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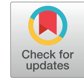
Bioremediation of Olive Mill Wastewater sediments in evaporation ponds through *in situ* composting assisted by bioaugmentation



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HIGHLIGHTS

- The community of polyphenol degrading microorganisms dominates OMW sediments.
- Novel fungal consortium isolated from OMW sediments is used for its bioremediation.
- *In situ* composting eliminated the ecotoxicity and phytotoxicity of OMW sediments.
- The fungal consortium accelerated the bioremediation efficacy of *in situ* composting.
- Compost from bioaugmented OMW sediments had an improved phytostimulant effect.

GRAPHICAL ABSTRACT

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ABSTRACT

The common method for the disposal of olive oil mill wastewater (OMW) has been its accumulation in evaporation ponds where OMW sediments concentrate. Due to the phytotoxic and antimicrobial effect of OMW, leaks from ponds can pollute soils and water bodies. This work focuses on the search for microorganisms that can be used as inocula for bioremediation of polluted matrices in OMW ponds by means of *in situ* composting. Two fungi isolated from OMW sediments, *Aspergillus ochraceus* H2 and *Scedosporium apiospermum* H16, presented suitable capabilities for this use as a consortium. Composting eliminated the phyto- and ecotoxicity of OMW sediments by depleting their main toxic components. Inoculation with the fungal consortium improved the bioremediation efficacy of the technique by hastening the decrease of phytotoxicity and ecotoxicity and enhancing phytostimulant property of compost produced. This procedure constitutes a promising strategy for bioremediation of OMW polluted sites.

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1. Introduction

The olive-oil processing industry is one of the most successful foodstuffs industries around the Mediterranean basin. In the last decades, the production of olive oil in the Mediterranean countries reached

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about 2.5 million tons per year, accounting for 90% of world production (Galliou et al., 2018). This agroindustry also produces large amounts of liquid and solid residues coming from the olive milling and further decantation, which poses significant environmental concerns and, therefore, should be adequately managed.

The characteristics of the olive oil industry residues vary depending on the system used in the olive oil extraction, which has changed over the time. Between the 1970s and the 1990s, one of the most used olive oil extraction system was the three-phase decanter, which generates olive oil, solid olive pomace residue and liquid olive mill wastewater (OMW—3P). From the 1990s, a more efficient extraction process was developed consisting of a two-phase decanter that allowed minimizing the volume of spent water and produces olive oil and semisolid olive mill waste (OMW—2P). Although the three-phase method is not being used as much as before, the large accumulation of OMW derived from these systems remains a challenge today. In addition, it is estimated that nowadays 10–30 million m^3 of OMW-3P are still generated each year (Souilem et al., 2017). The difficulty in handling and eliminating these wastes is linked to serious environmental issues, thus it is even now an unsolved problem (Zerva et al., 2017).

OMW-3P (OMW hereupon) is a liquid emulsion with a strong odor and dark brown to black color, which is composed of 50–94% water, 0.4–2.5% mineral salts, and 4–16% organic fraction (Hachicha et al., 2009). This fraction contains between 2 and 5% phenolic compounds, mainly phenols such as tyrosol and polyphenols such as secoiridoids and flavonoids (Dermeche et al., 2013). Although those compounds are lately known to have a wide number of applications upon extraction (Rahmanian et al., 2014), for example as antioxidants in the food industry (Galanakis, 2018), its uncontrolled disposal may cause serious environmental problems because of their high hydrophilicity, low biodegradability, and antimicrobial and phytotoxic effects (Senol et al., 2017).

The characteristics of OMW make difficult to apply a cost-effective treatment method (Galliou et al., 2018). The common practice for its management in Mediterranean countries has been the storage in evaporation ponds. However, with this management strategy, these effluents are transformed into a more polluting and recalcitrant solid sediment in which toxic compounds are concentrated. In addition, construction of evaporation ponds rarely meets engineering criteria for safe accommodation of liquid wastes, therefore, OMW often overflows or leaks and pollutes neighboring agricultural and underneath soils and surface- and groundwater (Kavvadias et al., 2017). Therefore, there is an urgent need to provide methods for the restoration of areas affected.

Besides the potential use of liquid OMW for agronomic applications (Belaqziz et al., 2016), several physical, physicochemical and biological strategies have been proposed for their treatment (Dermeche et al., 2013). However, most of them are too expensive and unsuitable for the treatment of sediments and soil matrices polluted in evaporation ponds, because they usually require the material to be collected and transported for *ex situ* treatment. Also, there is a lack of literature regarding the treatment and characteristics of the OMW sediments contained in evaporation ponds that are expected to be somewhat similar to the original liquid OMW from which they derive. Here we propose *in situ* composting of OMW sediments as a means of not only bioremediate polluted materials in evaporation ponds but also recycling them into compost. In fact, composting is an efficient method for the biotransformation of recalcitrant compounds such as phenols and polyphenols (Souilem et al., 2017) and it has been successfully applied for OMW treatment when co-composted with other organic wastes producing high quality compost (Asses et al., 2018). For this reason, the *in situ* composting of OMW sediments in evaporation ponds can be a sustainable way to re-use these residues along with other organic wastes while simultaneously solving their negative environmental effects.

Composting usually performs well with indigenous microbial communities. However, the insufficient quantity or low activity of these communities because of the low biodegradability or high concentration of toxic compounds in materials such as OMW may lead to low efficiency and poor compost quality (Xu et al., 2018). In order to cope with this constraint and to improve abatement of recalcitrant or toxic components from OMW, it has been proposed to implement this strategy by bioaugmentation with specific microorganisms (Parotta et al., 2016). Several fungal species have proven to be effective for the aerobic treatment of OMW, being *Aspergillus* one of the most prominent genera related to the reduction of the polyphenolic compounds, chemical oxygen demand and color of OMW (Salgado et al., 2016). Recently, this fungal genus along with *Mucor* and *Penicillium* has received considerable attention for their OMW bioremediation potential which is attributed to their enzymatic capabilities (Abd EL-Aziz et al., 2015). Despite the progress in the biological treatment of OMW, the search for novel microbial strains able to eliminate the toxic effects of phenolic compounds is of significant scientific, environmental and industrial interest.

The aim of this work was to select microorganisms that could be used in bioaugmentation strategies to improve bioremediation of polluted sediments and soil matrices in OMW evaporation ponds by means of *in situ* composting. In order to carry out this study, the following goals were established: (1) to isolate, select and characterize microorganisms from OMW sediments that could be suitable for bioremediation of OMW polluted sites, and (2) to prove the efficacy of selected inoculum for the improvement of bioremediation capabilities of *in situ* composting of polluted material from OMW evaporation ponds.

