Finally, the mixture was filtered and the volume of the extract was measured. All calculations were made with respect to this volume. The extracted pigments were diluted (tenfold the original volume) and measured spectrophotometrically at 665 and 649 nm against acetone as a blank [47].

The following empirical equations were used for the calculation of the chlorophyll content:

$$\text{Chlorophyll a } (\mu\text{g mL}^{-1}) = 11.63 \times A_{665} - 2.39 \times A_{649} \quad (4)$$

$$\text{Chlorophyll b } (\mu\text{g mL}^{-1}) = 20.11 \times A_{649} - 5.18 \times A_{665} \quad (5)$$

$$\text{Total Chlorophyll } (\mu\text{g mL}^{-1}) = 6.45 \times A_{665} + 17.72 \times A_{649} \quad (6)$$

Cultivation experiments were also carried out in the field (Union of Agricultural Cooperatives, Rethymno, Crete) to assess the OMW compost performance both alone and in comparison with traditional soil improvers, i.e., green waste (GW) compost and peat. Four farmers employed the OMW biotreatment product for the cultivation of tomato (*Solanum lycopersicum*, Solanaceae), cucumber (*Cucumis sativus*, Cucurbitaceae), melon (*Cucumis melo*, Cucurbitaceae) and watermelon (*Citrullus lanatus*, Cucurbitaceae) crops. The nurseries were planted in redhead on the line and the substrate in the plantation pit was shaped as follows (total substrate per plant 1000 g):

- 50 plants without any soil improvers (control).
- 50 plants with 250 g OMW compost added per plant.
- 50 plants with 250 g GW compost added per plant.
- 50 plants with 500 g OMW compost added per plant.
- 50 plants with 500 g GW compost added per plant.

The OMW biotreatment product was also employed in 100 flower beds of sensitive flora, i.e., palm trees and benjamin (*Ficus benjamina*, Moraceae) (Union of Agricultural Cooperatives, Rethymno, Crete). Two substrates were tested:

- 70% OMW compost: 30% redhead (v/v) (Substrate OMW).
- 70% peat: 30% redhead (v/v) (Substrate Peat).

The fertilization scheme stayed the same for all plants. All plants were irrigated with the use of an automatic watering system.

Again, in order to calculate the growth rate, stems above 2 cm were cut then dried at 105 °C for 24 h and weighed (dry basis). This was repeated several times during the first 40 d after plantation.

2.7. Statistical Analysis

The IBM SPSS Statistics v. 24.000 software package was employed for processing the experimental data. The statistical analysis included Pearson correlations and a one-way ANOVA at $p < 0.05$.

3. Results and Discussion

3.1. Physicochemical Characterization

The most important physicochemical parameters affecting OMW biotreatment were monitored systematically to determine the contribution of the biocatalyst to the process. The first thermophilic stage started shortly after the formation of the piles (2nd day), indicating the beginning of composting, and lasted about 13 days. Another four thermophilic stages were observed afterwards, influenced by the aeration and wetting of the pile that lasted about forty days in total (Figure 1). Thus, a considerable enhancement of the thermophilic stage and the concomitant bio-oxidative phase was achieved. Temperature increase effectuated the elimination of the pathogens.

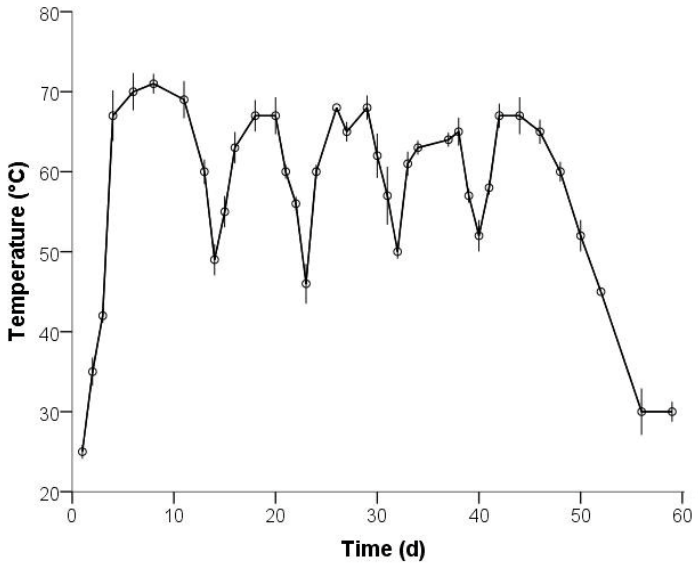

Figure 1. Temperature variance during OMW bioremediation. Error bars were placed at the 95% confidence level.

