J Ind Microbiol Biotechnol (2011) 38:1171–1178
DOI 10.1007/s10295-010-0892-5

ORIGINAL PAPER

An analysis of the bacterial community in a membrane bioreactor fed with photo-Fenton pre-treated toxic water

M. M. Ballesteros Martín · L. Garrido · J. L. Casas López · O. Sánchez · J. Mas · M. I. Maldonado · J. A. Sánchez Pérez

Received: 31 May 2010 / Accepted: 21 October 2010 / Published online: 9 November 2010

Abstract A photo-Fenton-membrane bioreactor (MBR) coupled system is an innovative tool for the treatment of wastewater containing high quantities of contaminants. In this paper, wastewater with 200 mg l^{-1} of dissolved organic carbon (DOC) of a selected mixture of five commercial pesticides: Vydate®, Metomur®, Couraze®, Ditimur-40®, and Scala® was treated by combining photo-Fenton and MBR. The effect of photo-treated pollutants on MBR operation was investigated by studying the population changes that occurred with time in the activated sludge of the biological system. Pre-treatment with photo-Fenton was carried out (only up to 34% of mineralization of DOC) and, after MBR treatment, 98% of biodegradation efficiency was obtained. During the biological treatment, little changes in the activated sludge population were detected by DGGE analysis, maintaining acceptable biodegradation efficiency, which points out the robustness of the MBR treatment versus changes in feed composition.

Keywords Advanced oxidation process (AOPs) · Membrane bioreactors (MBR) · Denaturing gradient gel electrophoresis (DGGE) · Genetic fingerprinting · Bacterial community structure

M. M. Ballesteros Martín · J. L. Casas López · J. A. Sánchez Pérez (✉)
Department of Chemical Engineering, University of Almería, 04120 Almería, Spain
e-mail: jsanchez@ual.es

L. Garrido · O. Sánchez · J. Mas
Departament de Genètica i Microbiología, Universitat Autónoma de Barcelona, 08193 Bellaterra, Spain

M. I. Maldonado
Plataforma Solar de Almería-CIEMAT, 04200 Tabernas, Almería, Spain

Introduction

Effluents of industrial activities often contain a considerable amount of toxic and non-biodegradable substances that are not able to be removed by conventional activated sludge treatments in wastewater treatment plants (WWTP). Numerous publications point to the suitability of combining advanced oxidation processes (AOPs) with biological oxidation to degrade these recalcitrant contaminants [18]. At the first stage, AOP is used to remove non-biodegradable substances to enhance wastewater biodegradability and the remaining organic matter is degraded using biological treatment in a subsequent step. This scheme diminishes the overall treatment costs since complete degradation using only AOP is expensive [19].

An interesting alternative aimed at economizing the AOP is the use of solar radiation as an energy source. In this sense, solar photo-Fenton has been demonstrated to be very effective for wastewater containing large quantities of contaminants such as pesticides [2, 3], even at an industrial scale [17]. In solar photo-Fenton reaction, HO· radicals (primary photoproduct) are generated by reaction of peroxide hydrogen and ferrous salt by using solar radiation. This non-selective oxidant species degrades the pesticide in the aqueous media, generating a wastewater containing more biodegradable intermediates that can be discharged into a biological system. However, photo-intermediate products lead to kinetic changes in the biological treatment, needing long acclimatization periods for an efficient carbon removal [3, 13, 26] and leaving, in some cases, a high organic load after biological treatment [21].

Membrane bioreactors (MBR) are a promising alternative to conventional activated sludge treatments for increasing biological efficiency and reducing biodegradation times [20]. In an MBR, wastewater is treated by

activated sludge in the bioreactor before being filtered, and the sedimentation stage of the conventional activated sludge process is replaced by a membrane filtration stage. As a result, microorganisms (some bacteria as coliforms, *Steptococcus faecalis*, *Salmonella* spp., bacteriophages and even viruses) are retained in the tank, obtaining excellent-quality treated water free of suspended matter and numerous micro-pollutants such as endocrine disrupting, pesticides, etc. [20]. This is especially relevant in developed countries with stringent regulations and a large demand of reused water, so MBR are being adopted in many areas as the next generation of biological water treatment technology.

However, although photo-Fenton and MBR technology is a worthwhile combination to treat toxic effluents such as pesticide-polluted wastewater, since biodegradation can be assessed in a short time, it is necessary to guarantee that bacterial community structure in the MBR is not affected by photo-treated intermediates present in the water. The effect of effluent from AOP treatments in activated sludge populations has not been previously studied, but there is evidence that some substances can influence, to some extent, the activated sludge stability of the WWTP. Thus, Kraigher et al. [11] observed that a mixture of pharmaceuticals at a concentration of 50 μg l^{-1} caused shifts in bacterial population and reduced bacterial diversity in bioreactors. Also, Lozada et al. [16] demonstrated that the addition of non-ionic surfactant (0.01%) could have a role in the shaping of the community structure.

Population changes in activated sludge can be monitored by denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments, one of the most frequently used methods for molecular fingerprinting. In this technique, electrophoretic separation of bands is based on the differences in the nucleotide sequence of the species present in the sample and it has been satisfactorily used for the assessment of changes in a large number of samples from complex communities along spatial and temporal gradients [4, 16, 27, 28]. Moreover, it allows the characterization of the diversity of the assemblage by subsequent sequencing.