2. Material and methods

2.1. Description of study area and sampling

The study was conducted in OMW evaporation ponds located in Mora municipality (Toledo, Spain) (39°40′ 07.4″ N 3°49′ 40.2″ W). The facility occupies 5 ha and includes eight evaporation ponds with dimensions between 2400 m^2 and 12,000 m^2, whose use for OMW disposal started in 1982 and ended in 2007. Currently, they are no longer used, most ponds contain solid concentrated OMW sediments, and leachates are expected to affect the surrounding area because some ponds are not waterproofed. In this work, OMW sediments from seven ponds that were dry enough to hold mainly sediments, were collected for analysis of microbiota (bacteria and fungi) and isolation of microorganisms that were further screened for inoculum selection for bioaugmentation experiments. In addition, one pond, containing the partially dried residue, having a total surface of 2400 m^2 was used as the experimental site for bioremediation through *in situ* composting. In order to characterize initial status of the material contained in this pond, the site (80 m × 30 m) was divided in a grid of 18 sampling points, each separated 10 m, and samples were collected at surface (0–45 cm) and depth (45–90 cm). Phytotoxicity, ecotoxicity and total water-soluble phenolic compounds were analyzed in these samples as explained in analytical.

2.2. Selection and characterization of inoculum for bioaugmentation

2.2.1. Counts of total and polyphenol-degrading microorganisms in ponds

Samples from seven pounds were used to estimate the ratio of microorganisms capable of biodegrade polyphenol compounds (microorganisms with polyphenol-oxidase activity) with respect to total microbial load. Five grams of each sample were suspended in 45 mL sterile saline solution (0.9% *w*/*v* NaCl) and shaken for 30 min at 120 rpm, after ten-fold serial dilutions, 100 μL of appropriate dilutions were spread out on Plate Count Agar (PCA) (Panreac) for total bacteria colony counts and Rose Bengal Chloramphenicol Agar (RB) (Panreac) for total fungi colony counts. For polyphenol-degrading microorganisms counts, dilutions were inoculated in PCA and Potato Dextrose Agar (PDA) (Panreac) added of tannic acid (Sigma) (0.5% *w*/*v*) for bacteria and fungi, respectively. In this case, colonies surrounded by brown halo were counted. After incubation at 30 °C for 48 h (bacteria) or

96 h (fungi), colonies were counted. The ratio of polyphenol-oxidase bacteria and fungi was expressed as a percentage of total bacteria and fungi counts, respectively.

2.2.2. Isolation and selection of OMW-degrading microorganisms

For the isolation of OMW-degrading microorganisms two procedures were used in parallel: i) direct inoculation of samples in different selective solid media; ii) preliminary enrichment of samples and further isolation in solid media containing tyrsosol. In the first case, microorganisms having polyphenol, phenol and lignin-related degrading capabilities were detected by spreading 100 μL of ten-fold serial dilutions of samples prepared as above on Petri dishes containing RBBR, PCA-TAN or PDA-TAN solid media. In the case of media containing tannic acid (PCA-TAN and PDA-TAN), the polyphenol-oxidase activity is detected, while in RBBR medium, it is the lignin degrading capability what is identified because the medium includes remazol brilliant blue R, an anthraquinone dye whose decolorization is related to the capability to degrade lignin (López et al., 2006). RBBR medium consists of (L^{-1}): 10 g glucose, 5 g peptone, 2 g yeast extract, 200 mg RBBR dye (Sigma), 20 g agar and 70 mL of trace element solution (Kirk et al., 1986). PCA-TAN and PDA-TAN contain the polyphenol tannic acid (0.5% *w*/*v*) and were prepared as previously mentioned. After incubation at 30 °C for 48 h (bacteria) or 96 h (fungi), colonies showing brown color around growth area in PDA-TAN and PCA-TAN media and those decolorizing blue RBBR medium were selected and pure cultures were obtained from each (Fig. 1). In parallel, isolates were also obtained by enrichment of samples in liquid medium and further streaking on tyrosol-containing solid media (TYR). For these enrichment cultures, 5 g of sample were placed in 250 mL Erlenmeyer flask containing 45 mL minimum mineral solution (MMS) (Janshekar et al., 1982) amended with (L^{-1}) 10 g glucose and 2 g yeast extract. Two enrichment cultures were prepared for each sample and incubated at 30 °C and 50 °C, respectively. After incubation at 120 rpm for 21 days, colonies were isolated by streaking on Petri dishes with TYR medium, which is composed of minimum mineral solution (MMS) added of (L^{-1}) 2 g yeast extract, 20 g agar and filter sterilized solution of tyrosol (Sigma) at a final concentration of 0.05% (*w*/*v*). After incubation at 30 °C or 50 °C for 48 h, pure cultures of colonies growing in this medium were obtained. Isolates included fungi and bacteria, from whom filamentous actinobacteria were differentiated by their visible pseudomycelial growth and microscopic features. All isolates were preserved on slants at 4 °C and cryoballs (Cryoinstant Deltalab) at −80 °C.

Pure cultures of the isolated microorganisms from the two procedures were tested for RBBR decolorization, polyphenol oxidase activity, ligninolytic activity and for the detection of several enzymes related to lignin and phenols degradation (laccase, peroxidase, tyrosinase, and extracellular oxidase) as described by López et al. (2006). Three replicates were used for each test.

In addition, the capability of isolates to use tyrosol, the main phenolic compound in OMW (Senol et al., 2017), as the sole carbon and energy source was quantified in liquid medium. For this, each isolate was inoculated in sterile 48 wells microplates (Thermo Scientific) containing 500 μL liquid medium composed of minimum mineral solution (MMS) added of filter-sterilized solution of tyrosol (Sigma) at a final concentration of 0.05% (*w*/*v*). Inoculum consisted of 2–3 colonies of bacteria cultured on PCA for 24 h or 1 cm^3 agar plug from fungi cultured on PDA for 5 days. Non-inoculated wells were used as blank control. Three replicates were prepared per isolate and blank. Microplates were incubated for 7 days at 30 °C and tyrosol concentration was quantified afterwards using Folin-Ciocalteau reagent according to modified protocol of Sánchez-Rangel et al. (2013). Briefly, 255 μL of each well were transferred to a 96 well microplate (Thermo Scientific) and added of 15 μL Folin-Ciocalteau reagent (Sigma) and 30 μL of 1N Na_2CO_3. After 1 h in darkness, the absorbance was measured at 625 nm in a microplate reader spectrophotometer EON (Biotek). Tyrosol concentration was obtained by using tyrosol as standard (0–500 μg mL^{-1}). The results were expressed as tyrosol degradation percentage by comparing the tyrosol concentration after incubation with those of blank controls.