EC, pH, and C/N are considered key chemical variables to assess the successive stages of composting and the quality of the final product [48]. EC values, being an indicator of prevailing soluble salts and ions, seem to increase with the inorganic content of the compost (Figure 2a).

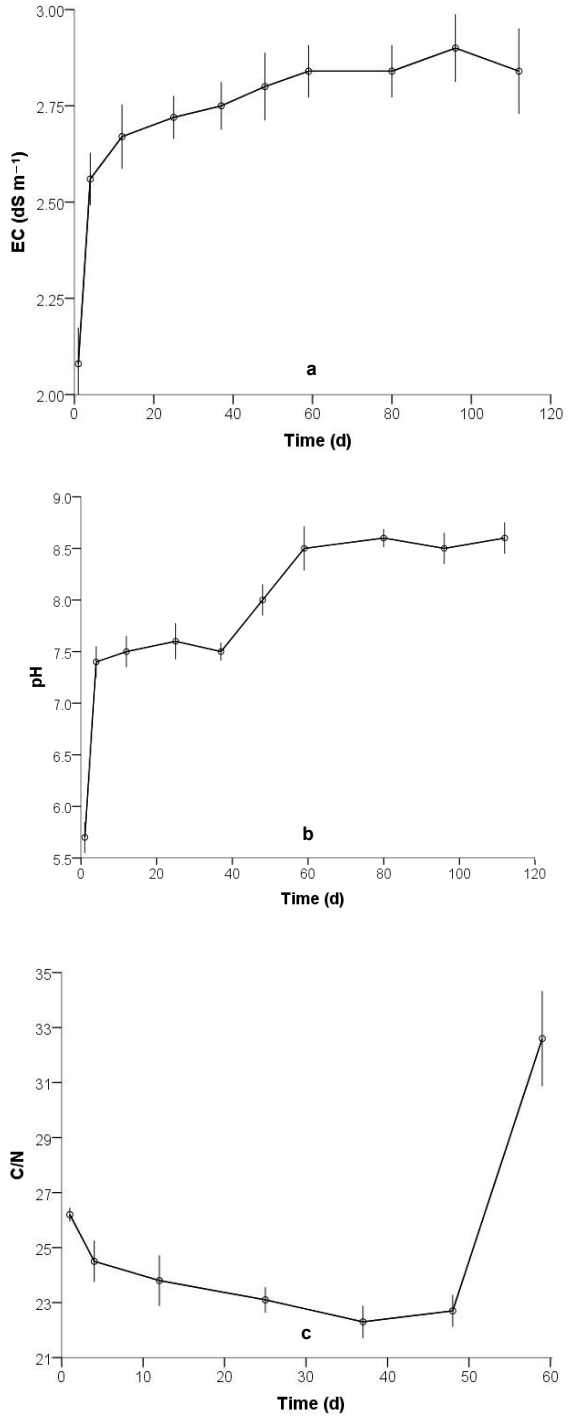

Figure 2. Time dependence of the EC (**a**), pH (**b**), and C/N ratio (**c**) values during bioremediation. Error bars were placed at the 95% confidence level.

This is to be expected as free ion availability increases with the presence of inorganic ions. In fact, EC values at about 2.8 dS m^{-1} after 60 days of processing were attributed mostly to slow release of nutrients. At this point, it is important to underline that high levels of salt near the root zone may inhibit both the growth and germination of plants, as the roots can no longer extract sufficient water from the environment solution. Salt tolerance varies among crops, for instance, bean, carrot, pea, and strawberry crops are affected by electrical conductivity of only 1 dS m^{-1}, while barley, cotton, and wheat can tolerate 8 dS m^{-1} of electrical conductivity before a reduction in yield is observed [49].

