Biotreatment of industrial olive washing water by synergetic association of microalgal-bacterial consortia in a photobioreactor

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Abstract

This study presents an effective technology for the olive processing industry to remediate olive washing water. A 14.5-L enclosed tubular photobioreactor was inoculated with a stable microalgal-bacterial consortium obtained by screening strains well adapted to olive washing water. The capacity of an enclosed tubular photobioreactor to remove toxic com- pounds was evaluated under photosynthesis conditions and without any external supply of oxygen. The results showed that the dominant green microalgae *Scenedesmus obliquus*, *Chlorella vulgaris* and the cyanobacteria *Anabaena* sp. and bacteria present in olive washing water (i.e. *Pantoea agglomerans* and *Raoultella terrigena*) formed a synergistic association that was resistant to toxic pollutants present in the effluent and during the initial biodegradation process, which resulted in the breakdown of the pollutant. Total phenolic com- pounds, COD, BOD_5, turbidity and colour removals of 90.3 ± 11.4, 80.7 ± 9.7, 97.8 ± 12.7, 82.9 ± 8.4 and 83.3 ± 10.4 %, respectively, were recorded in the photobioreactor at 3 days of hydraulic retention time.

Keywords Photobioreactor (PBR), Olive washing water (OWW), Microalgal-bacterial consortium, Synergistic relationships, Non-metric multidimensional scaling (MDS)

Introduction

The olive oil industry represents the major food sector for Mediterranean countries with the highest worldwide produc- tion, according to data from the International Olive Oil Council (IOOC 2015). There are more than 1700 olive mills currently authorized and operating in Spain (IOOC 2015). Moreover, 70 % of the olive oil produced during the 2013– 2014 campaign was obtained in Andalucía (Ochando-Pulido and Martinez-Ferez 2015). The significant growth of this in- dustrial sector has generated substantial amounts of wastewaters: olive washing water (OWW) and olive mill wastewater (OMWW) (Cerrone et al. 2011; Ochando-Pulido and Martinez-Ferez 2015).

Both wastewaters are quite similar, but OWW contains lower concentration (from 1/10 to 1/50) of pollutants such as chemical oxygen demand (COD) and phenolic compounds than OMWW (Cerrone et al. 2011; Maza-Márquez et al. 2014). A preliminary washing of the olives with potable water is necessary during the process, and this generates approximately 1 m^3 of OWW per processed ton (Pozo et al. 2007). The OWW has been associated with serious disturbances in natural ecology and long-term environmental destruction mainly due to the presence of phenolic compounds, organic and long-chain fatty acids, tannins and organohalogenated contaminants (Komnitsas and Zaharaki 2012; Maza-Márquez et al. 2013). Phenols and their derivatives are considered to have high environmental impact because they are phytotoxic recalcitrant pollutants to organisms even at very low concentrations (Maza-Márquez et al. 2014; Pozo et al. 2007). In this regard, OWW requires specific treatment prior to direct discharge in order to ensure environmental protection and for regenerated wastewater, according to the European Directive 2000/60/CE.

A wide range of chemical-physical methods has been developed to date for the treatment of OWW. However, these cost-ineffective methods do not completely resolve the pollution problem. Since olive oil industries are composed of small and geographically dispersed factories, they cannot endure the high cost associated to chemical-physical methods (Justino et al. 2012).

In contrast, biological treatment offers an effective, economical and simple solution for these small plants. Several studies had reported the use of microorganisms (individual or consortia) to degrade organic compounds present in OWW effluent (Cerrone et al. 2011; Maza-Márquez et al. 2014). Recently, Maza-Márquez et al. (2013, 2014) isolated and selected a tolerant, indigenous microalgae-bacteria consortium with the capacity to degrade phenolic compounds to produce an environmental discharge that meets specific guidelines and EU legislation. This biological treatment is possible since the microalgae/bacteria relationship is highly synergetic. High microalgae cell densities increased the availability of oxygen required for the degradation of phenol by the aerobic bacteria, resulting in an essential factor in the biotreatment of olive washing wastewater (Maza-Márquez et al. 2014). Phenol can be degraded into relatively safe bio-products due to these microorganisms (i.e. *Pantoea agglomerans* and *Klebsiella terrigena*) using the phenol as carbon and energy source (Maza-Márquez et al. 2013, 2014).

In this viewpoint, the microbial consortia (biofilms) appear to be an environmental friendly and cost-effective option to metabolize the phenol of wastewaters. These biofilms are composed of multiple species with different metabolic degra- dation pathways offering greater resistance from environmental stress (i.e. UV damage, desiccation, predation, biocides, solvents, toxic chemical and pollutants) (Mitra and Mukhopadhyay 2016).

One of the possible solutions to increase the reliability of the process is the use of a stable photobioreactor

(PBR) sys- tem. Biofilm PBR offers many advantages for OWW decontamination: high concentration and retention of biomass for long periods of time; enhanced metabolic activity; the biodegradation capacity; and, consequently, higher resistance to toxic compounds, large mass transfer area and coexistence of anoxic and aerobic metabolic activity. Furthermore, it reduces the health and environmental risk derived from the dispersion and evaporation of the contaminants. In addition, it also reduces the ecological footprint.

The goal of the present work was to examine the application of a biofilm PBR for the treatment of real OWW. PBRs were set up with a mix of microalgae and bacterial species with the ability to degrade phenolic compounds (Maza- Márquez et al. 2014). This is the first attempt to employ an enclosed tubular PBR system for the bioremediation of OWW. The ability of the PBR to degrade this toxic influent was assessed with and without inoculation of the microalgae- bacteria consortium under different operation conditions. In addition, shift of biofilm PBR community was analyzed by means of multivariate analyses (non-metric multidimensional scaling (MDS)). MDS reduces the complexity and permits visual appreciation of the relationship of a data set, making it more used than principal component analysis (PCA) analy- ses. Optimization and evaluation of the batch remediation process of PBR were also reported.

Materials and methods

Characteristics of wastewater

OWW was obtained from the olive oil factory Nuestra Señora de los Desamparados (Puente Genil, Córdoba, Spain). The pool (ca. 45 m^3) is 3 m deep with no agitation. Samples were collected at a depth of 1.5–2 m during the olive harvesting season (January to June 2014). Active carbon was used as a pretreatment in order to remove part of the colour of the in- fluent. The OWW was pumped through an active carbon column (0.5 kg) (pump 1 with variable speed depending on hy- draulic retention time (HRT), Table 1) with a contact time of 5 min. After long-term use (100 L OWW), part of the active carbon was regenerated (0.4 kg) to maintain its efficiency and reduce operation costs substantially. For cleaning, a carbon column was backwashed and then reused. The water produced during the cleaning of the active carbon was treated in the PBR.

