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Removal of Organic Load from Olive Washing Water by an Aerated Submerged Biofilter and Profiling of the Bacterial Community Involved in the Process

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Abstract The present work aims to use a biofilter technology (aerated submerged filters) for the aerobic transformation at laboratory-scale of olive washing water (OWW) generated in the first steps of olive oil processing, as well as the genetic profiling and identification to the species level of the bacteria involved in the formation of the biofilm, by means of TGGE. Chemical parameters, such as biological oxygen demand at five days (BOD_5) and chemical oxygen demand (COD), decreased markedly (up to 90 and 85%, respectively) by the biological treatment, and the efficiency of the process was significantly affected by aeration and inlet flow rates. The total polyphenol content of inlet OWW was only moderately reduced (around 50% decrease of the inlet content) after the biofilter treatment, under the conditions tested. Partial 16S rRNA genes were amplified using total DNA extracted from the biofilm and separated by TGGE. Sequences of isolated bands were mostly affiliated to the α-subclass of Proteobacteria, and often branched in the periphery of bacterial genera commonly present in soil (*Rhizobium*, *Reichenowia*, *Agrobacterium*, and *Sphingomonas*). The data obtained by the experimentation at laboratory scale provided results that support the suitability of the submerged filter technology for the treatment of olive washing waters with the purpose of its reutilization.

Keywords: Olive washing water (OWW), aerated biofilter, TGGE, soil bacteria

Olive oil production is a highly important agronomical industry for the economy of Mediterranean basin countries like Spain, Italy, Greece, Turkey, Morocco, and Tunisia. This industry is characterized by the production of several by-products with a high environmental impact (International Olive Oil Council, http://www.international-oliveoil.org).

The olive oil is obtained after a long and tedious process, which starts with the washing of the olives, needed to eliminate vegetable residues and soil particles, a step that is crucial to ensure good organoleptic properties and a high quality of the final product [12]. The water used in this step (50 l per 100 kg of olives) must be drinkable water, because of the use of olive oil for human consumption. The most common practice in the olive oil industry is the mixing of the olive washing water (OWW) with the wash waters of the secondary centrifuge, generated during the purification of virgin olive oil by the two-phase systems [26]. The pooled wastewaters compose the olive oil mill wastewater (OOMWW). OOMWW is commonly retained in ponds for water evaporation, and seldom undergoes treatment by physical, chemical, or biological processes [22]. Hence, the separated handling/treatment of OWW has received little attention, in comparison with OOMWW. However, the rural areas where the olive trees are grown are usually dry regions with frequent water deficiency problems, and consequently, the reuse of OWW is the most desirable future practice for sustainability.

Phenolic compounds are the chemical substances that make important contributions to the pollutant and recalcitrant characters of OWW and OOMWW [22]. Total phenolic compounds (Tph) are very toxic for some microorganisms (*i.e.*, methanogenic bacteria), limiting the use of the conventional anaerobic degradation processes for the treatment of these wastewaters. The content of Tph of OWW is 10–100 times lower than in OOMWW, but it is still high enough to make necessary the use of treatments for their depletion, if these wastes are to be reused, *i.e.*, for agricultural purposes. Some biological processes based on anaerobic fermentation have been proposed [6].

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In the last decades, aerobic-biodegradation processes have played an important role in the treatment of industrial, urban, and agricultural wastewaters [31]. Aerated submerged biofilter technology is a low-cost treatment system requiring minimum space and maintenance, which has been used often for the biological treatment of wastewaters contaminated with organic and inorganic pollutants [3, 8, 9, 17]. In recent years, molecular techniques have been increasingly applied to investigate the microbial community composition in various ecosystems, including biofilters and biotrickling filters. With the introduction of genetic fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE), multiple samples can be analyzed simultaneously, as it is required for studying the complex dynamics of microbial communities.