Measurements after the 60th day appear in Figures 2–5, indicating a mature compost. Thus, the piles were not further agitated.

The pH value affects composting by influencing the activity and nature of the microbial populations and by controlling the availability of nutrients to microbes. During the first forty days of the bioremediation process, the pH was mostly found to be near 7.5 (Figure 2b), a value considered optimal for most bacteria. The pH rise observed afterwards is attributed to NH_3 produced during the degradation of organic nitrogen and organic acids. Such a pH value, also indicating stability and low phytotoxicity, is important to the quality of the product, especially if the compost is intended for agricultural applications.

The initial C/N ratio (Figure 2c) was found to be 26 and fell into the 25 to 30 range, which is considered ideal for proper microbial action during composting [50], indicating availability of carbon and high decomposition rates to receive a qualitative end product. The C/N decrease with time accounted for the decline in the biodegradation rate. The remarkably increased C/N ratio received in the end can be ascribed to the nitrogen released by the compost, most probably as ammonia. Figure 3a,b exhibit a lucid rise of the bulk density and ash content values (both calculated on dry basis) with time. This is to be expected as, prior to the final stabilization, biodegradation involves processes which lead to condensation of the loose and bulky material as well as the mineralization of the compost [51].

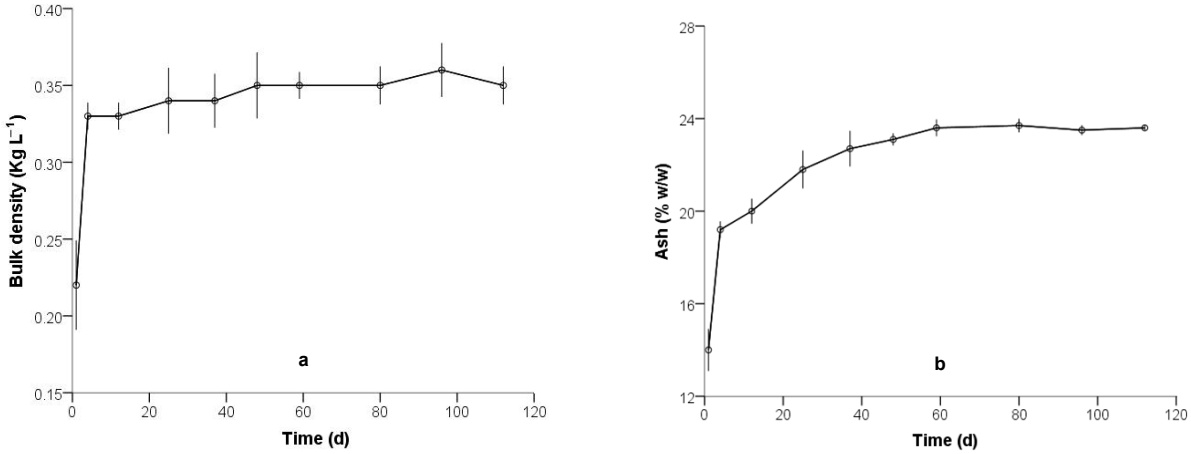

Figure 3. Variations in the bulk density (**a**) and ash content (**b**) of the biotreated OMW with time. Error bars were placed at the 95% confidence level.

The biodegradation progress is further observed in Figure 4a–c, showing the decline in the organic matter, total organic carbon, and total Kjeldahl nitrogen, respectively, also compatible with the above findings. The low TKN value (~1.3% w/w) at day 60 can explain the sudden C/N rise observed (Figure 2c). It is important to note that after day 60, both TOC and TKN were stabilized, and all three parameters, i.e., TOC, TKN and C/N, have been reported in the literature as maturation indices.