2.2.3. Identification of isolates

Selected isolates were identified by molecular methods based on amplification and sequencing of 16S rRNA gen for bacteria using universal primers 27F (50-AGAGTTTGATCATGGCTCAG-30) and 1492R (50-GGTTACCTTGTTACGACTT-30) and 5.8S-ITS ribosomal region for fungi using primers ITS1 (50-TCCGTAGGTGAACCTGCGG-30) and ITS4 (50 TCCTCCGCTTATTGATATGC-30) according to methodology used in Jurado et al. (2014). The forward and reverse sequences were edited, assembled and aligned using the programs Sequence Scanner v1.0 (Applied Biosystem), Reverse Complement (www.bioinformatics.org/sms/rev_comp.html), Clustal X v2.0.11, and MEGA 5 v5.2. The partial or nearly full-length sequences were compared for similar nucleotide sequences with the BLAST search of the National Center of Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

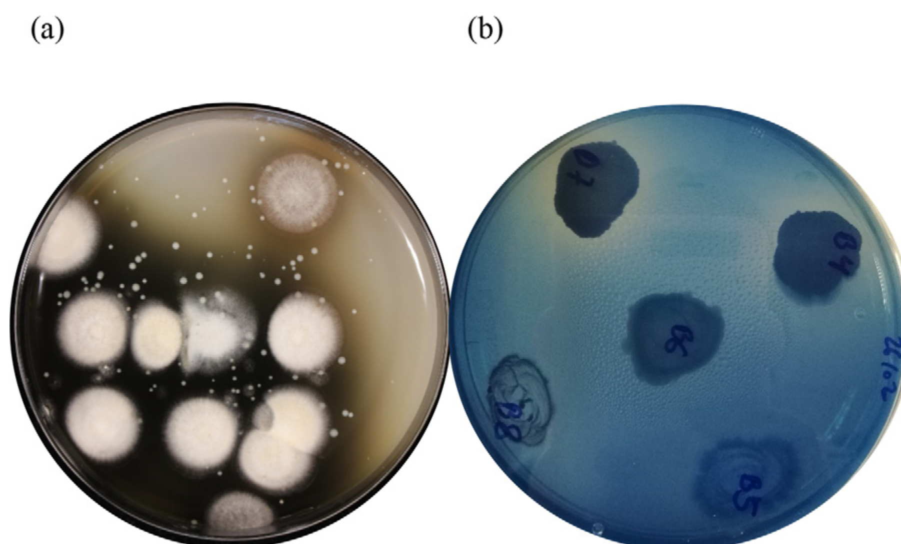

Fig. 1. a) Colonies of microorganisms showing polyphenol-oxidase activity is indicated by dark-brown color in PDA-TAN media; b) Colonies of RBBR-degrading microorganisms show a transparent halo in RBBR medium.

2.2.4. Characterization of inoculum for biaugmentation

Two fungi were selected for their use as inocula for bioaugmentation and a series of additional tests were performed in order to ascertain their compatibility and thermal growth range tests. In the case that more than one isolate was selected, it would be necessary to determine if there was any inhibitory effect of one isolate against the other for later jointly incorporation for bioaugmentation as a consortium. To ascertain this, dual confrontations of the isolates were performed on PDA media by placing a 0.5 cm diameter plug of a five-day old culture of each fungus on PDA. After incubation for 7–10 days at 25, 30, 40 and 50 °C, the presence or absence of mycelium overlaps was visually determined. In addition, the two fungi were incubated separately at the different temperatures and the mycelium diameter was measured after four days. Three replicates were used for each temperature and measurement.

2.2.5. Production of inoculum for biaugmentation

The selected strains were produced in large quantities in order to be applied for bioaugmentation strategies in field experiments. Therefore, the two selected fungi were cultured separately in sterile glass Petri dishes of 20 cm diameter with 80–200 mL of Tryptone Soy broth (TSB) (Panreac) which were inoculated with 5 plugs of fungus cultured on PDA for 5 days. After incubation in static culture for 7–10 days at optimum temperature for each isolate, the mycelium biomass was collected. The biomass collected from each microorganism was vacuum packed in aliquots of about 67 g dry weight. Viability of the inoculum was determined by suspension of 1 g biomass of each microorganism in 900 μL of sterile saline solution (0.9% *w*/*v* NaCl). After ten-fold serial dilutions, 100 μL of appropriate dilution were spread out on PDA and counts were obtained after incubation at 30 °C for 5 days.

2.3. Composting experiments in pond

The bioremediation strategies based on *in situ* composting were carried out in one evaporation pond, where the OMW sediment from the first 45 cm layer of two 7.5 m × 7.5 m plots was extracted with an excavator truck. The sediment was thoroughly mixed with organic residues available near the ponds *i.e.* spent-mushroom compost (SM), rabbit manure (RM), and chicken manure (CM) at the following *w*/w ratios 0.5:0.12:0.33:0.05 (OMW:SM:RM:CM), according to their C and N content, in order to reach a final C/N ratio of 25 (Table 1). Additional information about the chemical characterization of initial OMW, organic amendments and the initial mix used is shown in Table 1. This mixture was incorporated in the two previously excavated plots to form two piles of 7.5 m long × 3.75 m wide × 1.5 m high. A pile was inoculated at the beginning of the process by spraying 40 L of a suspension containing a mixture of equal amounts of mycelial biomass from the two fungi in water (total amount 135 g dry weight) that supposed an inoculum load of 7 × 10^7 CFU per m^3 of material. Inoculation was performed layer by layer each at 50 cm height during pile building up, adding 8 L of fungal suspension to each layer. Throughout the composting period, water was incorporated by irrigation system to maintain humidity around 50–60%. During the bio-oxidative phase, the piles were turned over according to the thermal profile. Samples were collected at the beginning of the process and at about 60 day intervals up to the end of composting at 204 days. Analysis of phytotoxicity, ecotoxicity and total phenolic compounds were performed in each sample, while temperature, as indicator of composting process, was monitored throughout the whole process. Each sample was a composite of 200 g subsamples collected from 5 different points of the entire pile.

Table 1
Initial mixture composition.

Material	Weight (t)	C (%)	N (%)	Moisture (%)	Apparent density (t/m^3)	Volume (m^3)
OMW	25	30	1.6	38.8	0.8	60.9
SM	12	46	0.6	74.8	0.35	34.3
CM	4	46	2.3	13.2	0.50	8.0
RM	32	48	1.2	62.8	0.40	79.5
IM	98	C/N = 25		50	0.6	183

SM: spent-mushroom compost; CM: chicken manure; RM: rabbit manure; IM: initial mixture.