The aim of the present work is to set up an aerated submerged biofilter (lab-scale) for the treatment of OWW. The performance of the treatment plant was evaluated by the study of the evolution of several parameters such as chemical oxygen demand (COD), biological oxygen demand at five days (BOD_5), and Tph of treated OWW samples under different laboratory work conditions (air and OWW inlet flows). Furthermore, in order to get an insight into the biological aspects of the process, the fingerprinting of the bacterial community in the biofilms was made by PCR amplification of the V3-hypervariable region of the 16S rRNA gene from total DNA extracted from the biofilm community, which was subsequently separated by TGGE. Such TGGE-based approaches have been commonly used in recent years for the genetic identification of the populations in complex environments and the study of their importance in biological processes for the removal of a variety of pollutants (hydrocarbons, heavy metals, phenols, pesticides) in several types of wastes [4, 7, 29, 16, 19].

Materials and Methods

Olive Washing Water (OWW) Samples

The OWW samples used in the aerobic degradation experiments were picked from poured pools outside the olive oil factory ("Torredonjimeno S.C.A.") in Torredonjimeno (Jaén, Spain), one of the most important areas of olive oil production worldwide. The water samples were dispensed in containers, and maintained under refrigerated conditions (4°C) until their analysis. Storage of OWW samples never exceeded 24 h.

Chemical, Physical, and Biological Analyses

The previous characterization of the OWW samples showed the following values: pH: 6.6; conductivity: 1.07 mS/cm; chemical oxygen demand (COD): 1,545 mg O_2/l; biological oxygen demand at five days (BOD_5): 725 mg O_2/l; total N: 10.0 mg/l; total polyphenol content (Tph, as mg of caffeic acid/l): 104.6 mg/l; total solids: 1.65 g/l; dissolved solids: 0.2 g/l; organic matter: 0.95 g/l; inorganic matter: 0.7 g/l; and mesophilic platable heterotrophic bacteria: 1.0×10^7 CFU/ml. The methodology used for these determinations was as described by *Standard Methods for the Examination of Water and Wastewater* [2].

Design of the Laboratory Plant

The laboratory-scale plant used in this study consisted of a glass cylinder column (0.50 m height, 6 cm diameter) packed with an inert material (expanded clay), registered as Filtralite. The inert material was used as support media for biofilm growth, packing the column up to a height of 0.25 m. The wastewater to be treated was pumped in at several rates (1.3, 2.3, and 4.2 l/day) using a piston pump. The olive washing water entered at the top of the glass cylinder and overflowed at the base. The air was supplied by a sliding vane blower to a single membrane air diffuser at the base of the glass cylinder (Fig. 1).

Experimental Conditions

The capacity of removal of organic matter by the biofilter was evaluated as reduction on COD, BOD_5, and total polyphenol contents. Two different air flows (0.05 and 0.1 l/min) and three different OWW flows (1.3, 2.3, and 4.2 l/day) were tested. The experiments were maintained until the system showed hydraulic load loss (11 days). The COD, BOD_5, Tph contents, and pH value of the treated effluent were measured daily. The nomenclature of the different treatments applied was the following: aeration (0.1 l/min) and OWW inlet flow of 1.3 l/day: F6; aeration

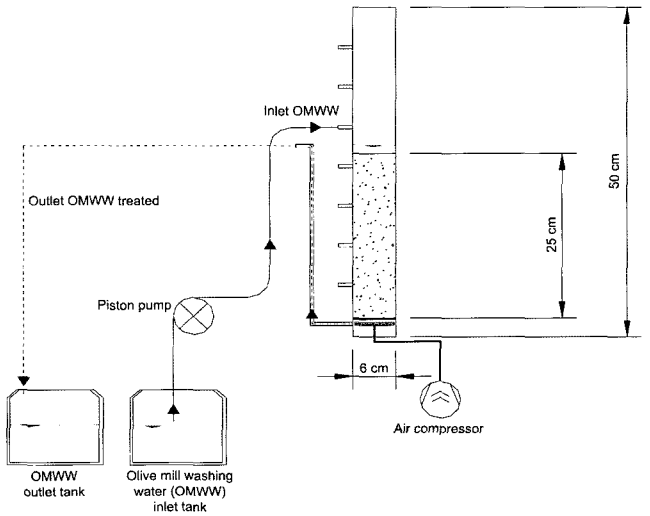

Fig. 1. Laboratory-scale plant: aerated submerged biofilter, OWW inlet and outlet tanks, and air compressor.
The laboratory-scale plant consisted of a glass cylinder column (0.50 m height, 6 cm diameter) packed with an inert material (expanded clay), registered as Filtralite. The inert material was used as support media for biofilm growth, packing the column up to a height of 0.25 m.