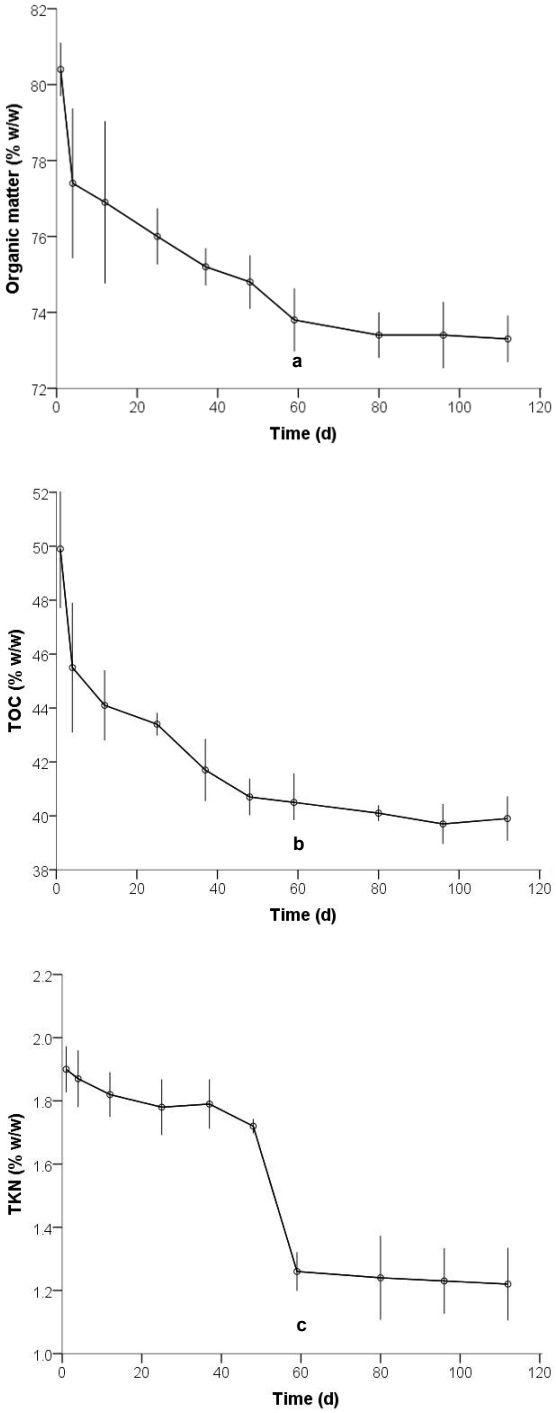

Figure 4. Variation in (**a**) the organic matter, (**b**) TOC, and (**c**) TKN values of the compost with time. Error bars were placed at the 95% confidence level.

Figure 5a displays a significant humic substance content (8.5% *w*/*w*) as a result of the humification processes occurring during the microaerobic biotreatment of the OMW. FTIR analysis verified that the composting of OMW includes a loss of aliphatic and peptidic structures and leads to an increase in the aromatic structures, confirming humification [52,53]. The highest humification rate was marked during the early stages of composting, a feature also observed in a previous study of green waste composting with the use of biocatalysts [26]. In traditional composting, the humification index increases at a later stage, during the maturation phase of the composting [54]. These facts demonstrate that not only the biocatalyst but also the periodic aeration and wetting all accelerate OMW composting.

Humic substances increased slowly towards the end of the biotransformation; thus, the humic substances content may serve as a maturity index [26]. To the authors' knowledge, this is one of the highest humic contents ever produced in an OMW bioremediation processes [26]. Humic substances are electron shuttling compounds ubiquitous in soils and sediments, accounting for almost one-third of global soil carbon. They are chemically resistant components that induce biostimulation effects in plant species, and play a vital role in sustainable agriculture as well as in immobilization and transport of nutrients and anthropogenic chemicals [55]. Regarding the transport of nutrients in particular, the great potential of HS in the field of matrix-assisted synthesis of metal-containing nanoparticles has been demonstrated [56] to produce nanoparticles with superparamagnetic properties of enhanced bioavailability to plants.