Microorganisms and culture conditions

Resistant strains adapted to live with strong environmental pollutants present in OWW were obtained by isolation from OWW where indigenous microorganisms have already been exposed to the target contaminants. Two bacterial strains (*Raoultella terrigena* and *P. agglomerans*) capable of degrading phenolic compounds, originally isolated from an OWW storage basin (Maza-Márquez et al. 2013), were coinoculated with two microalgae isolated from OWW (*Scenedesmus obliquus* and *Chlorella vulgaris*) (Maza- Márquez et al. 2014).

Microalgae were grown on synthetic OWW medium com- posed of 20 mg L^{-1} $NaNO_3$, 10 mg L^{-1} K_3PO_4 and trace minerals (Maza-Márquez et al. 2014). Culturing was accom- plished in 1-L Erlenmeyer flasks containing 300 mL of medi- um in a shaking incubator (HB 201 SF-L Hanbaek Scientific Co., Seoul, Korea) at 25 ± 1 °C, 160-rpm rotation and contin- uous illumination using a 60 μmol m^{-2} s^{-1} cool white fluo- rescent light. The initial concentration of the microalgae was controlled at an optical density (OD) of 0.24–0.25 at 560 nm (Wetherell 1961), which corresponded to 10^{12} cells mL^{-1} (1:1 mixture of each strain).

Two bacterial strains were grown in 1-L Erlenmeyer flasks containing 300 mL of synthetic OWW medium as previously reported by Maza-Márquez et al. (2013). The concentration of bacteria was 10^3 CFU mL^{-1} (1:1 mixture of each strain).

PBR pilot plant

The experiments were developed using a pilot-scale PBR, installed at the wastewater treatment facilities at the Institute of Water Research (University of Granada). A schematic diagram of the experimental plant is displayed in Fig. 1. The pilot plant is a semi-open system (16-L total working volume), which includes a mixing tank (0.4-m height, 0.3-m width, 0.2-m depth, 2-L operating volume), a tubular PBR (composed of five vertical tubes, per tube 1-m height, 0.04-m diameter, 2.1-L operating volume), a recirculation tank (0.4-m height, 0.3-m width, 0.2-m depth, 2-L operating volume) and a tank collector (0.2-m height, 0.2-m width, 0.15-m depth, 1.5-L operating volume). First, the mixed tank received the influent OWW (pump 1 with variable speed depending on HRT, Table 1) after pretreatment with active carbon. Second, the water passed through a tubular PBR composed of five colons of a meter. Third, the water enters the recirculation tank where one part was recycling (0.35 L min^{-1}) inside the PBR. Finally, the supernatant was pumped inside the tank collector. The mixing tank was equipped with an overhead

stirrer (200 rpm). Peristaltic pumps were used for influent feeding and effluent withdrawal (both Watson Marlow, USA). The tubular PBR was an enclosed jacketed 14.5-L glass tank (Afora, Spain) with a total working volume of 10.5 L. The tubular PBR was operated under continuous illumination at 10.000 lx using eight fluorescent lamps (Osram L, Germany, 40 W), arranged in a circular configuration. Fluorescent growth lights provided an average light intensity of 450 ± 50 μE/m^{-2} at the outer wall of the PBR. No mechanical aeration of the system was performed.

Experimental set-up

The PBR was initially filled with 16 L containing 14.5 L of synthetic medium for OWW (Maza-Márquez et al. 2014) and inoculated with 1.5 L of consortium. The inoculated consisted in 1 L (1:1, *v*/*v*) of the selected microalgae *C. vulgaris* and *S. obliquus* at a final concentration of 10^{12} cells mL^{-1} and with 500 mL (1:1, *v*/*v*) of the selected bacteria *P. agglomerans* and *R. terrigena* at a final concentration of 10^{3} CFU mL^{-1}.

The inoculum was created in order to reach an optimal microalgae/bacteria ratio of biodegradation of 10^{12}:10^{3} (Maza-Márquez et al. 2014). After 10 days of microbial inoculation, the PBRs were fed with real OWW. A control system was performed with a non-inoculated microbial consortium of OWW. It was initially filled with 16 L containing 14.5 L of synthetic medium OWW and inoculated with 1.5 L of OWW for 10 days following the same process as that for the inoculated system for comparison. The experiments were carried out on non-sterile OWW in order to simulate possible field application conditions. A 40-day batch experiment was car- ried out using a pilot-scale PBR. Since the consortia could be grown after 24 h in synthetic OWW medium (Maza-Márquez et al. 2014), 40-day experimental period was proposed, shorter than those of previous studies of wastewater treatment with PBR (Van Den Hende et al. 2014; Gonzalez et al. 2008). The PBR was operated at 5- and 3-day hydraulic retention times (HRTs) with a continuous internal effluent recycling (Watson Marlow M60617) of 0.35 L min^{-1} (Table 1). The HRTs were chosen according to typical values reported as optimum for wastewater treatment (de Godos et al. 2009). The PBR was operated as a complete-mix reactor due to the high recirculation rate/feed rate ratio imposed, which is appropriate when the wastewater to be treated exhibits a high inhibitory potential (Tchobanoglous et al. 2003). The recirculation tank was maintained under magnetic agitation (200 rpm) throughout the

entire experimentation.

Determination of microbial growth

Biomass concentration (as volatile suspended solids (VSS)) was used to monitor consortium growth. Biomass dry weight as suspended solids was determined gravimetrically according to Standard Methods (APHA-AWWA, WEF 2005). Phosphorus was measured as orthophosphate (P-PO_4) and nitrogen as the sum of nitrate and ammonium (N: N-NO_3 + N-NH^+). Total phosphorus (APHA 4500-P C) and total nitrogen (APHA 4500-NorgB) were quantified as described in the Standard Methods for the Examination of Water and Wastewater (APHA, AWWA, WEF 2005).

Analytical procedure

The samples of the influent and effluent from the PBR were collected periodically in order to monitor the colour, turbidity, COD, BOD_5, total phenols, pH and dissolved oxygen (DO). Methodologies used for determination were as described by the Standard Methods for the Examination of Water and Wastewater (APHA 2005), except for the total phenol deter- mination which was carried out spectrophotometrically (760 nm) by the Folin-Ciocalteu method (D'Annibale et al. 2006). Temperature and DO were monitored in situ using a multiple P4 Oxical-SL Universal Meter (WTW, Germany), while a Crison micropH 2002 (Crison Instruments, Spain) was used for pH determination.