In this work, this methodology was used to investigate the effect of photo-treated pollutants on MBR operation by studying the population changes that occurred with time in the activated sludge of the biological system.

Materials and methods

Photochemical reaction

Photo-Fenton experiments were carried out in a solar wastewater detoxification plant [3] with compound parabolic collectors able to treat up to 50 l of water (2.25 m^2 irradiated surface, 22 l irradiated volume). The absorber tube had an internal diameter of 50 mm. UV radiation was measured by a global UV radiometer (KIPP&ZONEN, model CUV 3, Delft, Netherlands), mounted on a platform tilted to 37° (local latitude), which provides data in terms of incident UV.

The plant was loaded with 50 l of distilled water containing a mixture of five pesticides [Vydate® (10% w/v oxamyl, $C_7H_{13}N_3O_3S$), Metomur®, (20% w/v methomyl, $C_5H_{10}N_2O_2S$), Couraze® (20%/v imidacloprid, $C_{16}H_{22}ClN_3O$), Ditimur-40® (40% w/v dimethoate, $C_5H_{12}NO_3PS_2$), and Scala® (40% w/v pyrimethanil, $C_{12}H_{13}N_3$)], with a dissolved organic carbon concentration of 200 mg l^{-1} corresponding to ~40 mg l^{-1} of DOC from each commercial pesticide. At the beginning of the process, the collectors were covered, the pH was adjusted to 2.7–2.9 with H_2SO_4, and ferrous iron salt ($FeSO_4 \cdot 7H_2O$) was added (Fe^{2+} concentration was 20 mg l^{-1}). The plant was well homogenized by turbulent recirculation after the addition of each reagent. Finally, the quantity of hydrogen peroxide necessary to achieve a mineralization percentage of 34% was added. Then, the collectors were uncovered and the photo-Fenton reaction began.

Biological treatment

Biological treatment with activated sludge was carried out in a 20-l working volume membrane bioreactor (MBR) provided with three A4 flat sheet membranes from Kubota Company (Tokyo, Japan) with a pore size of 0.4 μm, built in polyethylene with a working filtering area of 0.1 m^2, giving a total filtration area of 0.3 m^2. The MBR was equipped with two aeration systems, one to keep the membranes clean and another to supply additional oxygen. The total air flow was 19 l min^{-1}, equivalent to 0.95 vvm. Membrane aeration was introduced through a perforated tube located at the bottom of the reactor with a flow of 15 l min^{-1}, the rest of the flow was introduced through two porous spargers giving rise to smaller bubbles with higher specific surface, giving an oxygen transfer coefficient (K_La) measured with tap water, of 19.2 h^{-1}.

The bioreactor was fed with synthetic urban wastewater (SW) with biodegradable carbon sources and mineral salts, with a total DOC concentration of 500 mg l^{-1} until a steady state was achieved. Simulated wastewater contained 3.2 mg l^{-1} of beef extract, 6.4 mg l^{-1} of yeast extract, 16 mg l^{-1} of peptone, 0.8 ml^{-1} of glycerol (87% v/v) and mineral salts (16 mg l^{-1} NaCl, 0.5 g l^{-1} NH_4Cl, 0.5 g l^{-1} K_2HPO_4, 0.5 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$ and 10 ml l^{-1} of trace mineral solution [3]). The activated sludge was supplied by the municipal wastewater treatment plant of the city of

Almería, Spain (El Bobar, Almería, Spain). Volatile suspended solids (VSS) concentration throughout the experiment was 10 g l^{-1}.

When a steady stage was achieved, the influent of the MBR was constituted by the synthetic urban wastewater (SW) mixed with the wastewater containing phototreated pesticides (PTP) from the photo-Fenton reaction (132.5 mg l^{-1} of DOC) to achieve a final concentration of the effluent equal to 500 mg l^{-1} of DOC (SW + PTP). Pre-treated water with pesticide intermediates of the photo-Fenton reactions was previously neutralized to pH 7.0 ± 0.1 by adding 0.1 N NaOH and then mixed with the synthetic wastewater. In both cases, the influent flow rate was 2.4 l h^{-1} corresponding to a hydraulic residence time of 8.3 h and a transmembrane flux of 12 l h^{-1} m^{-2}. Temperature and pH remained constant at 17 ± 1°C and 6.5 ± 0.2, respectively.

Analytical methods

The five-pesticide solution was analyzed by HPLC (Shimadzu Lc10 with UV/Vis Shimadzu MX-10Av diode-array detector and autosampler, Kyoto, Japan) during photo-Fenton treatment to ensure that all active ingredients were completely eliminated in the pre-treated water before discharging into the biological system. A reverse-phase column (Sunfire™ Waters C_{18} 150-3 mm, 5 μm, Milford, USA) was employed. The mobile phase was acetonitrile (15%) and H_2O (85%) in a concentration gradient to 80% of acetonitrile and 20% of H_2O in 18 min (flow rate 0.5 ml min^{-1}). Detection was based on absorption at 210 nm for dimethoate and pyrimethanil, 234 nm for oxamyl and methomyl, and 270 nm for imidaclorid. Samples were diluted with acetonitrile (1:1) and filtered through 0.20-μm syringe filters (Millex®-GN, 25 mm, Millipore, Billerica, USA) before HPLC injection. The sample injection volume was 20 μl.