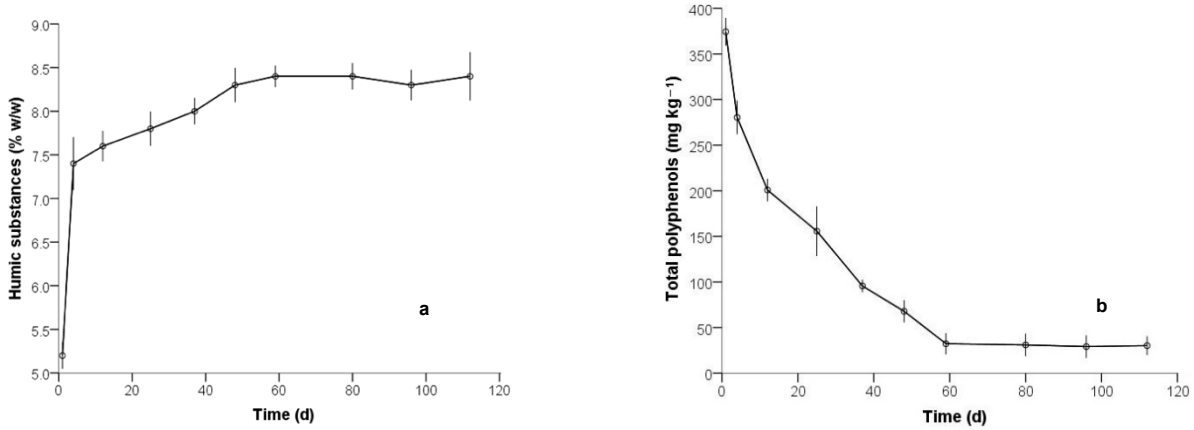

Figure 5. Humic substances (**a**) and polyphenols content (**b**) variation with time during bioremediation. Error bars were placed at the 95% confidence level.

In addition, the total polyphenols content was decreased substantially (Figure 5b). It is of significance that the polyphenols content was reduced more than 90% during the first 60 days of composting, a finding that can be attributed to the presence of the biocatalyst. Control samples run without biocatalyst did not show any phenol breakdown. As already mentioned, these samples did not bioremediate at all. Polyphenols represent the most severe pollutants, introducing negative effects to both soil properties and plant growth such as increase in the mineralization rate of native soil organic C, the induction of anaerobic conditions, the release of phytotoxic substances, and the microbial immobilization of plant nutrients [8,9]. Both the humic substances and polyphenols contents (Figure 5) may serve as maturation indices, indicating stabilization, and they are vital for the applicability of the end product to real field applications. Actually, the microbial activity induced to the composting mixture by the biocatalyst facilitates the breaking down of phenols and the bioremediation of the compost as a whole. In ECOLABEL and Greek regulations (Table 3), there are no limits regarding the polyphenol contents. However, target limits have been set in previous bioremediation studies [57], i.e., 57 mg kg^{-1}. Our experiments show (Figure 5b) that after 60 days of bioremediation, the polyphenols content (~30 mg kg^{-1}) was well below the limit mentioned above and, that it remained almost constant between day 60 and day 120.

Statistical calculations showed that all data presented above were normally distributed and had homogeneous variances. Since these assumptions are satisfied, the means were compared using one-way ANOVA. Table 4 demonstrates that all the data presented in Figures 1–5 present statistically significant differences. In addition, all experimental data were subjected to statistical analysis to detect any stealthy correlations among the physicochemical parameters determined. The results indicated that the humic substances content linearly followed the trend of the EC, ash, bulk density, and pH values, displaying high correlation coefficients ($r > 0.9$, $p \leq 0.005$) while it was inversely correlated to TKN, TOC,

organic matter, and phenols. These observations verify the increase of the humic substances content along with the composting process. The opposite trend was detected for polyphenols, i.e., they were found to be directly related to the TOC and organic matter contents, and inversely correlated with the EC, pH, ash, and bulk density, confirming a reduction in the polyphenols content over time. The bulk density, EC, pH, and ash were also found to be closely interrelated, showing similarly high correlation coefficients.