(0.1 l/min) and OWW inlet flow of 2.3 l/day: F9; aeration (0.05 l/min) and OWW inlet flow of 2.3 l/day: F12; and finally, aeration (0.1 l/min) and OWW inlet flow of 4.2 l/day: F15.

Biofilm Recovery

At the end of each cycle (11 days), biofilm was recovered from biofilters. The method used for biofilm recovery was as previously described [13]. Five g samples of the Filtralite material with biofilm adhered were placed in flasks with 50 ml of sterile saline (0.9% NaCl). These suspensions were sonicated for 1 min and then placed in an orbital shaker at 155 rpm for 1 h. The process was repeated twice. Finally, 100 ml of saline with suspended material from the biofilm was centrifuged at 1,500 ×*g* for 30 min. The pellets were immediately used for DNA extraction.

Microbial Community Analysis

DNA Extraction. Total DNA was extracted from the biofilm following a method based on the one previously described [30]. Briefly, pellets were suspended in 1 ml of Buffer I (10 mmol/l Tris-HCl [pH 8.0], 1 mmol/l EDTA, 0.35 mol/l sucrose, and 20 mg of lysozyme per ml) and incubated at 37°C for 10 min. After incubation, 1.5 ml of lysis buffer (100 mmol/l Tris-HCl [pH 8.0], 0.3 mol/l NaCl, 20 mmol/l EDTA, 2% [wt/vol] sodium dodecyl sulfate, 4 μg/ml proteinase K, and 2% [wt/vol] 2-mercaptoethanol) was added, and samples were incubated at 37°C for 1 h. Lysates were extracted twice with one volume of phenol-chloroform solution (1/1 vol/vol), incubated for 10 min with 125 μg/ml RNase, and extracted once with 1 volume of chloroform. Aliquots of 800 μl were precipitated with 1 volume of 2-propanol (30 min incubation at −20°C followed by centrifugation for 20 min at full speed in a tabletop centrifuge). The pellets were washed with 500 μl of a 70% ethanol solution before being resuspended in 50 μl of TE buffer and left to rehydrate at 4°C overnight. DNA was checked by agarose gel electrophoresis and stored at −20°C.

DNA was also extracted from OWW samples before they were treated by the laboratory-scale biofilter. Ten ml of OWW was filtered through a 0.22-μm sterile nitrocellulose filter (Millipore). The filter was then placed in a sterile tube, 2 ml of Buffer I was added, and the material on the filter was recovered by vortexing. Then, the material from the filter suspended in Buffer I was used for DNA extraction, following the same protocol described for DNA extraction from the biofilm pellets, scaling up the volumes as appropriate. This sample was named as "F0," and was included for comparison in further PCR and TGGE analyses.

PCR, TGGE, and Sequencing

For the amplification of the V3-hypervariable region of the 16S rRNA gene, a nested approach was used, using PCR conditions already described elsewhere [13]. The universal primers used were fD1 and rD1 (targeting almost the full-length 16S rRNA gene) for the first PCR [32], and P1-GC and P2 (targeting the V3-hypervariable region) for the nested PCR [23]. A Perkin Elmer GeneAmp PCR system 2400 (Perkin Elmer, Norwalk, U.S.A.) was used for all amplifications.