Isolation and identification of microalgal species from PBR

Several microalgae were isolated from the PBRs at 40 days of experimentation. For the isolation of strains, OWW samples (1, 2, 3 mL) were serially diluted in 1-L Erlenmeyer flasks which contained 300 mL of modified Rodriguez-López medium as described by Stainer et al. (1971). The medium contained $NaNO_3$ (1.5 g L^{-1}), K_2HPO_4 (0.04 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.075 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.036 g L^{-1}), citric acid (0.01 g L^{-1}), ferric ammonium citrate (0.006 g L^{-1}), Na_2 EDTA (0.001 g L^{-1}), Na_2CO_3 (0.02 mg L^{-1}) and 1 mL of trace metal solution per litre. The trace metal solution contained H_3BO_3 (61.0 mg L^{-1}), $MnSO_4$ (169.0 mg L^{-1}), $ZnSO_4 \cdot 7H_2O$ (287 mg L^{-1}), $CuSO_4 \cdot 5H_2O$ (2.5 mg L^{-1}) and

$(NH_4)_6MoO_4 \cdot 4H_2O$ (12.5 mg L^{-1}), essential micronutrients for microalgae growth (Maza-Márquez et al. 2014). The Erlenmeyer flasks were incubated at 25 ± 1 °C, 160-rpm rotation and 200 μmol m^{-2} s^{-1} illumination with a 16:8-h light dark cycle (Jeiotech, GC-300TLH, Lab Companion, Des Plaines, IL, USA) for 48–72 h.

The samples (0.1 mL) were serially diluted and spread on modified Rodriguez-López medium plates containing 4 % agar and incubated as previously described. Individual colonies were examined microscopically and isolated, and a partial sequence of 18S rRNA gene were obtained. The primers used to amplify the partial 18S rRNA gene of the selected isolates were forward primer Euk 1 (5′-CTGGTTGATCCTGCCAG-3′) and reverse primer Euk 516r (5′-ACCAGACTTGCCCTCC-3′) (Díez et al. 2001). Sequences were analyzed online by the European Bioinformatics Institute biocomputing tools (htpp:// www.ebi.ac.uk). The BLASTn program was used for analysis of sequence similarity, and ClustalX version 2.0.3 software was used for sequence alignment. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 1 (Kumar et al. 2001); the *p*-distance-based evolutionary tree was inferred using the neighbour-joining algorithm.

Statistical analysis

The Primer software (PRIMER-E v. 6.0, Plymouth, UK) was used to analyze the four sets of biotic data derived from the biomass microbial in the PBR samples (5-day HRT, 3-day HRT, control and influent OWW). Sample-resemblance ma- trices were generated using the Bray-Curtis coefficient of similarity. Based on these similarity matrices, cluster analyses, non-parametric multidimensional scaling (MDS) and analysis of similarity (ANOSIM) analyses were performed.The ANOSIM analyses was used to search significant differences between the groups of samples (5-day HRT, 3-day HRT, control and influent OWW). The MDS plots display the distribution patterns of the samples according to the similarity be- tween their biomass cultivations in the PBR. The stress level of the MDS plots indicates how well the variables' data fit into two-dimensional spaces, with values close to 0.2 giving a valid representation of their distribution (Clarke and Warwick 2001). Vectors in the MDS plots represent the Spearman rank correlations (ρ_S) of the different cultivation conditions on the PBR, illustrating their directional increase through the biological ordination.

Results and discussion

Characteristics OWW

The characteristics of the OWW used for the present investigation are shown in Table 2. OWW has an intense dark green colour resulting from the presence of recalcitrant polyphenolic compounds, which are often chemically linked to lignin and humic acid. OWW's dark colour reduces light transmission and may inhibit the growth of microalgae. For this reason, a pretreatment with active carbon was necessary to remove a portion of the colour and enhance microalgal growth. Pretreated OWW was applied directly to the culture consortium in the PBR, resulting in an overall colour and turbidity removal of 36.2 and 39.4 %, respectively (Table 2).

Optimization and evaluation of the PBR batch remediation process

In order to optimize the OWW bioremediation, a tubular PBR was used to create a homogenous condition during the treat- ment of toxic effluent and lower the risk of microalgal inhibition. According to Lee (2001), this configuration is the most efficient for wastewater bioremediation due to their high illuminated surface-to-volume ratio. In addition, tubular PBR was designed considering dark areas: dark green colour of OWW, turbidity and biofilm formation. The photosynthesis activity is dependent on the distribution and penetration of the light. In that context, PBR consisted in five tubular tubes of a meter of height, which increased the homogeneity of light distribution. Furthermore, the narrower diameter of tubes (40 cm) resulted in better hydrodynamic properties and improved the light penetration. Moreover, the internal recirculation provides some oxygen concentration. This, combined with the optimum microalgae/bacteria ratio, resulted in a high efficiency for pho- tosynthetic activity.

Moreover, closed PBRs create an enclosed growth environment for microbiota cultivation where the potential contaminant is concentrated and easier to manipulate and control. This configuration allowed the optimization of the essential variables and provided high photosynthetic efficiencies for bacterial degradation. In turn, this technology reduces the cost of mechanical aeration and mitigates the amount of CO_2 released by the bacteria, thereby preventing greenhouse gas emission (Muñoz and Guieysse 2006; Essam et al. 2014). In this regard, several studies have reported the greater potential advantages of PBRs for the treatment of industrial wastes. Muñoz et al. (2005) reported that a consortium of *Ralstonia*

basilensis- Chlorella sorokiniana completely degraded acetonitrile in a column PBR. According to Dhillon and Shivaraman (1999),

23 g L^{-1} day^{-1} acetonitrile was removed in a continuous column PBR inoculated with *C. sorokiniana* and a bacterial consortium. Likewise, Tamer et al. (2006) reported that a consortium of *C. vulgaris* and *Alcaligenes* sp. removed 100 % of the phenol in a continuous PBR with sludge recirculation.