Mineralization by photo-Fenton and biological degradation with activated sludge were followed by DOC determination in a Shimadzu-V_{CPH} TOC (Kyoto, Japan) analyzer calibrated with standard solutions of potassium phthalate. Hydrogen peroxide concentration was determined with ammonium metavanadate in an acidic medium, which forms a red–orange peroxovanadium cation with maximum absorbance at 450 nm. Biomass was determined gravimetrically by measuring VSS.

DGGE analysis of the consortium

DNA extraction and PCR-DGGE

During the course of the experiment, 50-ml samples were sterilely collected from the MBR and kept frozen for further DNA extraction. DNA was extracted using the PowerSoil™ DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, USA).

Fragments of the 16S rRNA gene suitable for DGGE analysis were obtained by using the bacterial specific primer 341fGC with a 40-bp GCclamp, and the universal primer 907RM, which amplify a fragment of approximately 560 bp long [28]. PCR was carried out with a Biometra (Göttingen, Germany) thermal cycler using the following program: initial denaturation at 94°C for 5 min; ten touchdown cycles of denaturation (at 94°C for 1 min), annealing (at 65–55°C for 1 min, decreasing 1°C each cycle), and extension (at 72°C for 3 min); 20 standard cycles (annealing at 55°C, 1 min) and a final extension at 72°C for 5 min. PCR mixtures contained 1–10 ng of template DNA, each deoxynucleoside triphosphate at a concentration of 200 μM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 μM, 2.5 U *Taq* DNA polymerase (Invitrogen, Carlsbad, USA) and PCR buffer supplied by the manufacturer. The volume of reactions was 50 μl. PCR products were verified and quantified by agarose gel electrophoresis with a low DNA mass ladder standard (Invitrogen, Carlsbad, USA).

DGGE was run in a DCode system (Bio-Rad, Hercules, USA) as previously described by Muyzer et al. [24]. A 6% polyacrylamide gel with a gradient of DNA denaturant was cast by mixing solutions of 0 and 80% denaturant agent (100% denaturant agent is 7 M urea and 40% deionized formamide). For each sample, 800 ng of PCR product was loaded and the gel was run at 100 V for 18 h at 60°C in 1× TAE buffer (40 mM Tris [pH 7.4], 20 mM sodium acetate, 1 mM EDTA). A linear gradient of 30–70% of denaturant agent was used. The gel was stained with SybrGold (Molecular Probes, Invitrogen, Carlsbad, USA) for 45 min, rinsed with 11× TAE buffer, removed from the glass plate to a UV-transparent gel scoop, and visualized with UV in the Fluor-S Multi-Imager (Bio-Rad, Hercules, USA). Prominent bands were excised from the gels, resuspended in Milli-Q water overnight and reamplified. Digitized DGGE images were analyzed with the Quantity One software (Bio-Rad, Hercules, USA). The software performs a density profile through each DGGE lane, detects the bands, and calculates the relative contribution of each band to the total band signal in the lane, after applying a rolling disk as background subtraction.

DNA sequencing

DNA purification and sequencing reactions from DGGE bands were performed by Macrogen Inc. (Seoul, Korea) with primers 907RM for bacterial DGGE bands. Sequences were subjected to a BLAST search to identify the phylogenetic affiliation, and to the Bellerophon program to

determine potential chimeric artifacts. Twenty 16S rRNA gene sequences obtained from the bioreactor were sent to the EMBL database (http://www.ebi.ac.uk/embl) and received the following accession numbers: FN669635 to FN669654.

Quantitative analyses

Digitized DGGE images were analyzed with Quantity One software (Bio-Rad, Hercules, USA). Bands occupying the same position in the different lanes of the gels were identified. A matrix was constructed for all lanes, taking into account the presence or absence of the individual bands and the relative contribution of each band (by percentage) to the total intensity of the lane. This matrix was used to obtain a dendrogram comparing samples using the Ward's method with the software SPSS.

Results and discussion

The five-pesticide mixture with an initial DOC concentration of 200 mg l^{-1} was treated with photo-Fenton by adding the appropriate dose of hydrogen peroxide ($\sim$300 mg l^{-1}) up to total reactive consumption to avoid damage to the biomass in the biological treatment. DOC concentration decreased to 132.5 mg l^{-1}, achieving a mineralization degree of 34% and total pesticide removal. It has previously been reported [3] that this value is the minimum percentage of mineralization to combine photo-Fenton pre-treatment with biological oxidation.