One μl (2–5 ng) of DNA extracted from the biofilms developed in aerated submerged biofilters was used as template for the first PCR, and subsequently, 1 μl of the first PCR product was used as template for the nested PCR. The amplification of final PCR products of the expected size was checked by electrophoresis in 3% agarose gels. Final PCR products were cleaned and/or concentrated (when required) using Microcon YM cartridges. Two to 5 μl was loaded in each well for TGGE.

The separation of the DNA fragments that are generated, based on differences in the melting properties of the individual molecules of DNA (TGGE analysis), was achieved using a Whatman-Biometra TGGE system. Denaturing gels (6% PAGE with 20% deionized formamide, 2% glycerol, and 8 mol/l urea) were made and run with 2×TAE buffer. The temperature gradient was optimized for efficient separation of bands at 43 to 63°C. The gels were run at 125 V for 18 h. Bands were visualized by silver staining using the Gel Code Silver Staining kit (Pierce). Portions of individual bands on stained TGGE gels were picked up with sterile pipette tips, placed in 10 μl of sterile PCR water, and directly used for reamplification with the appropriate primers. PCR products were purified by agarose gel electrophoresis, followed by DNA extraction with the Quiaex-II kit (Quiagen). DNA recovered from gels was directly used for automated sequencing in an Applied Biosystems 3100 Capillary automatic sequencer.

DNA sequences were analyzed using the biocomputing tools provided online by the European Bioinformatics Institute (http://www.ebi.ac.uk). The BLASTn [1] program was used for preliminary sequence similarity analysis. The ClustalX v 1.8 software [18] was used for the aligning of sequences. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 [20]. Gaps were excluded from the analysis. Bootstrap values below 50% are not shown in the tree.

RESULTS

The evolution of the COD, BOD_5, and Tph of OWW samples after being treated by the submerged aerated filter system was evaluated at different air and OWW flow conditions (Figs. 2, 3, and 4). The time necessary for the biofilm development on the system was 24 h. The pH values remained stable throughout the experiment in all the treatments, varying between 7.0 and 8.0. In contrast, the

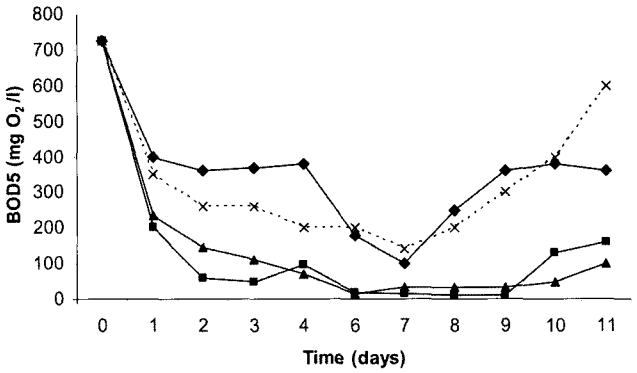

Fig. 2. Time-course experiments showing changes in BOD_5 values in OWW samples under aerated biofilter technology. The values are the mean of three replicates.
(▲): OWW samples under aeration (0.1 l/min) and water inlet flow of 1.3 l/day. (■): OWW samples under aeration (0.1 l/min) and water inlet flow of 2.3 l/day. (◆): OWW samples under aeration (0.05 l/min) and water inlet flow of 2.3 l/day. (×): OWW samples under aeration (0.1 l/min) and water inlet flow of 4.2 l/day.