PBR consortium development

The coselection of bacteria and microalgae to ensure microorganism compatibility has been previously reported (Maza- Márquez et al. 2013, 2014). The PBR was inoculated with a microalgal mixture including *S. obliquus* and *C. vulgaris* and a bacterial mixture of *R. terrigena* and *P. agglomerans*, considered as a stable microbial consortium capable of degrading phenolic compounds (Maza-Márquez et al. 2014). Figure 2 shows the development of microbial biomass inside the PBRs during the operational period at different HRTs (3 and 5 days). The PBR was operated for approximately 6 months (January to June of 2014) where the first 10 days were a period of microbial acclimatization with synthetic OWW and bioreactor start-up (Fig. 2a). Immediately after the PBR was fed with real OWW, a rapid biofilm growth occurred inside the plastic tube at 5 days of operation (Fig. 2b, c). The real OWW enhanced the development of the microalgae over that of the synthetic OWW, and the time necessary for the biofilm development in the system was approximately 6 days. The selected microorganisms are acclimated to this type of effluent, and OWW wastewater as the cultivation medium can offset the nutrients needed for the production of the microbial biomass. These results support those that previously demonstrated the growth of this consortium after 24 h (Maza-Márquez et al. 2014), which is an important factor for real applications. In that sense, extensive matrix biofilm formation was observed after the initial transfer of OWW into the PBR (Fig. 2b, c). The biofilm was strongly attached to the surface of the PBR, which resulted in optimal biodegradation conditions of the system. The PBR exhibited an intense green colouration of the tubes due to the high microalgal biomass throughout the experimentation process. In contrast, a biofilm did not develop in the control (non-inoculum PBR operated under same conditions as in the inoculated system) PBR.

Microbial growth in the PBR is illustrated in Fig. 3a. At operational conditions of 5-day HRT, the culture was in lag phase for the first 4 days when the microbial cells were slowly adapting to the new environmental conditions. However, at 3- day HRT, this lag phase was more prorogated until approximately day 10. This lag phase of growth was similar to that of the control at approximately 2–2.3 g L^{-1} (Fig. 3a). At 5-day

HRT, the density of the microbial biomass started to rapidly increase around the seventh day. However, at 3-day HRT, this exponential phase started 3 days after (approximately on day 10). Despite these differences, at the end of the experiment, the maximum biomass microbial growth was similar at different HRTs of approximately 8 and 8.5 g L^{-1}.

The MDS plots generated for the PBR samples on the basis of the consortia abundance of different experimental periods (OWW, control, 5- and 3-day HRTs) are shown in Fig. 3b, where the distance between any two sample points reflects their relatively similarity.

The vectors in the plots illustrate the direction of the linear relationships between the biotic variables (OWW, control, 5- and 3-day HRTs) and the sample's ordination based on the abundance of the microbial biofilm in the experimental period of PBR. The ANOSIM analysis also revealed that there were no significant differences of the microbial community be- tween two experimental phases ($R = -0.02$) (5- and 3-day HRTs, respectively), demonstrating a similar behaviour of the community despite the different HRTs. The nutrient re- moval performance of PBR is indicated in Fig. 4. The removal rates of total N and P for the microbes were 22.1 mg N L^{-1} day^{-1} and 8.5 mg P L^{-1} day^{-1} for 5-day HRT and

20.3 mg N L^{-1} day^{-1} and 8 mg P L^{-1} day^{-1} for 3-day HRT

in the PBR, respectively. The average effluent total N and P concentrations in the PBR were below 2 mg L^{-1}, with corre- sponding reductions of approximately 97 and 96 % (total N and total P, respectively). The data indicated that the removal of nitrogen and phosphorus corresponded with the consortium growth. However, the removal rates of total N and P for the control PBR were 3.8 mg N L^{-1} day^{-1} and 1.9 mg P L^{-1} day^{-1} corresponding to reductions of approximately 42.3 and

18.4 %, respectively (Fig. 4).

These experiments show that a microbial consortium can successfully grow on the OWW, which is favourable for reaching maximal biodegradation. Several studies had reported that the concentration of N and P pollutants in many kinds of wastewater can be reduced to a very low value through the assimilation of algal cells (Gao et al. 2014; Shi et al. 2014; Vasconcelos-Fernandes et al. 2015).

Biodegradation studies

The evolution of the colour, turbidity, COD, BOD_5, total phe- nol concentration and DO in the pilot-scale PBR was tested with different operational conditions (5- and 3-day HRTs, re- spectively) over a period of

40 days (Fig. 5 and Table 2).

Initially, we detected a lag phase of approximately 4 days for 5-day HRT and 10 days for 3-day HRT before the biodeg- radation commenced. The formation of the microbial biofilm coincided with a gradual improvement in the colour and tur- bidity. The maximal colour reduction obtained was 86 % after 17 days for 5-day HRT and 84.6 % after 30 days for the 3-day HRT experimental phase. A similar average turbidity removal of 83.2 and 82.9 % (5- and 3-day HRTs, respectively) was detected in both operational conditions. In addition, a continual decrease in the BOD_5 and COD parameters was observed with time. The treatment consortium at different HRTs drastically reduced BOD_5 values of the effluent water with averages of 98.4 % for 5 days and 97.8 % for 3 days. After 15 days of operation, the yields of BOD_5 removal observed were 98 % for both operational conditions. Total COD removal efficiencies reached approximately 81.3 % for 5 days and 80.7 % for 3 days from days 11 to 40, concomitantly with an increase in the effluent VSS concentration in the PBR (bio- film development) (Fig. 3a).

Finally, the total phenols exhibited a clear tendency after reaching stable conditions. The concentration of total phenols was drastically reduced, showing that the consortium can metabolize high phenolic compound concentrations. The average of acid phenols was significantly reduced under different conditions by 90.6 and 90.3 % (5- and 3-day HRT, respectively). The consortium was able to remove phenols at a maximum rate of 157.2 ± 12.8 and 145.5 ± 14.2 mg L^{-1} day^{-1}, (5- and 3-day HRTs, respectively). On the contrary, all of the measured parameters of the biodegradation process in the uninoculated PBR control were negligible (Fig. 5 and Table 2).

These results indicated a direct relationship between the resistant microbial consortium and the biodegradation activity (Figs. 2 and 5). In that context, inoculation of a stable microbiota-consortium leads to colonization of the abiotic PBR surface and formation of biofilm communities that are persistent to toxic pollutants present in the effluent and initially in the biodegradation process (Fig. 5 and Table 2). This conclusion was confirmed in the current study through the monitored DO content (Fig. 5, Table 2). The concentration of DO in the PBR was higher than 7 mg L^{-1} with inoculum, while the concentration of DO was below 5 mg L^{-1} in the uninoculated PBR (Fig. 5, Table 2). This result suggests that microalgae provide the oxygen for the efficient degradation because highly polluted wastewater like OWW contains an insufficient oxygen level. The results were homogeneous and consistent with those reported previously using the same consortium for the treatment of phenolic compounds (Maza-Márquez et al. 2014). Previously, we determined the optimal microalgae/bacterial ratio for a synergistic relationship. This synergism is based on the use of microalgae as suppliers of O_2 as the key electron acceptor of heterotrophic bacteria during aerobic biodegradation.