2.4. Analytical techniques

Phytotoxicity was determined by the analysis of germination index in seeds of *Lepidium sativum*, according to slight modifications of method proposed by Zucconi et al. (1985). Briefly, the material was moistened until reaching 60% humidity, allowed to stand for 30 min and further diluted with distilled water up to 10% (*w*/*v*). The aqueous extract was separated from solids by filtration through a cellulose membrane filter of 0.45 μm. Then, 4 mL of the aqueous extract was added in square dishes of 12 cm containing a filter paper on which 25 seeds of L. *sativum* were placed. Controls with distilled water were used as reference. Four replicates were used for each sample with a total of 100 seeds for determination. After incubation at 25 °C for 48 h in darkness, the number of seeds germinated was counted and the radical length was measured. Germination Index (GI) was calculated by multiplying the germinated seed number (G) by length of roots (L) and expressed as percentage (GI %) with respect to the control as follows: $GI = [(G_S\% \times L_S) / (G_{dw}\% \times L_{dw})] \times 100$; where: GI: Germination Index, G_S%: percentage of germinated seeds in the presence of the sample, G_{dw}%: percentage of germinated seed in the presence of distilled water, L_S: mean of radicle elongation (mm) in the presence of the sample, L_{dw}: mean of radicle elongation (mm) in the presence of distilled water.

Ecotoxicity was measured by determining the bioluminescence inhibition of *Aliivibrio fischeri* according to Jarque et al. (2016). Kinetic bioassays were performed in microplates using modified described by Bláha et al. (2010). Sample suspensions were prepared at concentration of 100 mg dry weight (dw) mL^{-1} in 2% NaCl, pH 7.0, mixed by vortex for 5 min and serial dilutions (1:2) of each sample were directly prepared into microplate. Final concentrations tested were 100, 50, 25, 12.5, and 6.75 mg dw mL^{-1}. The Aboatox kit (1243-500 BioTox kit) was used, following the protocol established for reconstitution and adequated dilution of *A. fischeri* (strain NRRL-B-11177). The luminescence measurements were made in Luminoskan Ascent Luminometer (Thermo Fisher Scientific). The inhibition of luminescence was calculated as follows: $INH\% = 100 - (IT15/IT0) \times 100$; where IT15 is the luminescence of the test sample after the contact time (15 min) (mV); and IT0 is the initial luminescence (initial luminescence peak) of the sample (mV). For the calculation of the EC50 (concentration of the sample that causes a 50% reduction of the light emitted by the bacteria), the linear regression lines of concentration *versus* INH% were obtained. From this factor, the toxicity units (TUs) were calculated according to the formula $TU = [1/(EC50)] \times 100$ (Sprague and Ramsay, 1965).

Water-soluble phenols were quantified using Folin-Ciocalteau reagent according to protocol of Beltrán et al. (1999). Spectrophotometric readings at 765 nm were collected and absorbance was compared against gallic acid as a standard.

2.5. Statistical analysis

Analytical were performed at least in triplicate and data are presented as the mean. A one-way analysis of variance (ANOVA) and multiple comparison tests of Fisher's least significant difference (LSD) at a 95% confidence level were used to test for significant differences between factor levels. Normality and homogeneity of the variances were checked using the Shapiro–Wilk and Levene tests, respectively, before ANOVA. Analyses were carried out using Statgraphics Centurion XVII version 17.1.1 (Stat-Point, Inc.).

3. Results and discussion

3.1. Selection and characterization of inoculum consortia for bioaugmentation

One of the main objectives of this work was the isolation of microorganisms that could be used for bioaugmentation in bioremediation strategies of OMW contaminated sites such as abandoned evaporation ponds. The best source of samples for this purpose is OMW sediments from the ponds themselves. Thus, in order to determine the potential microbial resources available in OMW ponds for inoculum isolation, the proportion of polyphenol degrading bacteria and fungi with respect to total microbial load was analyzed in sediments collected from seven evaporation ponds. Results of these analyses are shown in Fig. 2. The capability to degrade polyphenol is of special interest for OMW bioremediation because these compounds are the main responsible for the toxic effects of OMW (Dermeche et al., 2013). Thus, >40% of total bacteria and fungi were polyphenol degraders in most ponds and they dominated the bacterial and fungal population in ponds 3 and 6, reaching 84.5% and 100% of the total microbial group population, respectively. This high load of specialized microorganisms can be explained by adaptation phenomena because of the prolonged exposure. The indigenous microorganisms have successfully tolerated the presence of microbial inhibitory substances in OMW, such as polyphenol compounds, and would have developed metabolic abilities related to their degradation (Arous et al., 2018). Consequently, this preliminary study demonstrated the suitability of the samples for the inoculum isolation.

OMW are very complex in composition and the selection of microorganisms for the bioremediation of matrices contaminated with them requires the search of a quite high number of degradation activities in order to guarantee depletion of polluting compounds. Thus, the procedures for the screening and selection of microorganisms for bioremediation were designed to cover a wide range of degrading activities of compounds likely found in OMW, and also taking into account they were destined for bioaugmentation in composting. Polyphenols, as the main responsible of the toxic effects of OMW, were the primary target. These polymers are characterized by the presence of a wide variety of phenol structural units. Thus, culture media used for isolation included tannic acid as polyphenol model compound or tyrosol as polyphenol structural unit. In addition, capability to degrade lignin was also explored because of its connection with polyphenol activity. Decolorization of antraquinone dye RBBR (Palmieri et al., 2005) as well as the production of several lignin and phenol-degrading enzymes (peroxidase, ligninase, laccase, oxidase, tyrosinase) were tested. In addition, incubations were performed at two different temperatures, 30 °C and 50 °C, in order to ensure the isolation of microorganisms that could resist thermal fluctuations during composting. One strategy for the isolation of microorganisms involved the direct inoculation of selective media with samples of OMW sediments. In parallel, in order to favor the growth of microorganisms which were in low proportion or whose development was slow, other strategy consisted on enrichment of samples in liquid medium followed by isolation in selective media constituted by tyrosol, as a phenol model. Both procedures allowed obtaining a final collection of 60 strains, including 36 bacteria, 4 actinobacteria and 20 fungi. A total of 37 microorganisms were isolated from the enrichment cultures and the remaining came from the direct isolation procedure, being 14 strains from APHA-TAN and PDA-TAN and 9 strains from RBBR.