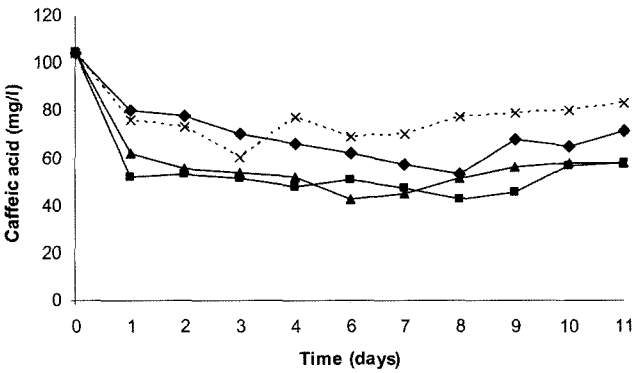

Fig. 4. Time-course experiments showing changes in total polyphenol content in OWW samples under aerated biofilter technology. The values are the mean of three replicates.
(▲): OWW samples under aeration (0.1 l/min) and water inlet flow of 1.3 l/day. (■): OWW samples under aeration (0.1 l/min) and water inlet flow of 2.3 l/day. (◆): OWW samples under aeration (0.05 l/min) and water inlet flow of 2.3 l/day. (×): OWW samples under aeration (0.1 l/min) and water inlet flow of 4.2 l/day.

different treatments promoted significant changes in the ability of the system for COD and BOD_5 removal.

Regarding the BOD_5 removal (Fig. 2), a continuous decrease of this parameter with time was observed in all cases. Biofilters drastically reduced BOD_5 values of effluent water in all treatments assayed. The increase of the aeration flow positively affected the reduction of this parameter in the treated effluent. In this context, the best results on BOD_5 reduction were obtained with an aeration flow of 0.1 l/min. However, an increase in OWW flow (1.3 l/day to 4.2 l/day) had negative effects on BOD_5 reduction.

COD values of treated OWW (Fig. 3) were also significantly affected by the modifications of the OWW flow. Our results showed that optimum yields of COD removal were observed with an OWW flow of 1.3 l/day, achieving a reduction in this parameter of 85% after 6 days of plant operation.

Finally, the Tph content in the treated OWW (Fig. 4) was significantly reduced in all the treatments tested, as soon as 1 day after the start of plant operation. From this point to the end of the experiment, the reduction on this parameter varied widely, depending mainly on the OWW flow tested. With OWW flows of 1.3 and 2.3 l/day, the final Tph content of effluent samples was around 50 mg/l (50% reduction), whereas at an OWW flow of 4.2 l/day, the final Tph content was 80 mg/l. These data suggest that the level of reduction of this parameter was affected by the OWW inlet flow, and the microbiota developed on the inert support was not able to remove all these recalcitrant substances under the experimental conditions tested through the study.

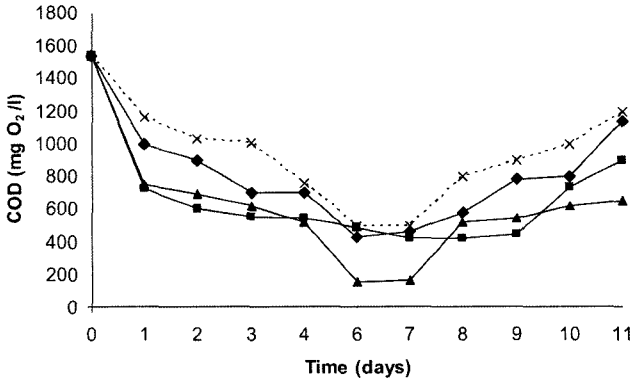

Fig. 3. Time-course experiments showing changes in COD values in OWW samples under aerated biofilter technology. The values are the mean of three replicates.
(▲): OWW samples under aeration (0.1 l/min) and water inlet flow of 1.3 l/day. (■): OWW samples under aeration (0.1 l/min) and water inlet flow of 2.3 l/day. (◆): OWW samples under aeration (0.05 l/min) and water inlet flow of 2.3 l/day. (×): OWW samples under aeration (0.1 l/min) and water inlet flow of 4.2 l/day.

The analysis of the diversity of the bacterial community of aerated submerged biofilms was done using PCR amplification with universal primers, followed by TGGE analysis. The bacterial DNA from OWW samples without treatment was also included in the TGGE analysis. Fig. 5 shows the TGGE profiles based on the V3-hypervariable region of the 16S rRNA gene corresponding to treatments F6, F9, F12, and F0 (OWW samples previous to treatment). "M" is a mixed-species control ladder. The TGGE banding pattern showed the disappearing of some of the initial mayor bands, while new bands absent in the F0 sample predominated (*i.e.*, bands 34 and 35). Nevertheless, some of the initial biofilm genotypes remained dominant in the biofilm throughout all the treatments (*i.e.*, bands 22, 23, 33). The variations of the biofilm community fingerprint due to the changes of air and OWW flows were only slight.