The combination of phototrophic and heterotrophic organisms enhances the degradation potential of the whole consortium (Fig. 5 and Table 2). Several studies have shown that photosynthetic oxygenation supports the aer- obic treatment of toxic wastes containing pollutants such as black oil, phenantrene, phenol, acetonitrile, napthalene, benzopyrene or azo compounds (Borde et al. 2003; Essam et al. 2014; Mahdavi et al. 2015; Muñoz and Guieysse 2006; Muñoz et al. 2005; Ryu et al. 2015; Tang et al. 2010). It is well known that OWW causes serious disturbances in the natural ecology and represents one of the major pollutants found in wastewater and underground, in particular due to the colour and phenols. Phenolic compounds are included in the US Environmental Protection Agency (EPA) list of the 129 specific priority pollutants (Singh et al. 2013). In order to protect human and environmental health, many biological and chemical approaches have been investigated. Chemical techniques include a diverse range of treatments, including simple precipitation to more advanced methods such as electrochemical oxidation (Belaid et al. 2013), catalytic oxidation using Fenton's reagent (El-Gohary and Tawfik 2009) or ozonation (Chedeville et al. 2009). However, these methods are normally characterized as too expensive (Babuponnusami and Muthukumar 2013; Hussain et al. 2015; Justino et al. 2012), inefficient (Busca et al. 2008) and capable of generating hazardous by-products (Hussain et al. 2015; Liotta et al. 2009). In contrast, our proposed PBR technology offers several advantages over the chemical methods. PBR could be a cost-effective system due to the absence of aeration (provided by microalgae). It is well documented in literature that oxygen aeration represents more than 50 % of the energetic biological treatment (Delrue et al. 2016). Moreover, only a single inoculum is necessary for start-up. Its versatility leads to modifications to adapt to local needs and provides minimal controls from qualified personnel, making it a low-cost system. There is minimal possibility of the production of by-products and the maintenance of the neutral pH, which differs from some chemical methods (i.e. pH Fenton is around 3). The pH of the effluent generated by the biological treatment was always 7.0 from the original 5.0 (Table 2), which constitutes an important factor in the case of discharge or reuse as a fertilizer.

In addition, the results presented here indicate that this simple technology significantly reduced the colour and phenolic compounds in the pollutants by approximately 85 and 90 %, respectively, without dilution of OWW (Fig. 5 and Table 2). As a consequence, the effluent generated could be reused in ferti-irrigations and/or it could be sent to direct dis- charge (according to the region-specific regulations). Furthermore, a simple treatment such as membrane technology could also be applied to water recycling. The yields obtained by the consortium were higher than those reported in studies with aerobic bacterial biofilter technology (50 % of phenol elimination from OWW; Pozo et al. 2007) and higher than yields previously reported for *Trametes versicolor* in a continuous bubble column bioreactor process after OWW dilution (65 % of colour and 89 % of phenol; Cerrone et al. 2011).

Interestingly, the results obtained in our PBR system demonstrated some better degrading efficiencies than those ob- served in other biological studies based on similar and com- plex effluents (Ganzenko et al. 2014). Phenol efficiencies ranging from 64 to 90 % have been reported in biological treatment (Bastos et al. 2000; González et al. 2001; Tambekar et al. 2013). Microalgae consortium was able to remove 58 % of COD and 84 % of colour from a diluted pulp and paper industry wastewater (Tarlan et al. 2002). Approximate reductions of 65–81 % for colour and of 84–87 % for COD have been reported in dye effluents depending on the aerobic or anaerobic treatment (Gnanapragasam et al. 2011; Libra et al. 2004; Smith et al. 2007; Ong et al. 2010). Biological treatment petroleum effluents were able to eliminate 76 % of phenol from coke oven (Chakraborty et al. 2010) or around 90 % of phenol under anaerobic conditions from petrochemical wastes (Guo et al. 2015). These data suggest that the synergistic microalgae-bacteria consortium used in our study could be successfully applied to the treatment of other common and problematic pollutant effluents such as dyes and textile wastewater, olive oil mill wastewater, pharmaceuticals and/or hospital wastewater. However, further re- search should be conducted to explore the potential applications in the treatment of complex industrial wastes and, therefore, to reduce the HRT. In that sense, studies based on full-scale PBR are in progress. The system will be composed of eight modules with ten tubes each (per tube 2-m height, 0.25-m diameter, 98.17-L working conditions), able to treat around 1374 or 3120 L day^{-1} (5- and 3-day HRT, respectively). During the improvements in the testing period, research will be focused on the optimization of the hydraulic operation conditions operated under extreme conditions such light, temperature or pollutant concentration and the HRT can be even lower than 3 days. Moreover, the system will be performed under sunlight, avoiding the light cost. The technology is based on factors of light penetration and microalgae concentration to supply the oxygen necessary to remove hazardous compounds. In addition, microalgae inhibition may occur at extreme-light or low-light intensities, high levels of biomass and high pollutant concentrations if the process is not properly controlled. In that context, the complex biofilm microalgae-bacteria formation will be studied by next-generation sequencing. This will be investigated in future experiments.

Isolation and identification of microalgal species from PBR

Microscopic images of microalgae revealed the two strains of the consortium and one major cyanobacterium (Fig. 6a). The taxonomic affiliations of the strains are shown in Fig. 6b. A comparison with database

sequences indicated that strain 1 was *C. vulgaris* (99 % identity, accession number KX618655), strain 2 was *S. obliquus* (99 % identity, accession number KX618656), and strain 3 was *Anabaena* sp. (99 % identity, accession number KX618657). Cyanobacteria are common in some extreme environments, and the ecological requirements depend on the genera or the strain (Markou and Georgakakis 2011). Thus, *Anabaena* sp. can grow in a wide range of wastewater conditions and can make a significant contribution to the treatment of agro-industrial wastes by significantly reducing the inorganic and organic pollutants (Olguín et al. 1997). Moreover, several studies reported that *Anabaena* sp. utilizes CO_2 at a higher rate than chlorophyceae (González López et al. 2009), which contributes to mitigating or removing CO_2 as an environmental pollutant (Park et al. 2010).