Table 3. Evaluation parameters for the bioremediated OMW compared to those of the Greek (Governmental Ministerial Decision 56366/4351, 2014) and ECOLABEL (Commission Decision (EU) 2015/2099 of 18 November 2015) regulations for soil conditioners.

Parameter (Dry Basis)	OMW Soil Conditioner	Greek Regulations	ECOLABEL Regulations
Pb (mg kg^{-1})	0.05	300	100
Cd (mg kg^{-1})	0.18	3	1
Cr (mg kg^{-1})	0.10	250	100
Cu (mg kg^{-1})	40	400	100
Ni (mg kg^{-1})	28	100	50
Zn (mg kg^{-1})	123	1200	300
Hg (mg kg^{-1})	-	2.5	1
As (mg kg^{-1})	-	10	
PCBs (mg kg^{-1})	-	0.4	
PAHs (mg kg^{-1})	-	3	6
Salmonella spp. (c.f.u. g^{-1})	0	0	0
Admixtures > 2 mm (%)	1.8	3	
Moisture (% *w*/*w*)	36	40	

Table 4. Statistical processing of data in Figures 1–5.

Parameter	t [1]	df [2]	Sig. (2-Tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Temperature (°C)	66.233	189	0.000	57.053	55.35	58.75
pH	64.224	49	0.000	7.7900	7.546	8.034
Bulk density (kg L^{-1})	57.598	49	0.000	0.33200	0.3204	0.3436
Ash (% *w*/*w*)	51.065	49	0.000	21.5200	20.673	22.367
Organic matter (% *w*/*w*)	228.59	49	0.000	75.4600	74.797	76.123
TOC (% *w*/*w*)	92.345	49	0.000	42.5500	41.624	43.476
TKN (% *w*/*w*)	37.821	49	0.000	1.58300	1.4989	1.6671
C/N	43.208	34	0.000	25.02857	23.8514	26.2058
Humic substances (% *w*/*w*)	58.121	49	0.000	7.7800	7.511	8.049
Total polyphenols (mg kg^{-1})	7.855	49	0.000	129.7290	96.540	162.918
EC (dS m^{-1})	80.590	49	0.000	2.70000	2.6327	2.7673

[1] The test statistic of the one-sample t test, denoted t. [2] The degrees of freedom for the test.

To further establish the applicability of the compost in agricultural cultivation, the final product was tested for heavy metal content. Heavy metals vary with the origin of wastes to be processed, remain unaffected during biotreatment, and increase in content with the volume reduction of wastes during biodegradation, thus having adverse effects on living organisms. Table 3 demonstrates the concentrations of heavy metal ions in comparison to Greek Government limits. It should be emphasized that all metal contents fell within the permissible limits, another fact that makes the OMW biotreatment product suitable for biological agriculture applications.

In summary, the physicochemical parameters studied indicate that OMW were successfully bioremediated after a 60-d treatment. Thus, biodegradation time is considerably shortened, and no need for OMW pretreatment, dilution or oxidation exists. The method proposed requires no reactors of any type, is simple and economic, is easily upgradable at a large scale, and applicable in situ employing conventional agricultural equipment (Scheme 2). Finally, this process produces neither the waste sludge of the aerobic processes nor the toxic compounds of anaerobic digestion [7]; instead, it ends up producing a first-class soil conditioner that qualifies for the ECOLABEL and can be readily applied in organic farming. The product obtained can be bagged and stored in sheltered and ventilated places at ambient temperature for more than a year. During this period of time, the physicochemical characteristics of the product obtained remain unaffected.

Scheme 2. Production of soil conditioner from OMW bioremediation on the large scale.