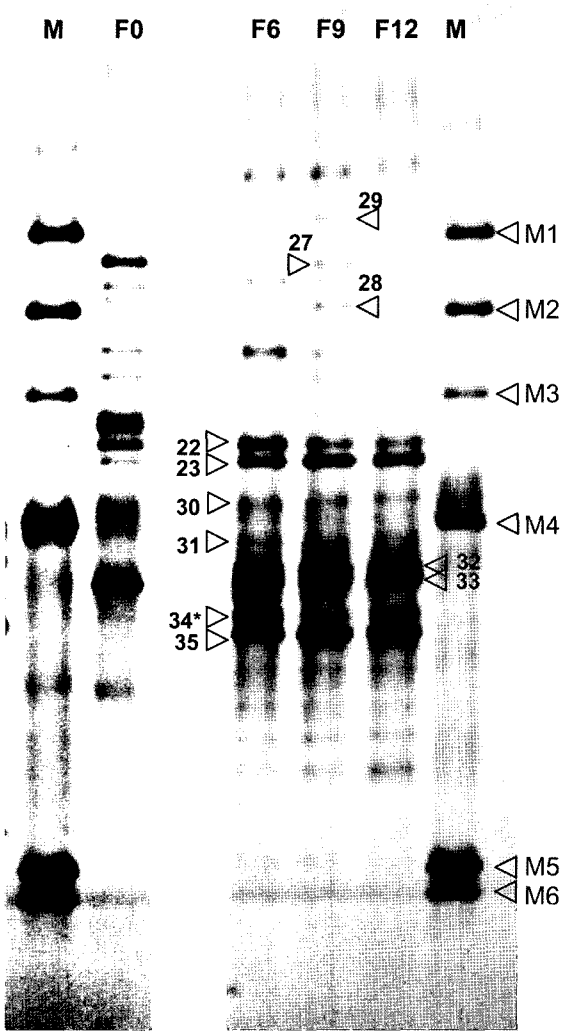

Fig. 5. Community profiles of bacteria in the biofilms taken from the lab-scale submerged biofilter for the treatment of OWW.

Profiles are based on amplification and TGGE separation of the V3-hypervariable region of the 16S rRNA gene. Numbered arrows point to bands that were reamplified and sequenced for the phylogenetic analysis shown in Fig. 6. The different treatments are indicated above each lane (see text). M: six-species marker, made by amplification of V3-region from DNA of the following culture collection strains: M1, *Staphylococcus aureus* ATCC 25923; M2, *Pseudomonas putida* ATCC 8750; M3, *Acinetobacter calcoaceticus* ATCC 15308; M4, *Escherichia coli* DH5α; M5, *Nocardia corynebacterioides* ATCC 21253; M6, *Micrococcus luteus* ATCC 9341. * This band was excluded from the phylogenetic analysis shown in Fig. 6.

Eleven dominant TGGE bands from treatments F6, F9, and F12 were successfully reamplified and sequenced (Fig. 6). The sequence of band 34 yielded ambiguities in nucleotides crucial for species identification, and therefore was excluded from the phylogenetic analysis. Sequences of the other 10 bands were affiliated to the α-subclass of Proteobacteria, and often branched in the periphery of genera of bacteria commonly present in soil (*Rhizobium*, *Reichenowia*, *Agrobacterium*, and *Sphingomonas*), as showed in the corresponding phylogenetic tree (Fig. 6).