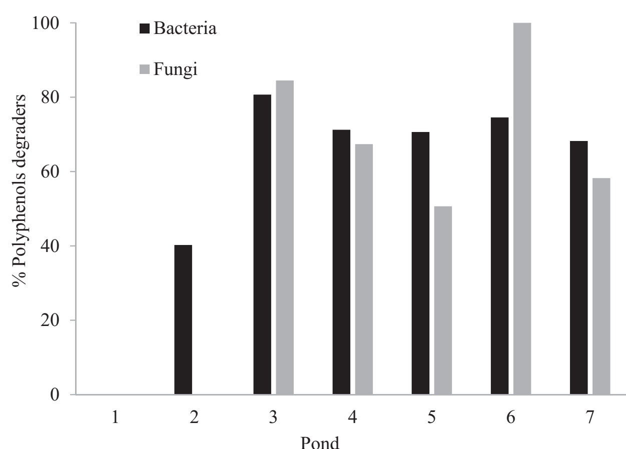

Fig. 2. Proportion of polyphenol-degrading bacteria and fungi with respect to total microbial load in samples collected from evaporation OMW sediments ponds.

A battery of qualitative (lignin and phenol-degrading activities) and quantitative (tyrosol depletion) tests was used for all strains isolated in order to demonstrate the expression of enzymes and activities directly or indirectly involved in the degradation of the OMW compounds. The number of strains giving positive results for the tests performed is shown in Table 2. All the strains were able to grow in a medium with tyrosol. The peroxidase activity was detected in 43 strains. These enzymes help in the degradation of lignin and other aromatic compounds by using hydrogen peroxide as mediator. Thus, Zerva et al. (2017) demonstrated its production by two fungal species during OMW degradation showing high capacity of this enzyme for phenolic compounds oxidation. Polyphenol oxidase activity tested in media with tannic acid was detected in 22 strains. This enzyme is of interest due to its ability to hydroxylate phenolic compounds. The number of strains showing activities related to lignin degradation, such as RBBR dye decolorization and ligninase (LIG), laccase (LAC), oxidase (OXY) and tyrosinase (TYRase) production was quite low, only 6 to 8 strains exhibited some of these activities, though they are of great interest. Martínková et al. (2016) tested the potential ability of laccases and tyrosinases as degrading enzymes of the wide variety of phenolic compounds present in OMW. Laccase has also been studied by Sharma et al. (2018) who reported it as a powerful enzyme for the bioremediation of a wide range of pollutants such as phenolic compounds, aromatic heterocyclic compounds and amine containing aromatic compounds. Thereby, microorganisms capable of degrading lignin have metabolic capacities to degrade a wide range of aromatic compounds found in OMW (Zerva et al., 2017). In reference to the quantitative test based on the efficiency for tyrosol degradation, only 9 strains degraded between 80 and 100% of tyrosol, 6 of them depleted between 50 and 80% of tyrosol, while the rest of strains removed <50%. Fungi and actinobacteria were more active for tyrosol depletion than bacteria. While the best strains of fungi and actinobacteria eliminated >90% of such compound, bacteria barely reached 30%. In contrast, other researchers demonstrated a high phenol degradation ability for bacteria (Arous et al., 2018). These authors found a bacterial consortium exhibiting a 64% phenolic compounds elimination efficiency. In addition, many aerobic bacteria seem to be very effective against some phenolic compounds and relatively ineffective against others, while fungi are more effective than bacteria in degrading both simple and complex phenolic compounds (Ayed et al., 2017).

The qualitative and quantitative analytical tests allowed the preselection of 8 strains, 6 fungi, 1 actinobacteria and 1 bacterium whose identification and metabolic capabilities are shown in Table 3. This

Table 2
Number of isolates that showed positive results for the qualitative tests and degraded >50% tyrosol.

Microorganism group	RBBR	PO	TYR	LAC	OXY	TYRase	PER	LIG	>50% TYR degradation
Actinomycetes	2	0	4	1	1	0	3	1	2
Bacteria	11	4	36	0	0	0	32	11	0
Fungi	13	18	20	6	5	8	8	2	13
Total	26	22	60	7	6	8	43	14	15

RBBR: Remazol Brilliant Blue reactive decolorization; PO: polyphenoloxidase; TYR: growth in tyrosol-containing media; LAC: laccase; OXI: oxidase; TYRase: tyrosinase; PER: peroxidase; LIG: ligninase; % TYR degradation: percentage of tyrosol degradation.

Table 3
Identification and metabolic capabilities of selected strains.

Id	Species	Sequence bp	Accession number	Similarity %	RBBR	PO	TYR	LAC	OXI	TYRase	PER	LIG	%TYR DEGR
A4	*Streptomyces koyangensis*	869	KM678242.1	99.77	0	0	1	0	0	0	1	0	96.6
B14	*Lysinibacillus fusiformis*	854	FJ174591.1	100	1	0	1	0	0	0	1	1	28.4
H1	*Aspergillus micronesiensis*	623	KP987080.1	99.52	1	1	1	0	0	0	0	0	96.6
H2	*Aspergillus ochraceus*	581	KT803068.1	100	1	1	1	0	0	1	1	1	76.6
H10	*Pleurostora richardsiae*	380	MG966406.1	100	1	1	1	1	1	1	1	0	73.8
H16	*Scedosporium apiospermum*	593	KP132631.1	99.66	0	1	1	1	1	1	0	0	96.6
H18	*Aspergillus terreus*	596	MK541019.1	100	1	1	1	0	0	0	0	0	96.6
H21	*Cosmospora viridescens*	592	MF782760.1	99.83	0	0	1	0	0	0	1	0	96.6

Id: A: actinobacteria; B: bacteria; H: fungus; bp: base pairs; S%: similarity percentage; RBBR: Remazol Brilliant Blue reactive decolorization; PO: polyphenoloxidase; TYR: growth in tyrosol-containing media; LAC: laccase; OXI: oxidase; TYRase: tyrosinase; PER: peroxidase; LIG: ligninase; % TYR DEGR: percentage of tyrosol degradation.

selection was carried out based on the number of activities related to phenols degradation (three or more positive activities), microbial group and ability to degrade tyrosol. According to these criteria, the 8 strains showed a range of metabolic activities and physiological capacities that make them potentially suitable for the bioremediation of OMW polluted environments. Focusing on the selection of microorganisms for bioaugmentation in bioremediation of OMW by composting, the fungi H2 and H16 were selected taking into account the range of activities related to the metabolism of recalcitrant compounds as well as the capability to degrade tyrosol. H16 had five activities and degraded 97% tyrosol, while H2 expressed six relevant activities and degraded 77% tyrosol. Other interesting strain was H10, that showed seven activities but it was not selected due to its slow growth, low sporulation, and lower ligninolytic activity than the selected ones, however, it will be an interesting candidate for further studies.