In this work, it was concluded that the biofilm formation structure inside the PBR was highly stable and no cells from the biofilm were detected into the tank collector. However, an important factor to consider is the production of toxins by cyanobacteria, known as cyanotoxins (Codd et al. 2005). An additional post-treatment such as activated carbon could be applied for removing soluble toxins and cyanobacteria cells. Further work is required to elucidate this aspect.

The community biofilm in PBR systems is diverse, and the role of several algal-bacterial interactions can be complex and difficult to analyze. Several studies had reported that each microalga and bacterial species in the polyculture remove a specific amount of pollutants, resulting in an increase in the overall removal of pollutants (Muñoz and Guieysse 2006; Ghasemi et al. 2011). However, it is impossible to find a single consortium that can degrade a mixture of different pollutants (Maza-Márquez et al. 2014). In general, microalgae and cyanobacteria play an important role in consortia since they supply molecular oxygen to heterotrophic partners and support the initial steps of degradation (Muñoz and Guieysse 2006), while the heterotrophic microorganisms supply CO_2 to their photosynthetic partners. According to Park et al. (2008), the coexistence of bacteria and microalgae would have positive synergistic effects on mixotrophic microalgal growth. Previous studies had reported that bacteria mixed with microalgae as in our experiment excrete active metabolites to help pollutant degradation (Muñoz et al. 2005). Moreover, it was recently established that bacteria increase the floc size of algae, thereby enabling settlement (Bester et al. 2012). However, the mechanism and the role of extracellular poly-saccharide substances (EPS) in the floc formation are not clear. In that context, more research is required to better understand the complex biology and interactions of microalgae and bacteria.

Conclusions

Biofilm PBRs represent innovative technology for OWW de- contamination. This study demonstrates a clear positive cor- relation between the consortium inoculum and the pollutant removal. The influence of the biofilm formation on the surface of the PBR is an essential factor contributing to the success of the depuration using this technology. Moreover, no significant differences in biodegradation were observed when the consor- tium was cultured at 5- or 3-day HRT, suggesting that a 3-day HRT biotreatment is sufficient for OWW. The obtained results highlight the potential of biofilm PBR systems in the environ- mental engineering panorama.

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Figures and Tables

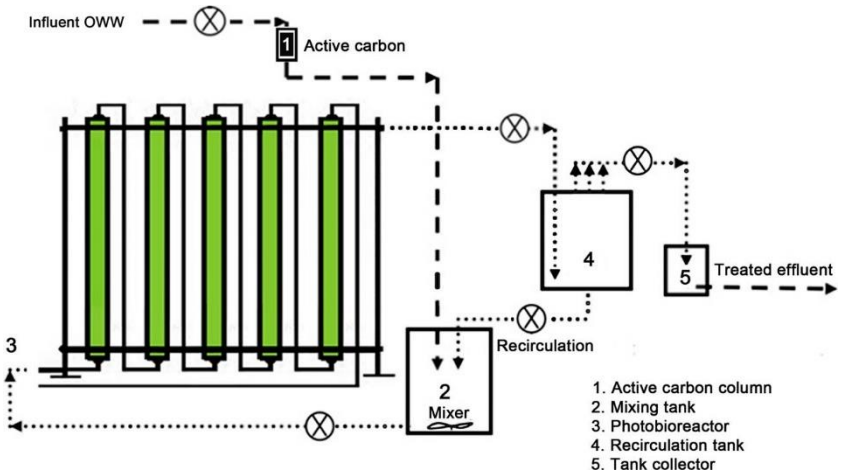

Fig. 1 Schematic diagram of the pilot-plant photobioreactor

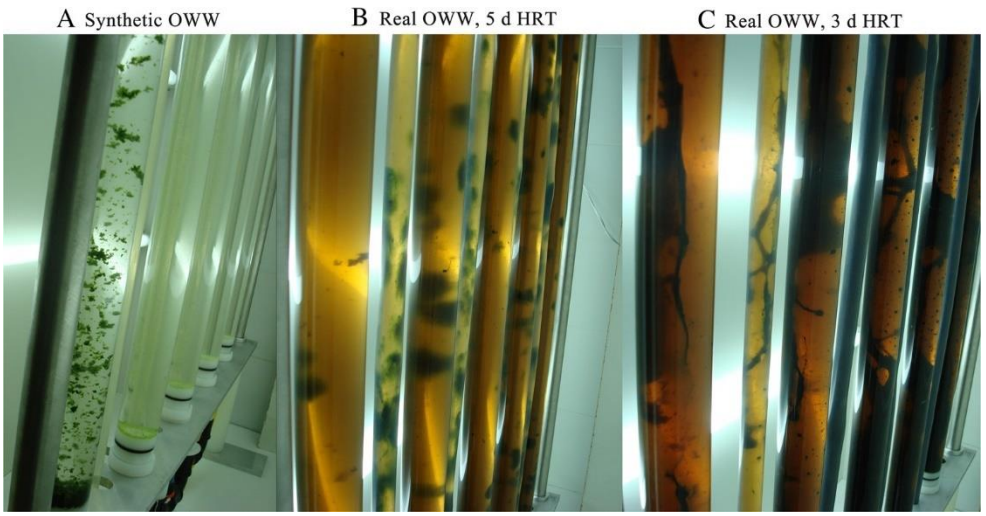

Fig. 2 Development of a microbial biofilm in a tubular PBR. a Microbial biofilm at start- up in synthetic OWW. b Microbial biofilm in real medium OWW at 5-day HRT conditions. c Microbial biofilm in real medium OWW at 3-day HRT conditions.

