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Phenol removal from hypersaline wastewaters in a Membrane Biological Reactor (MBR): Operation and microbiological characterisation

J. Dosta [a], J.M. Nieto [b], J. Vila [b], M. Grifoll [b], J. Mata-Álvarez [a,*]

[a] Department of Chemical Engineering, Faculty of Chemistry, University of Barcelona, Martí i Franquès, 1, 6th Floor, 08028 Barcelona, Spain
[b] Department of Microbiology, Faculty of Biology, University of Barcelona, Av. Diagonal, 646, 08028 Barcelona, Spain

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ABSTRACT

In this study, two Membrane Biological Reactors (MBR) with submerged flat membranes, one at lab-scale conditions and the other at pilot-plant conditions, were operated at environmental temperature to treat an industrial wastewater characterised by low phenol concentrations (8–16 mg L^{-1}) and high salinity (~150–160 mS cm^{-1}). During the operation of both reactors, the phenol loading rate was progressively increased and less than 1 mg phenol L^{-1} was detected even at very low HRTs (0.5–0.7 days). Membrane fouling was minimized by the cross flow aeration rate inside the MBRs and by intermittent permeation. Microbial community analysis of both reactors revealed that members of the genera *Halomonas* and *Marinobacter* (*gammaproteobacteria*) were major components. Growth-linked phenol degradation by pure cultures of *Marinobacter* isolates demonstrated that this bacterium played a major role in the removal of phenol from the bioreactors.

1. Introduction

Although phenolic compounds are hardly present in municipal wastewaters, they are frequently found in some industrial wastewaters, such as pulp and mill, textile and petrochemical industries and refineries. Phenols are chemical compounds consisting of a hydroxyl group bonded directly to an aromatic hydrocarbon group. Several methods can be used for phenol degradation, such as advanced oxidation processes (AOPs) and/or biological processes. For economic reasons, biological treatments are preferred when the studied wastewater does not contain toxic compounds that inhibit biomass activity.

Some industrial wastewaters containing phenol can be efficiently treated by means of a biological Sequencing Batch Reactor (SBR) due to its flexibility of operation (resistance to changes in phenol concentration), compactness and easy control (Brenner et al., 1992; Buitrón and Ortiz, 1997; Macé and Mata-Álvarez, 2002). Since microorganisms responsible of phenol degradation have a relatively low growth rate, biomass is usually attached in porous materials to improve biomass retention in the digesters (Puhakka and Järvinen, 1992; Buitrón and Ortiz, 1997; González et al., 2001; Sá and Boaventura, 2001; Sgountzos et al., 2006). However, the application of Membrane Biological Reactors (MBR) could assure the retention of phenol degrading microorganisms without risk of an eventual wash-out of biomass (Cornel and Krause, 2006; Lesjean and Huisjes, 2007).

In fact, the MBR technology has lead to a very good biomass retention capacity in reactors working with microbial consortiums characterised by slow growth rates (Trigo et al., 2006; Lesjean and Huisjes, 2007). Very few experiences are reported for phenol biodegradation in MBRs: Barrios-Martinez et al. (2006) studied the treatment of synthetic wastewater representative of petrochemical effluents from a refinery (1.01 mg phenol L^{-1}). These authors operated a MBR with an external membrane module configuration obtaining phenol removal efficiencies around 100%. On the other hand, Marrot et al. (2006) examined phenol biodegradation in a MBR (submerged hollow fiber membrane module) for the treatment of synthetic wastewater with high phenol concentration (0.5–3.0 g phenol L^{-1}) obtaining high removal rates. Ahn et al. (2008) used an MBR (submerged hollow fiber membrane module) to treat a phenol loaded wastewaters, reporting good removal efficiencies when phenol was fed at low (0.1 g L^{-1}) and high (1.0 g L^{-1}) concentration levels. Moreno-Andrade et al. (2008) studied the treatment of a synthetic wastewater with 4-chlorophenol (600 mg L^{-1}) in a sequencing batch MBR (submerged tubular membrane module) obtaining removal efficiencies higher than 99%. Recently, Carucci et al. (2010) reported high removal efficiencies (99–100%) of 50 mg L^{-1} of 4-chlorophenol in a lab-scale MBR (3.1 L of capacity, hollow fiber membrane unit) operated as a sequencing batch reactor at Hydraulic Retention Times (HRT) between 12 and 24 h, depending on cycle length.