Therefore, fungi H2 and H16 were selected as prominent candidates to constitute an interesting set of microorganisms to use in bioaugmentation strategies because they cover a wide spectrum of metabolic capabilities. The fungi selected were identified as *Aspergillus ochraceu*s H2 (Accession number NCBI KT803068.1) and *Scedosporium apiospermum* H16 (Accession number NCBI KP132631.1), with a similarity percentage of 100% and 99%, respectively.

S. apiospermum is a fungus belonging to the phylum *Ascomycota*, which has been isolated from various environments, such as effluents of wastewater treatment plants (Rougeron et al., 2018). The fact of finding this species commonly in contaminated soil and water led to extensive research on its use as bioremediation agents. Discoloration experiments conducted with strains of *Scedosporium* isolated from leachates and effluents from a wastewater treatment plant demonstrated that this fungus is a good candidate for bioremediation (Rougeron et al., 2018). This fungus has also been reported as a hydrocarbon degrading microorganism, it is able to degrade polluting compounds such as phenol, p-cresol, phenylbenzoate and its derivatives, toluene, polycyclic aromatic hydrocarbons (PAHs), long-chain aliphatic hydrocarbons, and mixtures of these contaminants (Clauben and Schmidt, 1998) and offers an opportunity to propose its use in controlled bioremediation or bioaugmentation processes. *Scedosporium* species are adequate for bioaugmentation since they are thermotolerant and have the ability to survive at extreme conditions. For this reason, Rougeron et al. (2018) proposed inoculation of plant residues during composting with *Scedosporium* cellulolytic species and demonstrated that it promoted the activation of the process.

The genus *Aspergillus* has also been extensively studied for use in bioremediation because of its versatility to biotransform or cometabolize a variety of toxic compounds such as textile dyes, aromatic compounds, pesticides *etc.* (Sharma et al., 2018). The species *Aspergillus ochraceus* has been proposed for the treatment of OMW due to its capability to growth on phenols (Bevilacqua et al., 2017). *A. ochraceus* is also able to decolorize and degrade textile dye Reactive blue-25 mediated by the production of oxidative enzymes such as lignin peroxidase, laccase and tyrosinase (Parshetti et al., 2007). In the same way, Saratale et al. (2006) also reported that *A. ochraceus* could effectively decolorize several textile dyes such as malachite green and cotton blue.

In order to determine some practical aspects related to the combined application of *A. ochraceus* H2 and *S. apiospermum* H16 in field composting experiments, the thermal growth range of both microorganisms, their compatibility and potential synergistic degrading capabilities were tested. *A. ochraceus* H2 grew *in vitro* at 25 °C and 30 °C, with greater vigor at this last temperature, as it has also been reported by other authors (Palacios-Cabrera et al., 2005). *S. apiospermum* H16 grew *in vitro* between 25 °C and 40 °C at the maximum, as it was also reported by Clauben and Schmidt (1998). In general, no inhibitory effect

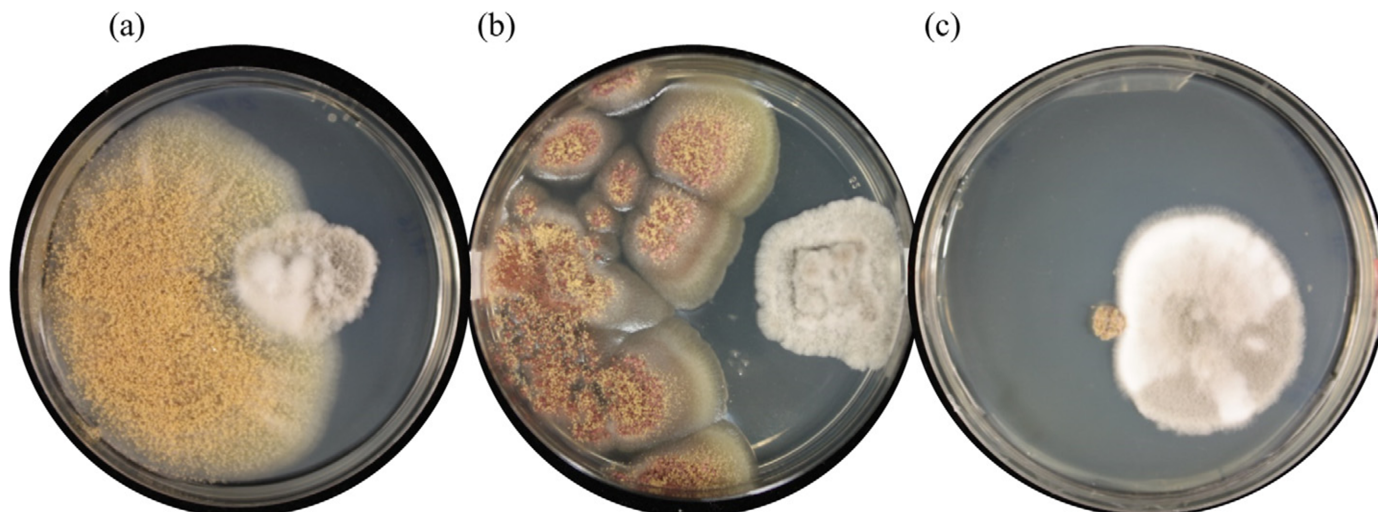

Fig. 3. Confrontation of *A. ochraceus* H2 (left: yellow mycelium) and *S. apiospermum* H16 (right: white mycelium) after 10 days of culture at (a) 25 °C, (b) 30 °C, and (c) 40 °C.

of one fungus against the other was observed, as reflected by the overlapping of mycelium of both fungi at 25 °C (Fig. 3). The two fungi were cultivated in liquid medium with tyrosol, separately and together, in order to determine the existence of synergistic effect in the degradation of this compound. This study revealed that the fungi H2 and H16 separately degraded 96% and 77% of tyrosol, respectively (Table 3), while together they completely eliminated the tyrosol (100%). According to these results, a mixed culture of both fungi can be grown and applied as a consortium since they are compatible and synergistically deplete toxic compounds. Moreover, the fact that both jointly covered a temperature growth range between mesophilic and thermotolerant conditions clearly benefits their persistence throughout thermal fluctuations during composting process. In fact, fungi are decisive for the proper development of composting, mainly because of their capability to produce a wide range of extracellular enzymes that breakdown polymers. They are present in all the different stages of the process (López-González et al., 2015). Mesophilic fungi persist in the outer layers of composting piles during the thermophilic stages and re-colonize when the temperature drops sufficiently (Gaur et al., 1982). Thus, after thermophilic stage, during the second part of the process, fungi can be found as the most significant decomposers (Sánchez et al., 2017). Although the consortium employed in this work was applied at the beginning of the composting, during the thermophilic stages they can persist in the out-layer or as spores and further colonize the whole material during turning operations allowing their propagation in the following stages. For this reason, while the optimum temperature for the growth of H2 and H16 are 30 and 40 °C respectively, it is expected that they will be present throughout the entire composting process.