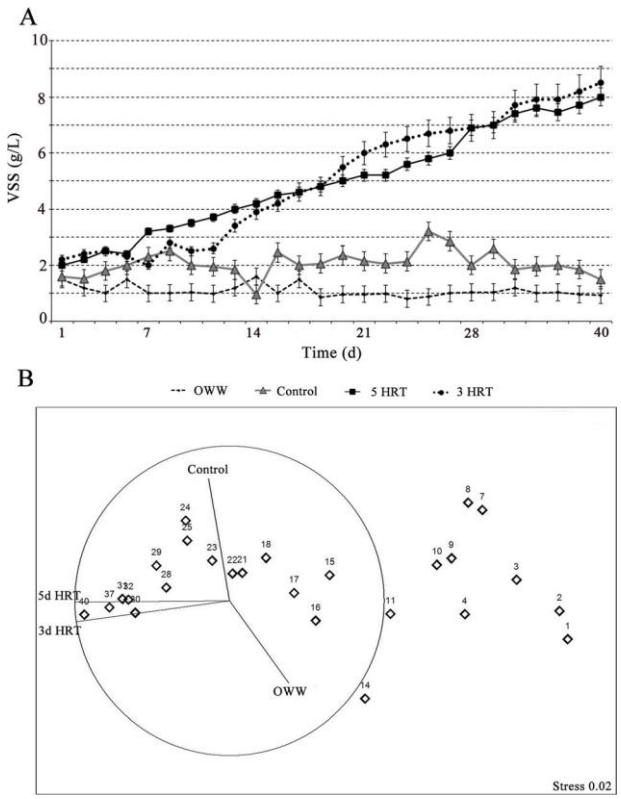

Fig. 3 a Growth curves of microbial consortium in the influent OWW (*dashed lines*), control PBR (*triangles*), 3-day HRT PBR (*circles*) and 5- day HRT PBR (*squares*). b Non-metric multidimensional scaling (MDS) plots illustrating the distribution of the consortium biofilm abundance samples retrieved from the photobioreactor (PBR) throughout the experimental period (5-day HRT, 3-day HRT, control and influent OWW), according to the relative similarity of their community structure. The vector represents the direction along the samples of each set of biotic data derived from the microbial biomass (5-day HRT, 3-day HRT, control and influent OWW), showing the role that each of them played in the distribution of the samples

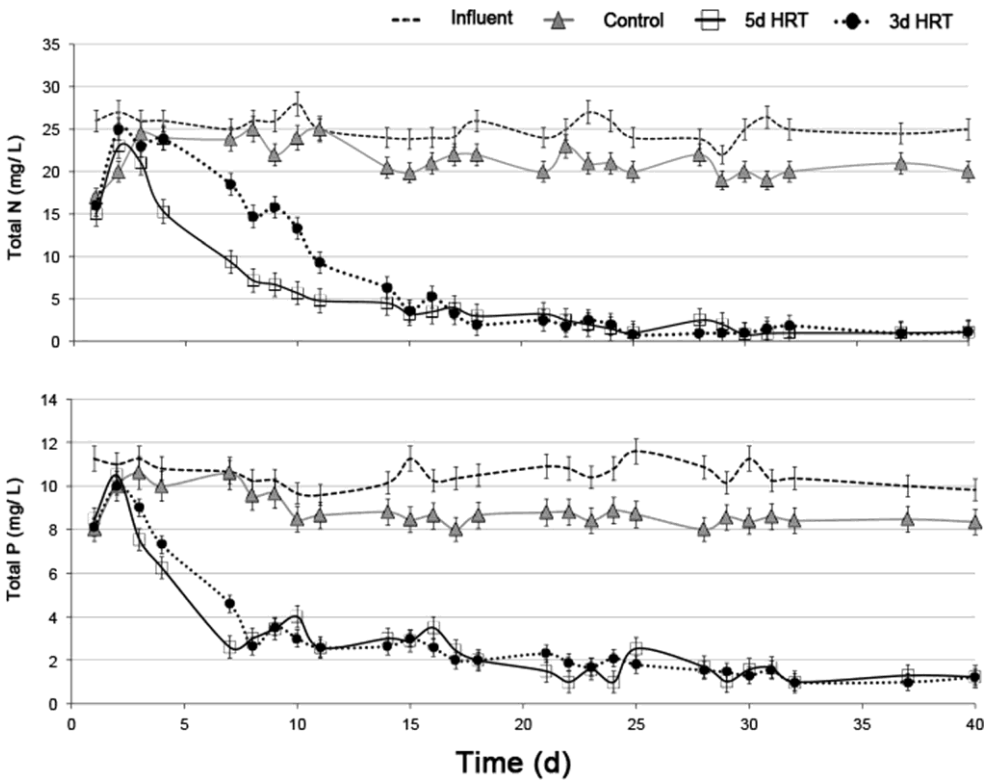

Fig. 4 Total nitrogen and total phosphorus concentrations in the influent OWW (*dashed lines*), control PBR (*triangles*), 3-day HRT PBR (*circles*) and 5-day HRT PBR (*squares*)