MBR was fed with a synthetic wastewater (SW) with an initial dissolved organic carbon of 500 mg l^{-1} until a steady state was achieved. During this phase, VSS was 10 g l^{-1} and DOC concentration in the MBR effluent was 5 mg l^{-1}, which indicates that the carbon removal percentage was higher than 99%. Once steady state was attained, the bioreactor was fed with wastewater containing 500 mg l^{-1} of DOC (26.5% from pesticide pre-treated by photo-Fenton and 73.5% from synthetic wastewater) according to previous work [3]. Mixing the photo-Fenton effluent (from the treatment of the same five-pesticide mixture) with a biodegradable carbon source increased the biological oxidation efficiency (over 90%) in a stirred tank reactor [3]. Other authors have combined pre-treated wastewater by AOPs with a biodegradable carbon source obtaining high biodegradation efficiencies. Thus, Oller et al. [26] achieved similar efficiencies $\sim$92% of the initial COD with immobilized activated sludge for the degradation of an effluent pre-treated by photo-Fenton containing a mixture of pesticides methomyl, dimethoate, oxamyl, cymoxanil, and pyrimethanil (DOC = 113 mg l^{-1}) and glucose (DOC = 142 mg l^{-1}) as additional carbon source requiring 5 days of treatment. Lin and Chang [15] also combined electro-Fenton pretreated wastewater with a chemical oxygen demand (COD) of 295 mg l^{-1} with urban wastewater and 90% of the initial COD was eliminated.

Figure 1 represents the response of the MBR to this change in the feeding composition, starting from synthetic wastewater (SW) and then adding photo-treated pesticides (SW + PTP). Photo-Fenton intermediates caused a change in the biodegradation capacity of the sludge, giving rise to a sharp increase in DOC concentration of the MBR effluent up to 30 mg l^{-1}, followed by an acclimatization period of 10 days. The final steady DOC concentration in the outgoing stream was $\sim$10 mg l^{-1} (98% of biodegradation efficiency).

High efficiencies in pesticide degradation by combining photo-Fenton and other biological systems have been reported in the literature [3, 4, 6–8, 17]. However, in all previous studies, organic load in the final effluent was higher than in the MBR treatment. For example, remaining dissolved organic carbon values around 20–30 mg l^{-1} were found in the treatment of mixtures of pesticides (with an initial DOC of around 200 mg l^{-1}) in a sequencing batch reactor [2, 3]. SBR was also applied to treat wastewater containing the pesticides diuron and linuron pretreated by photo-Fenton up to 32% of mineralization with a global efficiency below 70% [8]. Furthermore, an immobilized biomass reactor (IBR) was used by other researchers [26] in the biodegradation of wastewater-containing pesticides at the same percentage of mineralization by photo-Fenton, although lower global efficiencies than with MBR were obtained. The same configuration of bioreactor achieved a degradation of 80% with a previous treatment by photo-Fenton of two pesticides [21]. This

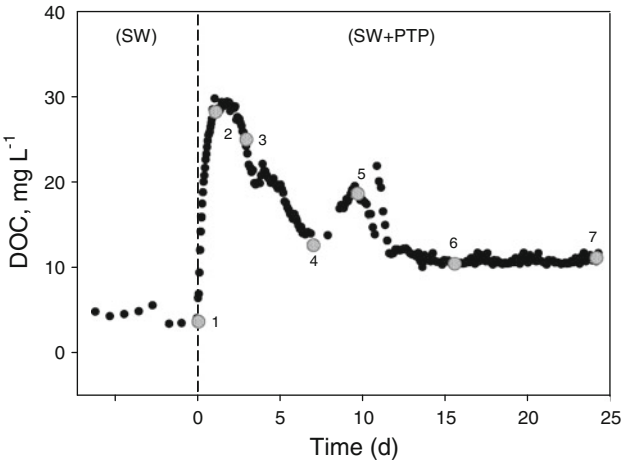

Fig. 1 Operation of MBR fed with synthetic wastewater (SW) and the mixture of photo-treated pesticides (SW + PTP). Seven replicated samples were taken (*gray circles*) during the acclimatization period

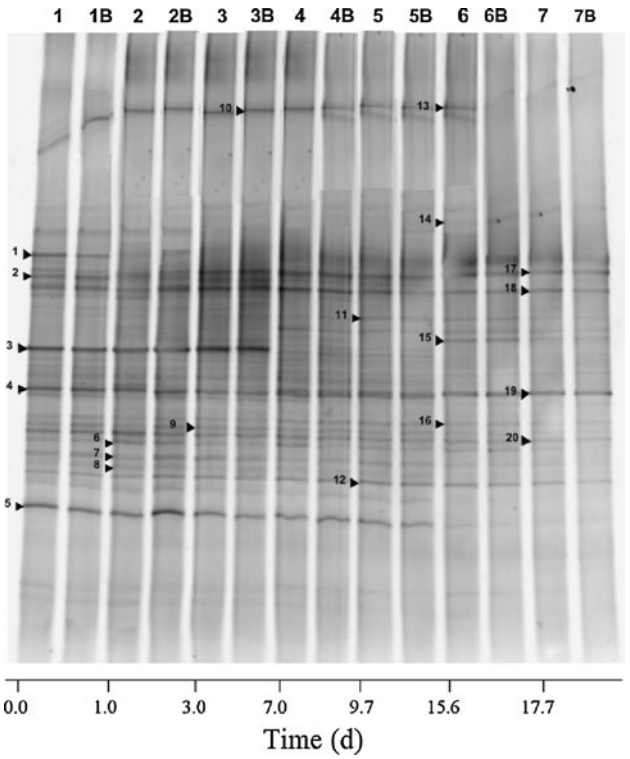

Fig. 2 DGGE fingerprints of the seven activated sludge samples (*B*: replicated sample) during MBR treatment. 1–20 numbers represent band positions excised in order to be sequenced