Discussion

The results of this study demonstrate the suitability of the aerated submerged filter system as an efficient biological, low-cost technology for OWW decontamination. Our results have proven the effectiveness of the lab-scale plant for COD and BOD_5 removal (over 85% COD and 90% BOD_5 after six days, under optimal air and inlet flows). The efficiency of the COD removal was in the range of 61–85% (Fig. 3). These yields are similar to those reported [6] for a biomass-immobilized fluidized bed reactor anaerobically treating OWW. These authors obtained a 50–90% elimination of the COD present in the OWW for organic loading rates of 0.46 to 2.25 g O_2/l per day. Remarkably, the organic loading rates used in our experiments were significantly higher (2.00, 3.55, and 6.49 g O_2/l per day) at the 3 inflow rates tested (1.3, 2.3, and 4.1 l/day, respectively). The results show that the aerobic treatment of OWW in a submerged biofilter requires lower hydraulic retention times than the anaerobic digestion in a fluidized bed reactor, whereas they allow for the achievement of a similar COD removal efficiency in a shorter time.

The submerged biofilter showed only moderate efficiency in the elimination of the total content of polyphenolic compounds (around 50% decrease of the initial OWW content). These yields are equivalent to those reported for the anaerobic digestion of diluted OOMWW (60 mg/l of Tph as caffeic acid) in immobilized biomass reactors [26]. These authors reported a 53% elimination of the phenols present in the diluted OOMWW, for an organic loading rate of 2.25 g O_2/l COD per day, and could achieve an efficiency of removal of over 90% only by extremely long hydraulic retention times (25 days). In the submerged biofilter described in this paper, testing of inflow rates lower than 1.3 l/day will be considered in future studies, with the aim to improve the efficiency of phenol removal.

In our experiments, the colonization of the inert support of the biofilter by bacteria naturally present in the OWW samples in order to form the biofilm was a rapid (24 h) and simple process, and no external inoculation was needed to initiate the biofilm. In contrast, most investigations regarding the biological treatment of wastes generated in olive oil processing, under aerobic or anaerobic conditions, are based on the use of active biomass taken from other wastewater treatment systems [26] or inoculation with selected microorganisms with previously proven phenolic acid removal capacities [3, 5, 10, 11, 14, 28]. Phylogenetic analysis derived from TGGE separation confirmed the dominance of α-Proteobacteria as colonizers of the biofilms formed in the submerged aerated biofilter used in the