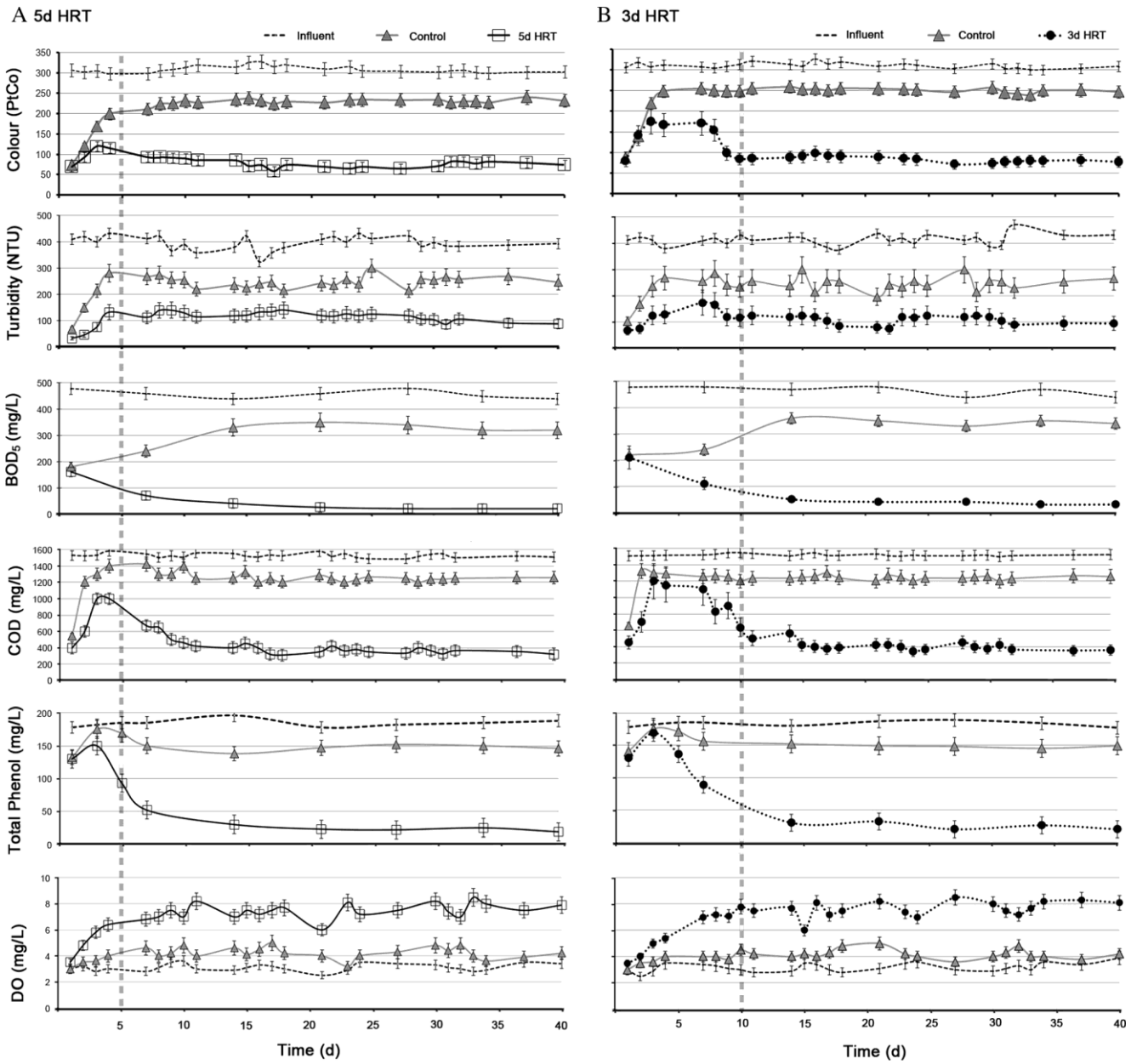

Fig. 5 Time course of colour, turbidity, BOD_5, COD, total phenols and DO concentration in the influent (*dashed lines*), control PBR (*triangles*), 3-day HRT PBR (*circles*) and 5-day HRT PBR (*squares*). *Vertical dashed lines* separate the stability phase

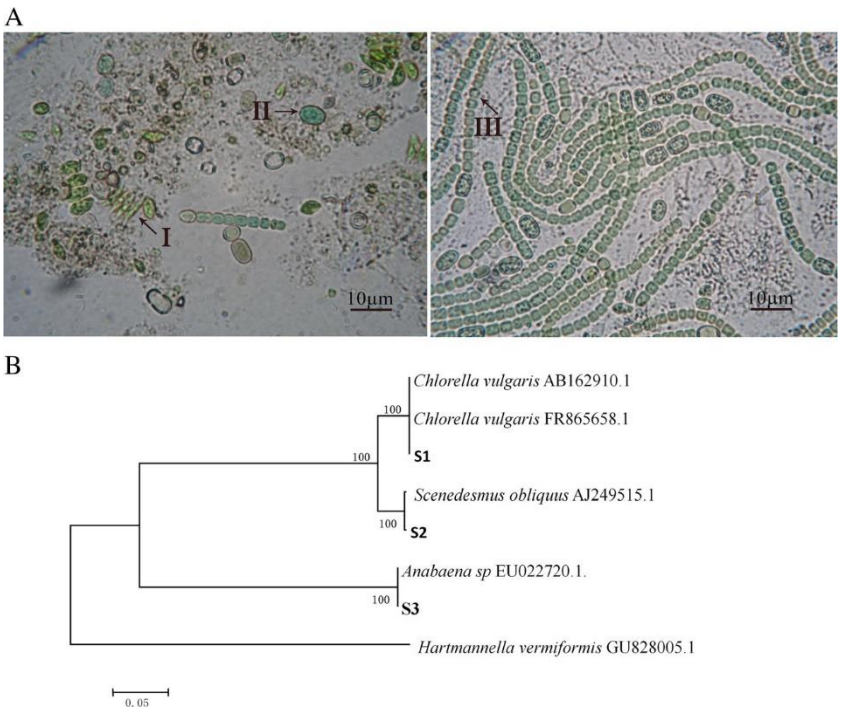

Fig. 6 Microscopic images of microalgae. a Complex morphological types found in PBR (×1000). b Phylogenetic neighbour-joining tree of the sequences from the microalgae isolated. Sequences retrieved from the EMBL database are indicated with their corresponding accession number. Bootstrap values below 50 are not shown. Outgroups included *Hartmannellids vermiformis* (GU828005.1)

Table 1 Operational conditions during the evaluation of the performance of the PBR

Stage	Time (days)	HRT (days)	Feed flow (L c y^{-1})	Recirculation flow (L min^{-1})	Inoculum
Control I	40	5	2.9	0.35	No
Control II	40	3	4.83	0.35	No
I	40	5	2.9	0.35	Yes
II	40	3	4.83	0.35	Yes

Table 2 Physicochemical characterization of olive washing water (OWW) and removal rates of the system after pretreatment with active carbon and photobioreactor treatment uninoculated or inoculated with the microbial consortium

Parameter	OWW	Influent	Effluent		
		Pretreatment	PBR experimental conditions		
			Uninoculated Control	Inoculated 5-day HR Γ	Inoculated 3-day HRT
Colour (PtCo)	487 ± 12	310.7 ± 22.6	225.5 ± 19.8	70.1 ± 15.6	81.8 ± 16.3
Turbidity (NTU)	654 ± 36	396.3 ± 33.5	237.4 ± 31.8	109.9 ± 24.7	111.8 ± 26.4
BOD_5 (mg/L)	558 ± 19	475.9 ± 12.4	316.9 ± 10.6	8.9 ± 5.1	12.3 ± 7.1
COD (mg/L)	1632.6 ± 33.1	1508.5 ± 44.6	1240.8 ± 51.6	305.3 ± 25.4	315.1 ± 26.7
Phenol (mg/L)	202.6 ± 6.1	185.8 ± 12.7	147.3 ± 10.7	19 ± 5.4	19.6 ± 4.2
Total P (mg/L)	15.6 ± 4.2	10.6 ± 3.6	9 ± 3	0.3 ± 0.1	0.5 ± 0.2
Total N (mg/L)	28.2 ± 4.1	25.2 ± 5.6	23 ± 6.8	0.5 ± 0.1	0.8 ± 0.2
DO (mg/L)	3.2 ± 0.3	3.6 ± 0.6	4.1 ± 0.5	7.1 ± 0.6	7.1 ± 0.4
pH	5.77 ± 0.4	5.74 ± 0.5	5.3 ± 0.4	7.5 ± 0.6	7.2 ± 0.2