* Corresponding author.
E-mail addresses: mgrifoll@ub.edu (M. Grifoll), jmata@ub.edu (J. Mata-Álvarez).

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Phenol contaminated industrial wastewater holds microorganisms belonging to different phylogenetic taxons (Van Schie and Young, 2000; Watanabe et al., 1998). Particularly, gammaproteobacteria have been found to constitute a major part of the phenol-degrading bacterial population in some industrial phenol waste bioremediation systems (Whiteley and Bailey, 2000), however, the role of specific bacterial components in the removal of phenol from those bioreactors has scarcely been addressed.

In this study, a lab-scale MBR and a pilot plant MBR were tested for the treatment of an industrial wastewater characterised by low phenol concentrations and high salinity. When both reactors worked under steady state conditions and high phenol degradation, their microbial composition was studied by culture independent and culture dependent methods and phenol degrading isolates were obtained and characterised.

2. Methods

2.1. Lab-scale Membrane Biological Reactor (MBR)

The lab-scale MBR (5 L) used in this study is schematised in Fig. 1a. It was operated as a SBR. The digester was a rectangular methacrylate reactor containing a submerged flat membrane (0.3 μm) and a pH probe (Crison pH 25). Aeration was supplied by means of two air blowers (300 L h^{-1}) connected to a porous stone to assure a homogeneous oxygen distribution in the mixed liquor. Two peristaltic pumps (Ismatec REGLO and Selecta PERCOM-1) performed the feeding and the permeation of the MBR. The operation of the lab-scale reactor was controlled by means of a Programmable Logic Controller (PLC; Siemens LOGO!).