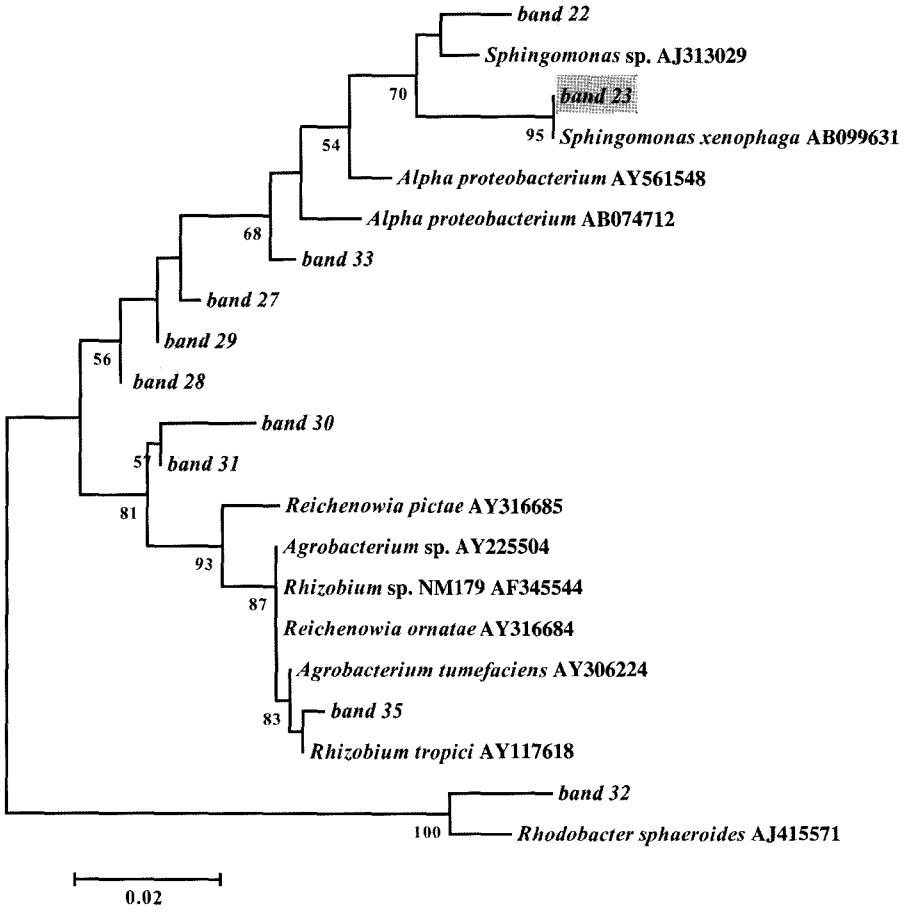

Fig. 6. Phylogenetic neighbor-joining tree of the 16S rRNA gene sequences (V3-hypervariable region) from the TGGE bands shown in Fig. 5.

Sequences retrieved from the EMBL database are indicated with their corresponding accession numbers. Bootstrap values below 50 are not shown.

study. The microorganisms were mainly grouping in the periphery of bacterial species of the *Rhizobiaceae* (bands 30, 31, 35) and *Sphingomonadaceae* (bands 22, 23, 27, 28, 33). These bacteria are universally found in the soil and rhizosphere of temperate regions (such as the Mediterranean area), and commonly lack special growth requirements. Members of the *Sphingomonadaceae* are common inhabitants of soil and freshwaters, and *Sphingomonas*-related strains are often specialized in the degradation of aromatic chemicals (PHAs, dioxin compounds, and chlorinated phenols) or phenolic acids at low concentrations [24], showing promising biotechnological properties [10, 27, 33]. Di Gioia *et al.* [10] reported that several *Sphigomonas* strains isolated from OOMWW were able to aerobically degrade some monocyclic OOMWW compounds such as veratric acid, 4-hydroxybenzoic acid, syringic acid, cinnamic acid, caffeic acid, or 4-hydroxyphenyl acetic acid.

The submerged biofilter technology has been previously successfully applied for the simultaneous removal of ammonia and phenol from contaminated sea water [7]. TGGE community analysis of the biofilm in such systems showed the dominance of aerobic marine α-Proteobacteria, but several sequences closely related to the genera *Reichenowia* and *Agrobacterium* were also present in the biofilm, confirming the ubiquity of the *Rhizobiaceae* members in environments contaminated with aromatic compounds, as well as their potential for biotechnological applications targeting the remediation of these pollutants. Most aromatic substrates are aerobically catabolized via the catechol or protocatechuate branch of the β-ketoadipate pathway in members of the family *Rhizobiaceae*, although these metabolic capacities are a strain-specific trait. In this sense, some strains of *Rhizobium leguminosarum* have the ability to use catechol, whereas other strains lack this ability [25]. It must also be taken into consideration that the removal of Tph may include not only biological degradation but also passive adsorption of the compounds to the cell biomass (biosorption).

Although the inoculation of the biofilter was shown to be not essential for the good performance of the plant, the possibility of increasing the removal of Tph by using bacterial strains isolated from OWW and selected according

to their ability to degrade polyphenols should also be considered. As previously discussed, the rates of COD, BOD_5, and phenol removal by the microbiota present in the biofilms of the treatment system were also significantly affected by variations of the plant operation parameters, and the removal of the Tph content from OWW could also be improved by their modification. These facts will be explored in future research using a pilot-scale treatment biofilter.

In conclusion, the experimentation at laboratory scale has provided data that support the possibility of applying the submerged biofilter technology at full scale for the treatment of OWW, with the purpose of its reutilization. However, the aerobic submerged biofilter should be followed by a complementary treatment, in order to be safely used in further olive washing steps, or for agricultural purposes. This quality can be reached by applying advanced physicochemical technologies, such as ultra- and nanofiltrations [21].

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