3.2. Microbial Populations

Total mesophilic aerobic and spore-forming bacteria were calculated at 2.3×10^7 c.f.u. g^{-1} at 60-d of treated OMW bioremediation product. This cenose plays a crucial role in soil vitality, nutrient immobilization and release, the retention of soil structure, suppression of plant diseases, resistance and resilience of land, water balance into soil, degradation of organic matter, humus accumulation, greenhouse effects, and C sequestration. These are of importance, given that a typical fertile agricultural soil contains about 5×10^5 c.f.u. g^{-1} of bacteria and fungi, respectively [58], i.e., the product obtained contains at least 100-fold the microorganisms of typical fertile agricultural soil. Additionally, cultivation experiments to determine *E. coli* and *Salmonella* populations revealed the absence of pathogens in the original pile. Only 48 c.f.u. of *E. coli* per 100 g were counted in the stabilized compost (dry basis), a fact showing that the compost obtained is readily applicable in real field applications. The lack of pathogens can be attributed to both the prolonged thermophilic stage and polyphenol content that suppresses the viability of those populations.

3.3. Germination/Cultivation Experimentes

Germination experiments were carried out to monitor the effect of the biotreated OMW in Lactuca sativa L. The compost was added to perlite at the following ratios (v/v): 0, 33, 50, 100%. Three basic parameters were studied, i.e., germination, total chlorophyll and the growth rate. Germination and total chlorophyll content were measured 10 d after the seeds were placed in the containers, while the growth rate was calculated 20 d afterwards. Both the germination and growth rate results (Figure 6a,c) demonstrate that the substrate containing 33% bioremediated OMW shows slightly decreased performance compared to perlite within the 10-day period of study. Nevertheless, after 17 d, all seeds in 100%

OMW compost germinated. This was not the case for the total chlorophyll (Figure 6b). The substrates with 100 and 33% OMW compost contents, respectively, exhibited the highest chlorophyll contents.

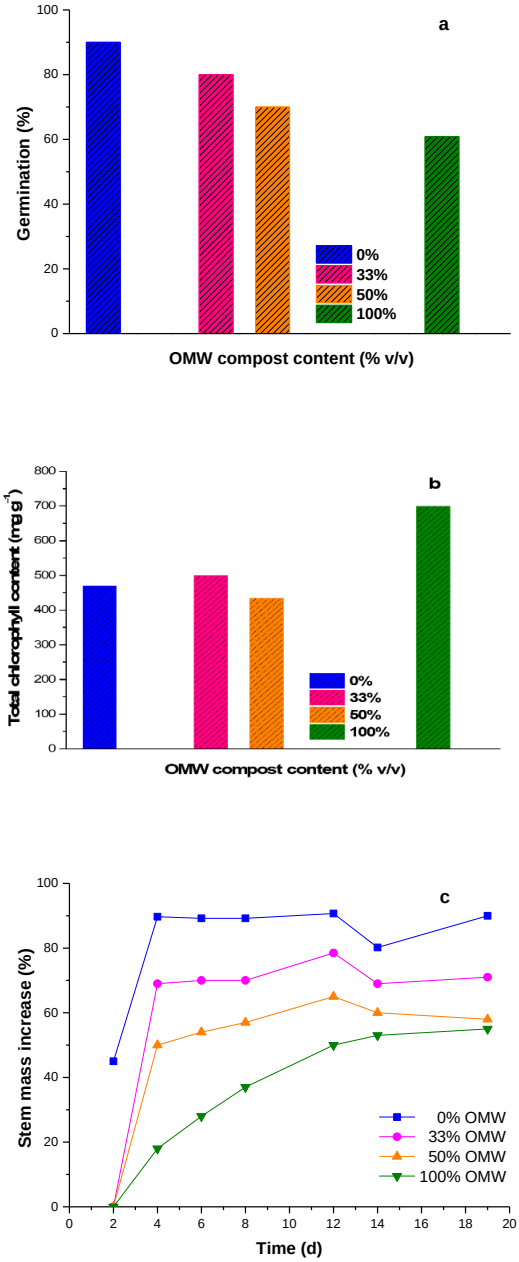

Figure 6. Germination (**a**), total chlorophyll content (**b**), and growth rate (**c**) of *Lactuca sativa* L. in substrates with different OMW biotreatment contents.

Statistical calculations showed that all sample germination, total chlorophyll content and growth rates were normally distributed and had homogeneous variances. Since these assumptions are satisfied, the means were compared using one-way ANOVA. In all cases, a statistically significant difference was observed (Tables S1–S3).