Mycelial biomass of the two fungi was produced at large scale for bioaugmentation experiments whose results are described below. Before the vacuum packaging, the viability of the fungi was analyzed and counts were made. The inoculum of *A. ochraceus* H2 had a load of 7.9×10^7 UFC g^{-1} and in the case of *S. apiospermum* (H16) it was 1.1×10^8 UFC g^{-1}.

3.2. OMW ponds bioremediation through composting: bioaugmentation with fungal consortium

In order to demonstrate the ability of the selected fungal consortium for improving bioremediation of OMW evaporation ponds by *in situ* composting, a field experiment was conducted in one pond in which two composting piles were built. Bioremediation capability was monitored by measuring phytotoxicity, ecotoxicity and total polyphenols. The analysis of the material contained in the pond revealed that contamination was limited to the upper 45 cm layer. Phytotoxicity and ecotoxicity were higher in the upper layer (0–45 cm) than in depth (45–90 cm), in fact, both effects were absent in the deeper layer. The upper layer had 66% GI and the deeper layer 107% GI. The results concerning ecotoxicity were 1.2 TU and 2.6 TU in deeper and upper layer, respectively. The total water-soluble polyphenolic compounds were very high, with an average value of 3.4 g kg^{-1} dw. These components were more abundant in the superficial layer, where a concentration of 4.7 g kg^{-1} dw was obtained, than in the deep one (2.2 g kg^{-1} dw). This indicated the existence of an interrupted vertical flow, as well as capillarity and evaporative phenomena, which allow a higher concentration in superficial layer. Consequently, for *in situ* composting only the first 45 cm layer of sediment was recovered, mixed with organic waste amendments (spent-mushroom compost, rabbit manure and chicken manure) and placed in the two sub-plots in the pond to build up two piles, one was inoculated with fungal consortium and the other was not inoculated.

During composting, the thermal profile of the two piles (inoculated and non-inoculated) was similar. Material reached thermophilic phase (>50 °C) 3 days after piles were built and temperature increased up to 75 °C at day 18. Five turnings were applied at about biweekly intervals, according to thermal register, each performed once temperature showed a decreasing trend for more than three consecutive days. The bio-oxidative phase, with increases of temperature to values higher than 50 °C after each turning, lasted for 115 days. During the first 90 days, temperature values higher than 50 °C were maintained for >7 days. After 115 days, material cooled down and did not warm even

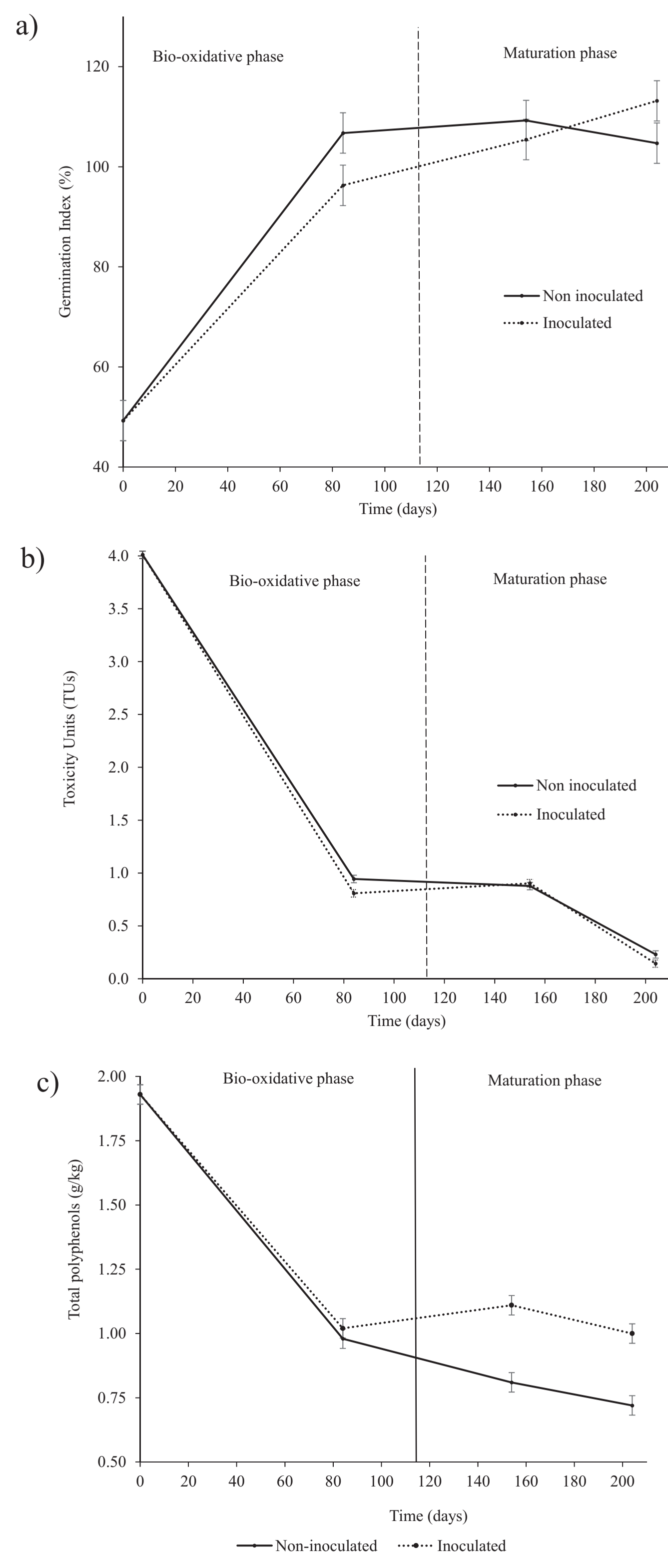

Fig. 4. Evolution of phytotoxicity (a), ecotoxicity (b) and total polyphenols (c) in OMW composting piles non-inoculated and inoculated with fungal consortium. Main phases of composting are indicated. Results are the mean of three replicates. Errors bars represent the LSD Fisher interval ($p < 0.05$).