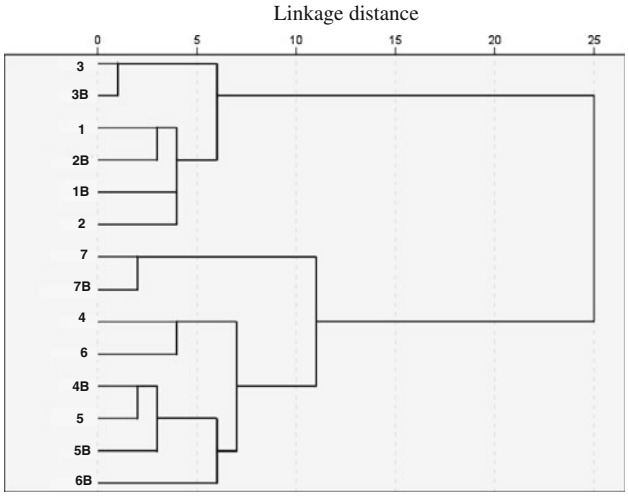

Fig. 3 Dendrogram generated from the DGGE profiles of the seven samples taken during the MBR operation, determined by the Ward's method using average linkage (between groups)

percentage was also obtained when a mixture of five pesticides was degraded coupling photo-Fenton and biological oxidation with a packed-bed bioreactor [13].

The higher C-removal efficiency found in the MBR treatment is related to sludge acclimatization caused by the exposure of the microbial community to the less biodegradable compounds generated during the photo-Fenton step. LaPara et al. [12] demonstrated that activated sludge in an MBR fed with simple synthetic wastewater adapted their enzyme activities primarily for the nutrients provided. Nonetheless, there is a lack of knowledge about the effect of PTP on sludge composition or microbial metabolism. In this sense, DOC concentration values are not appropriate to explain if adaptation was due to acclimatization of microorganisms to the new influent or to changes in the microbial population [9]. Subsequently, replicated samples of sludge were taken during the MBR operation and analyzed by DGGE to study changes in the bacterial community structure.

In general, DGGE analysis (Fig. 2) showed that there was little change in band numbers over the biological treatment time, although variations in band intensity could be observed (samples 1 and 1B were taken as reference). Most of the bands (2–20) that appear in the first sample (before adding photo-treated pesticides) were also present in the following lanes. Therefore, it can be supposed a priori that the influent (SW + PTP) did not significantly affect the composition of microorganisms in the activated sludge. Bands were preserved but with variations in intensity in bands 12, 17, 18, and 19, where intensity increased, meanwhile it decreased in bands 2, 3, 5, 7–10, 13, 15, and 16. However, these variations in intensity do not contradict the fact that, in general, the community of the sludge was stable throughout the experiment since limited changes in band number were observed. The variations in band intensity indicate a priori changes in the relative proportion of species but not in composition (there is a positive correlation between band intensity and species concentration, considering the same amount of DNA in all lanes). Therefore, a cluster analysis (Fig. 3) was carried out in order to compare the similarity between samples taking into account the presence or absence of the individual bands as well as their relative intensity.

As it can be observed in Fig. 3, two principal linkage groups were obtained; one for samples 1–3 and another for the remaining samples. Therefore, the dendrogram showed a great similarity between samples 4, 5, and 6, which exclude the fact that the decrease in the efficiency of biodegradation shown between days 7–11 (Fig. 1) is attributable to a change in the composition of the biomass. Probably, the increment in DOC at 9.7 d (sample 5, Fig. 1) was the result of an accumulation of non-rapidly biodegradable organic carbon, which was finally degraded.

A total of 20 band positions were excised and sequenced in order to determine their phylogenetic affiliation. The closest matches (and percentages of similarity) for the sequences retrieved were determined by a BLAST search (Table 1). The number of bases used to calculate each similarity value is also shown in Table 1, as an indication

of the quality of the sequence. Similarities ranged between 83.1 and 99.8%. It is remarkable that the majority of sequences retrieved from the activated sludge sample corresponded to uncultured microorganisms of different phyla, a problem that usually arises when the diversity of environmental samples is analyzed. In particular, it has been estimated that the fraction of isolated microorganisms in activated sludge ranges between 1 and 15% [4].

We could find different members from the Beta- and Gamma- subclasses of Proteobacteria, as well as from Bacteroidetes, Actinobacteria, and Firmicutes. These taxonomic groups are typically found constituting the biomass of activated sludge in MBR [6, 7] and they are responsible of the degradation of many pollutants. In general, the Bacteroidetes are involved in biopolymer degradation [6], and filamentous members of this group have been commonly observed in activated sludge samples originating from both municipal and industrial WWTPs, where they can occasionally cause bulking. Several of the retrieved bands corresponded to Bacteroidetes, and specifically two of them (bands 10 and 13) were close at the genus level (between 95 and 97% similarity) to Sphingobacteriales bacteria; members of this group are recognized by their capability to degrade a wide variety of refractory environmental pollutants; sphingomonads have been identified in situ by FISH in activated sludge samples and have been found to be rather abundant, accounting for about 5–10% of the total cells [25].