Finally, real field cultivation experiments were carried out to assess the performance of biotreated OMW in comparison with traditional soil improvers, i.e., green waste compost and peat. Figure 7a exhibits clearly that, for identical compost quantities, bioremediated OMW provides similar results to those obtained from green waste compost, i.e., both products proved equally beneficial to plants. The same conclusion stands, also, in the

case of flower cultivations (Figure 7b) where bioprocessed OMW and peat exhibited comparable efficacy.

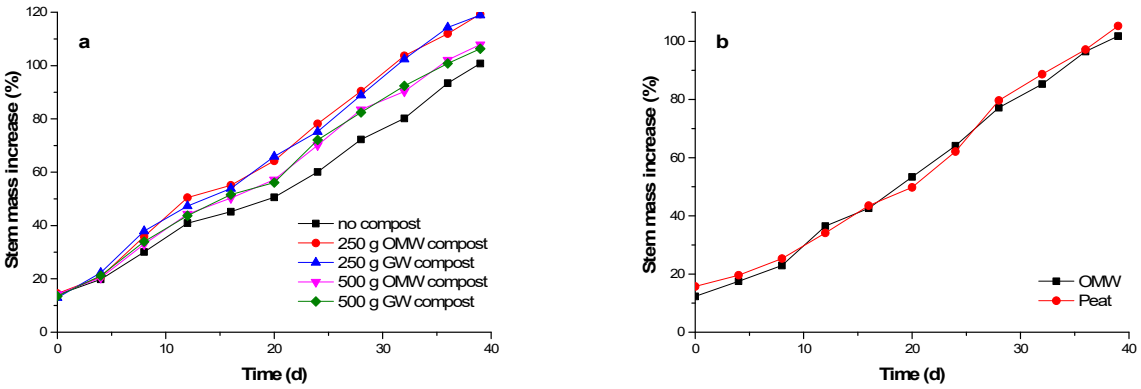

Figure 7. Comparison of stem mass increase in (**a**) vegetables, (**b**) flowers for real field application experiments.

The above experimental data were statistically processed; all samples were normally distributed and the means were compared using one-way ANOVA (Table S4). The results demonstrate that stem mass increase in vegetables cultivated in substrates containing green waste compost did not show any statistically important difference compared with vegetables grown in substrates containing the same quantity of OMW. Analogous results were obtained also in the case of stem mass increase for flowers cultivated in peat or OMW.

Thus, the valorization of degraded and toxic OMW biomass was achieved; bioremediated OMW can be used as a first-class soil conditioner in real field applications, suitable for biological cultivations and organic farming.

4. Conclusions

A new single-stage green biotechnological treatment of actual OMW was developed. The innovations of this method include both the application of microaerobic composting processes and the addition of a tailor-made biocatalyst that is extremely rich in soil microorganisms, operates at a wide pH range, prolongs the thermophilic stage, and accelerates biochemical reactions during OMW bioremediation. Zeolite was also added.

The most significant physicochemical parameters, i.e., electrical conductivity, pH, C/N ratio, specific weight, ash, organic matter, total organic carbon, total Kjeldahl nitrogen, and humic substances were measured to systematically monitor the composting process. The proposed method requires no OMW pretreatment, is simple and rapid, of low cost in both investment and operation, is easily scalable, and provides an integrated solution for OMW valorization.

The OMW compost received after a 60-day biotreatment was stable and detoxified, free of phenols and pathogens. Most importantly, it afforded a rich cenose and high humic substances content, vital for soil fertility. The application of biotreated OMW, both in the laboratory and in field cultivations, confirms that this product can be useful as a first-class soil conditioner suitable for organic farming.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11061114/s1, Table S1: Statistical processing of lettuce germination data using One-Way ANOVA, Table S2: Statistical processing of lettuce chlorophyll data using One-Way ANOVA, Table S3: Statistical processing of lettuce growth rate data using One-Way ANOVA, Table S4: Statistical processing of real field growth rate data using One-Way ANOVA.

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