Table 4
Bioremediation effect of *in situ* inoculated and non-inoculated composting of OMW sediments in evaporation ponds.

Treatment	Phytotoxicity (GI%)	Ecotoxicity (TU)	Water soluble phenols (g kg^{-1} dw)
Untreated OMW sediments	65.97a	2.57a	22.89a
In situ non inoculated composting	104.71b	0.23b	0.72b
In situ inoculated composting	113.17c	0.14c	1.00c

Values are the mean of three replicates. Values with same letter in column are not significantly different (LSD, $p < 0.05$).

after turning, so maturation phase started and ambient temperatures were maintained up to the end of composting on day 204. Samples were collected for analysis at the beginning (0 days), at the middle of bio-oxidative phase (84 days), at maturation phase (154 days) and at the end of experiment (204 days).

The phytotoxicity evolution, expressed as germination index (GI), is shown in Fig. 4a. Values of GI below 50% indicate high phytotoxicity, GI above 80% indicate absence of phytotoxicity, while values above 100% indicate phytostimulant effect (Zucconi et al., 1985; Emino and Warman, 2004). The mixture used for composting was more phytotoxic (GI 49%), than untreated OMW sediments in the pond (GI 66%), considered as initial reference value. This is due to the addition of fresh organic matter (spent mushroom, chicken and rabbit manure) to the OMW sediments that causes lack of oxygen as result of intense microbial activity leading to phytotoxicity (Hachicha et al., 2009). GI values increased gradually during composting in the two piles, surpassing 100% at bio-oxidative phase (84 days) in the non-inoculated pile and at maturation phase (154 days) in the inoculated pile. This means that the materials were phytostimulants. Thus, the composting process stabilized organic matter and depleted phytotoxic compounds; as a result, compost derived from OMW is suitable for use as organic amendment. Although the absence of phytotoxicity was reached before in non-inoculated pile, the GI of final compost after 204 days was significantly higher in the inoculated pile (113%) than in the non-inoculated (105%). These results showed that the inoculum had a positive effect by providing a final compost of higher quality with respect to its phytostimulant character. Similarly, Parotta et al. (2016) found that the treatment of composting piles with inoculants belonging to the genus *Serratia* and *Pantoea*/*Enterobacter* isolated from OMW led to an increase in the GI.

The results obtained in the evaluation of the ecotoxicity during composting are shown in Fig. 4b. The ecotoxicity values were expressed as toxicity units (TU). According to Persoone et al. (2003), the ecotoxicity can be classified into one of the following categories: TU > 100, very high acute toxicity; 10 < TU < 100, high acute toxicity; 1 < TU < 10, acute toxicity; 0.4 < TU < 1, slight acute toxicity; and TU < 0.4, no acute toxicity. As previously described, OMW is a residue rich in polyphenols that causes high ecotoxicity and has a strong antibacterial effect. Thereby, untreated OMW sediment had acute toxicity (2.6 TU) and similar to what was found for phytotoxicity, an increase in ecotoxicity was obtained when organic matter was added to the untreated material (4 TU). These levels progressively decreased since the beginning of composting. Both materials, inoculated and non-inoculated, reached TU < 1 at 84 days, being significantly lower in inoculated (0.8 TU) than in non-inoculated (0.9 UT) pile. Considering the toxicity classification mentioned above, the material had slight acute toxicity after 84 days composting. Between the bio-oxidative phase (84 days) and the middle of maturation phase (105 days), ecotoxicity did not change significantly but decreased afterwards reaching final values TU < 1 which were lower in inoculated pile (0.14 TU) than in non-inoculated pile (0.23 TU). Thus, the final compost obtained from both treatments were non-ecotoxic.

The decrease of phytotoxicity (increase in GI) and ecotoxicity of OMW sediments during composting above discussed may be partially explained by the decrease in water-soluble phenolic compounds, whose results are shown in Fig. 4c. These compounds are known to be the main responsible of OMW toxicity (Duarte et al., 2011). The untreated OMW material had 22.89 g kg^{-1} dw of water-soluble phenolic compounds and when it was mixed with organic waste amendments the content decreased to 1.93 g kg^{-1} dw, likely because of dilution. During composting, in the non-inoculated compost pile, these compounds steadily decreased reaching a concentration of 0.72 g kg^{-1} dw in the final product. The inoculated compost pile performed similarly during bio-oxidative phase, in which a 50% phenolic compounds were depleted and their concentration at day 84 (1.02 g kg^{-1} dw) was close to that in non-inoculated pile. However, no further decreases in phenolic compounds were obtained for inoculated pile during maturation. In fact, they slightly increased up to 1.11 g kg^{-1} dw at maturation phase (154 days). This phenomenon has also been reported by Jurado et al. (2015) during composting of bioaugmented lignocellulosic waste. This can be due to the release of phenols from the lignocellulose matrix of organic waste amendments as consequence of a more intense microbial degradation of lignin fraction in inoculated pile at the end of composting. However, despite the higher phenolic content in final material of inoculated pile with respect to non-inoculated one, they did not lead to higher toxic effects, as previously discussed. This relates to the fact that toxic effect of phenolic compounds involves a complex interplay between different phenolic substances from OMW in dependence on their concentrations, mixture composition and toxicity mechanisms (Buchmann et al., 2015).

Table 4 summarizes the bioremediation effect of *in situ* inoculated and non-inoculated composting of OMW sediments in evaporation ponds. As stated above, composting eliminated the toxicity of OMW sediments by depleting almost completely (>95%) their main toxic components and leading additionally to a phytostimulant and ecotoxicity-free compost product. Furthermore, the fungal consortium used for bioaugmentation in inoculated composting had the ability to enhance the bioremediation, as noticed by the higher phytostimulant and lower ecotoxic values in final compost in comparison to those coming from non-inoculated composting. This compost could be used for agronomic applications as it has been also proposed elsewhere for compost obtained from olive mill wastes (Asses et al., 2018).

4. Conclusions

OMW sediments from evaporation ponds are naturally enriched in polyphenol-degrading microorganisms. The fungi isolated from these materials, *Aspergillus ochraceus* H2 and *Scedosporium apiospermum* H16, presented a range of metabolic and physiological capabilities suitable for their use as consortium in bioremediation. The *in situ* composting of OMW sediments in evaporation ponds is a sustainable way to recycle them along with organic waste, while simultaneously solving their negative environmental impact. The use of fungal consortium for bioaugmentation of composting improves the bioremediation efficacy of the technique by speeding up the decrease of phytotoxicity and ecotoxicity and enhancing phytostimulant property of compost produced.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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