On the other hand, Proteobacteria have been found to cause biofouling in MBR treatments or bulking in industrial WWTP [14, 32], and in many works where the study of activated sludge diversity has been addressed by molecular techniques, either in WWTP or in laboratory-scale reactors, the dominance of the Beta subclass of the class Proteobacteria has been described [31]. In the present study, two bands (bands 9 and 19) corresponded to

Table 1 Phylogenetic aphiliation of sequence obtained from DGGE bands, with closest uncultured and cultured matches

Band	Closest match	% Similarity (no bases)[a]	Taxonomic group	Accession no (GenBank)	Cultured closest match (% similarity)
SP_MBR1	*Clostridium tyrobutyricum*	98.0 (492)	Firmicutes	M59113	
SP_MBR2	*Clostridium* sp. T2	88.7 (400)	Firmicutes	FJ435965	
SP_MBR3	Uncultured bacterium clone T4 1 30	98.6 (504)	Firmicutes	EU828369	*Clostridium* sp. (97.1)
SP_MBR4	*Stenotrophomonas rhizophila*	90.6 (470)	γ-proteobacteria	FJ982872	
SP_MBR5	Uncultured bacterium clone W2-12	98.2 (496)	Actinobacteria	FJ545597	*Micropruina glycogenica* (96.4)
SP_MBR6	*Arthrobacter* sp.	90.7 (456)	Actinobacteria	FN377737	
SP_MBR7	Uncultured actinobacterium clone 2-73	91.4 (465)	Actinobacteria	GQ144770	*Microbacterium* sp. (91.2)
SP_MBR8	*Arthrobacter* sp.	86.9 (443)	Actinobacteria	FJ894229	
SP_MBR9	*Methylibium petroleiphilum*	89.2 (451)	β-proteobacteria	FJ269076	
SP_MBR10	Uncultured bacterium clone HH5 e5	98.4 (508)	Bacteroidetes	AY218692	Sphingobacteriaceae bacterium BR5-29 (95.7)
SP_MBR11	Uncultured bacteroidetes bacterium clone IS017B86	86.0 (424)	Bacteroidetes	AY806781	*Prevotella salivae* (85.6)
SP_MBR12	*Mycobacterium* sp.	99.4 (512)	Actinobacteria	AB251601	
SP_MBR13	Uncultured bacterium isolate DGGE gel band LK1_21	98.1 (516)	Bacteroidetes	GQ336901	Sphingobacteriales bacterium 1-HG42 (96.7)
SP_MBR14	Uncultured bacterium clone A194	91.3 (483)	Bacteroidetes	FJ660602	*Chitinophaga* sp. (88.6)
SP_MBR15	Clostridiaceae bacterium	87.9 (444)	Firmicutes	AB298726	
SP_MBR16	Uncultured bacterium clone UTFS-R12-214-63	83.1 (398)	γ-proteobacteria	GQ871665	*Frateuria aurantia* (82.5)
SP_MBR17	Uncultured *Fluviicola* sp. clone bf1-11	98.7 (517)	Bacteroidetes	CV927760	*Pedobacter* sp. (88.0)
SP_MBR18	Uncultured bacterium clone nbw425g04c1	90.3 (474)	Bacteroidetes	GQ093843	Bacteroidetes bacterium ONB11 (89.0)
SP_MBR19	*Comamonas testosteroni*	99.8 (540)	β-proteobacteria	CP001220	
SP_MBR20	Uncultured bacterium clone A88	99.8 (542)	γ-proteobacteria	FJ660577	*Dokdonella* sp. (98.2)

[a] The *numbers in parentheses* are the numbers of bases used to calculate the levels of sequence similarity

Beta-proteobacteria and were close to *Methylibium petroleiphilum* and *Comamonas testosteroni* respectively. *Methylibium petroleiphilum* is a microorganism involved in biodegradation of xenobiotic compounds such as methyl-tert-butyl ether [5], while *Comamonas testosterini*, originally identified in membrane-coupled bioreactors fed with simple synthetic wastewater [12], has been found to be related to the biodegradation of *p*-toluene sulphonic acid and acrylonitrile from wastewaters [1, 11].

Also, the high G + C Gram-positive bacteria (Actinobacteria) have been reported when analyzing the bacterial diversity of activated sludge [29]. These microorganisms are believed to be involved in several important processes because they include bacteria implicated in the EPBR processes (enhanced biological phosphorous removal), foam formation, and other solid–liquid separation problems, and have been found in a range of plant designs. Thus, they can markedly influence plant performance. One of the retrieved bands (band 12), which was present during the whole experiment, was affiliated to *Mycobacterium* sp. at the species level (99.4% similarity), a microorganism studied in WWTP showing enhanced phosphorous removal [10]. Another sequence (band 5) had a cultured closest match similar to *Micropruina glycogenica*, a glycogen-accumulating organism (GAO), originally isolated from an activated sludge reactor also showing enhanced biological phosphorus removal activity.

As previously discussed, intensity in bands 12, 17, 18, and 19 increased during the experiment. These bands correspond to microorganisms that degrade recalcitrant compounds. Thus, this fact could indicate an enhancement of some species along the reactor operation contributing to the biodegradation of the xenobiotics present in the influent.

Microbial community diversity in an MBR treatment can be modified by changes in operational parameters in the bioreactor or feed composition. Pholchan et al. [27] showed that reactor configuration (sequencing batch reactor, SBR or completely stirred tank reactor, CSTR) had a determinant effect on microbial diversity while the effects of organic loading rates and feed composition were less marked. Nonetheless, Stamper et al. [30] observed large modifications in population structure attributable to operational changes in MBR operation, particularly variations in feed strength. Miura et al. [22] suggested that influent wastewater composition had a high impact on bacterial community structures. Other authors [23] used polymerase chain reaction-temperature gradient gel electrophoresis (PCR-TGGE) with the objective of monitoring a pilot scale MBR used for the aerobic treatment of domestic wastewater. They determined that significant differences were found in bacterial communities depending on operation parameters and environmental factors. Therefore, all investigation in this area verifies that changes in operational parameters or feed affect the microbial structure of the sludge. Moreover, some authors have confirmed that low concentrations of non-biodegradable or toxic substances affect the bacterial assemblage [11, 16].

In the present work, all operational parameters and environmental variables (e.g., temperature or pH) remained constant throughout the experiment and the only modification included was the composition of the influent. Despite this change, the population of the activated sludge was not strongly altered, maintaining acceptable biodegradation efficiency, and the enzymes developed by microorganisms in the adaptation phase were enough to give a high-quality effluent. These results support the initial hypothesis that the strategy of mixing AOP effluent with a biodegradable carbon source provides high efficiency in removing pollutants by MBR. Furthermore, if the activated sludge population is not strongly affected by the influent, it is possible to decrease the mineralization percentage in the photo-Fenton stage, reducing overall costs. In any case, further research is needed to find the minimum photo-Fenton treatment mineralization degree required to avoid changes in bacterial community.

In general, selection of the biological treatment technology determines overall efficiency of the combined system, although the effect of AOP intermediates on biodegradation kinetics must be taken into account for proper operation of the bioreactor.

Conclusions

This paper shows the viability of the AOP-MBR coupled system to treat wastewater containing high concentrations of pesticides. When pre-treatment with photo-Fenton was carried out (only up to 34% of mineralization of pesticides), DGGE analysis showed that the discharged effluent in the MBR did not cause significant changes in bacterial community structure. This fact shows the robustness of the MBR treatment versus changes in feed composition, which allows a potential reduction in the photochemical mineralization degree of toxic compounds.

Acknowledgments This research was supported by the Consejería de Innovación, Ciencia y Empresa de la Junta de Andalucía (Andalusian Regional Government) (FOTOMEM project, P08-RNM-03772) and PAIDI (BIO-263). We appreciate the collaboration of the El Toyo (Aqualia) WWTP in Almería for the activated sludge supply.

References

1. Artem K, Paul P (1996) Biodegradation of *p*-toluene sulphonic acid by *Comamonas testosteroni* in an aerobic countercurrent

structured packing biofilm reactor. Water Sci Technol 34:257–266
2. Ballesteros Martín MM, Sánchez Pérez JA, Casas López JL, Oller I, Malato S (2009) Degradation of a four-pesticide mixture by combined photo-Fenton and biological oxidation. Water Res 43:653–660
3. Ballesteros Martín MM, Sánchez Pérez JA, García Sánchez JL, Casas López JL, Malato S (2009) Effect of pesticide concentration on the degradation process by combined solar photo-Fenton and biological treatment. Water Res 43:3838–3848
4. Boon N, De Windt W, Verstraete W, Top EM (2002) Evaluation of nested PCR–DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. FEMS Microbiol Ecol 39:101–112
5. Chen D, Chen J, Zhong W, Cheng Z (2008) Degradation of methyl tert-butyl ether by gel immobilized Methylibium petroleiphilum PM1. Bioresour Technol 99:4702–4708
6. Chen R, LaPara TM (2006) Aerobic biological treatment of low-strength synthetic wastewater in membrane-coupled bioreactors: the structure and function of bacterial enrichment cultures as the net growth rate approaches zero. Microbial Ecol 51:99–108
7. Duan L, Moreno-Andrade I, Huang CL, Xia S, Hermanowicz SW (2009) Effects of short solids retention time on microbial community in a membrane bioreactor. Bioresour Technol 100:3489–3496
8. Farré MJ, Maldonado MI, Gernjak W, Oller I, Malato S, Domènech X, Peral J (2008) Coupled solar photo-Fenton and biological treatment for the degradation of diuron and linuron herbicides at pilot scale. Chemosphere 72:622–629
9. González S, Müller J, Petrovic M, Barceló D, Knepper TP (2006) Biodegradation studies of selected priority acidic pesticides and diclofenac in different bioreactors. Environ Pollut 144:926–932
10. Kampfer P, Erhart R, Beimfohr C, Bohringer J, Wagner M, Amann R (1996) Characterization of bacterial communities from activated sludge: culture-dependent numerical identification versus in situ identification using group- and genus-specific rRNA-targeted oligonucleotide probes. Microbiol Ecol 32:101–121
11. Kraigher B, Kosjek T, Heath E, Kompare B, Mandic-Mulec I (2008) Influence of pharmaceutical residues on the structure of activated sludge bacterial communities in wastewater treatment bioreactors. Water Res 42:4578–4588
12. LaPara TM, Klatt CG, Chen R (2006) Adaptations in bacterial catabolic enzyme activity and community structure in membrane-coupled bioreactors fed simple synthetic wastewater. J Biotechnol 121:368–380
13. Lapertot M, Ebrahimi S, Dazio S, Rubinelli A, Pulgarin C (2007) Photo-Fenton and biological integrated process for degradation of a mixture of pesticides. J Photochem Photobiol A: Chem 186:34–40
14. Levantesi C, Beimfohr C, Geurkink B, Rossetti S, Thelen K, Krooneman J, Snaidr J, van der Waarde J, Tandoi V (2004) Filamentous alphaproteobacteria associated with bulking in industrial wastewater treatment plants. Syst Appl Microbiol 27:16–727
15. Lin SH, Chang CC (2000) Treatment of landfill leachate by combined electro-Fenton oxidation and sequencing batch reactor method. Water Res 34:4243–4249
16. Lozada M, Basile L, Erijman L (2007) Impact of non-ionic surfactant on the long-term development of lab-scale-activated sludge bacterial communities. Res Microbiol 158:712–717
17. Malato S, Blanco J, Maldonado MI, Oller I, Gernjak W, Pérez-Estrada L (2007) Coupling solar photo-Fenton and biotreatment at industrial scale: main results of a demonstration plant. J Haz Mat 146:440–446
18. Malato S, Fernández-Ibáñez P, Maldonado MI, Blanco J, Gernjak W (2009) Decontamination and disinfection of water by solar photocatalysis: recent overview and trends. Catal Today 147:1–59
19. Mantzavinos D, Psillakis E (2004) Enhancement of biodegradability of industrial wastewaters by chemical oxidation pretreatment. J Chem Technol Biotechnol 79:431–454
20. Melin T, Jefferson B, Bixio D, Thoeye C, De Wilde W, De Koning J, van der Graaf J, Wintgens T (2006) Membrane bioreactor technology for wastewater treatment and reuse. Desalination 187:271–282
21. Mendoza-Marín C, Osorio P, Benítez N (2010) Decontamination of industrial wastewater from sugarcane crops by combining solar photo-Fenton and biological treatments. J Haz Mat 177:851–855
22. Miura Y, Hiraiwa MN, Ito T, Itonaga T, Watanabe Y, Okabe S (2007) Bacterial community structures in MBRs treating municipal wastewater: relationship between community stability and reactor performance. Water Res 41:627–637
23. Molina-Muñoz M, Poyatos JM, Sánchez-Peinado M, Hontoria E, González-López J, Rodelas B (2009) Microbial community structure and dynamics in a pilot-scale submerged membrane bioreactor aerobically treating domestic wastewater under real operation conditions. Sci Total Environ 407:3994–4003
24. Muyzer G, Brinkhoff T, Nübel U, Santegoeds C, Schäfer H, Wawer C (1998) Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. In: Akkermans ADL, van Elsas JD, Brujin FJ (eds) Molecular microbial ecology manual, vol 344. Kluwer Academic Publishers, Dordrecht, pp 1–27
25. Neef A, Witzenberger R, Kämpfer P (1999) Detection of sphingomonads and in situ identification in activated sludge using 16S rRNA-targeted oligonucleotide probes. J Ind Microbiol Biotechnol 23:261–267
26. Oller I, Malato S, Sánchez-Pérez JA, Maldonado MI, Gassó R (2007) Detoxification of wastewater containing five common pesticides by solar AOPs–biological coupled system. Catal Today 129:69–78
27. Pholchan MK, Baptista JC, Davenport RJ, Curtis TP (2010) Systematic study of the effect of operating variables on reactor performance and microbial diversity in laboratory-scale activated sludge. Water Res 44:1341–1352
28. Sánchez O, Gasol JM, Massana R, Mas J, Pedrós-Alió C (2007) Comparison of different denaturing gradient gel electrophoresis primer sets for the study of marine bacterioplankton communities. Appl Environ Microbiol 73:5962–5967
29. Seviour RJ, Kragelund C, Kong Y, Eales K, Nielsen JL, Nielsen PH (2008) Ecophysiology of the *Actinobacteria* in activated sludge systems. Anton Leeuw 94:21–33
30. Stamper DM, Walch M, Jacobs RN (2003) Bacterial population changes in a membrane bioreactor for graywater treatment monitored by denaturing gradient gel glectrophoretic analysis of 16S rRNA gene fragments. Appl Environ Microbiol 69:852–860
31. Wagner M, Loy A, Nogueira R, Purkhold U, Lee N, Daims H (2002) Microbial community composition and function in wastewater treatment plants. Anton Leeuw 81:665–680
32. Xia S, Guo J, Wang R (2008) Performance of a pilot-scale submerged membrane bioreactor (MBR) in treating bathing wastewater. Bioresour Technol 99:6834–6843