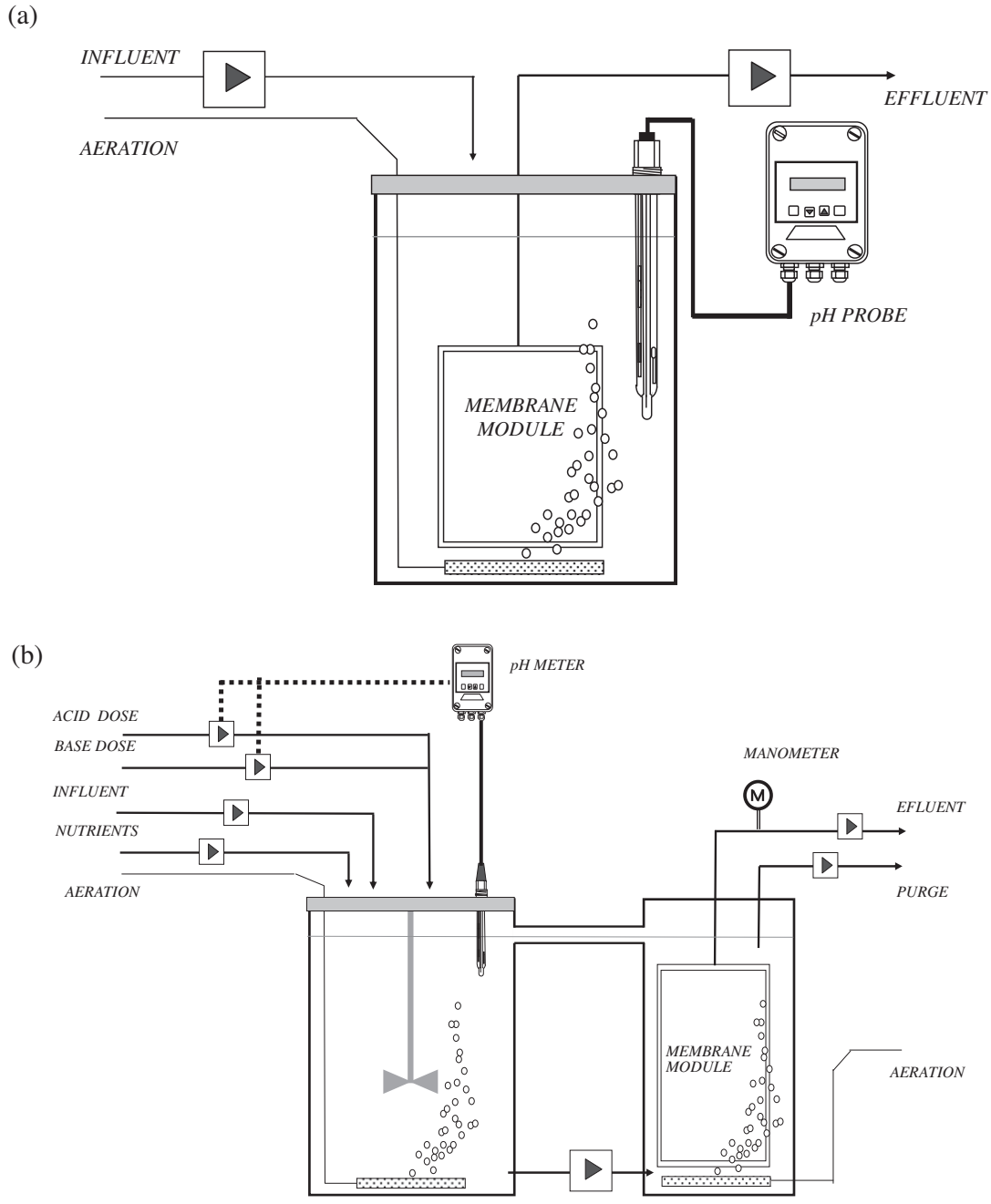

Fig. 1. Scheme of the lab-scale MBR (a) and the pilot plant MBR (b) used in this study.

2.2. Pilot plant scale Membrane Biological Reactor (MBR)

The MBR at pilot plant scale (8 m^3) had a separated flat membrane module configuration (see Fig. 1b). The reaction tank had 4 m^3 of capacity and was equipped with a pH probe. pH was controlled in the range 7.0–8.5. If pH was higher or lower than these values, acid (HCl, 0.1 N) or base (NaOH, 0.1 N) were added. Aeration was performed by two air blowers in both the reaction tank and the membrane tank. Both tanks were connected by a centrifugal pump that continuously pumped the mixed liquor from one tank to the other one. Wastewater feeding, nutrients addition, permeation and sludge purge were also performed by centrifugal pumps. A manometer was situated before the permeate pump, in order to clean the membrane when transmembrane pressure (TMP) was above 0.4 bars.

2.3. Chemical and physico-chemical analysis

The analysis of total solids (TS), volatile total solids (VTS), suspended solids (SS), volatile suspended solids (VSS) and alkalinity were performed according to the *Standard Methods* (APHA, 1998). Phenol concentration was assessed by reverse phase High Performance Liquid Chromatography (HPLC; *Waters Corporation*, Massachusetts, EUA). The Total Organic Carbon (TOC) was measured in a TOC analyser (Dohrman DC-190). Conductivity was measured by a conductimeter (Crison CM 35). Polyphenols and polyanilines concentrations were analysed by High Performance Liquid Chromatography–Mass Spectrometry (HPLC–MS). Polyformaldehyde concentration was analysed by Nuclear Magnetic Resonance (NMR; Bruker DMX500).

2.4. Media and culture-dependent microbiology methods

Artificial sea water (ASW) medium was prepared with ASW salts (Scharlau, Barcelona, Spain) (per liter, NaCl, 24.53 g; Na_2SO_4, 4.11 g; $MgCl_2$, 11.2 g; $CaCl_2$, 1.16 g; $SrCl_2$, 0.042 g; KCl, 0.7 g; $NaHCO_3$, 0.2 g; KBr, 0.1 g; H_3BO_3, 0.0029 g; NaF, 0.003 g) supplemented with nutrients (NH_4NO_3 10 mM and Na_2HPO_4 1 mM) and metals (per liter, $MgSO_4{\cdot}7H_2O$, 0.2 g; $FeSO_4{\cdot}7H_2O$, 12 mg; $MnSO_4{\cdot}H_2O$, 3 mg; $ZnSO_4{\cdot}7H_2O$, 3 mg; $CoCl_2{\cdot}6H_2O$, 1 mg; nitrilotriacetic acid disodium salt, 0.123 g). ASW agar plates were prepared with ASW medium solidified with purified agar (Pronadisa, Madrid, Spain). ASW-LB1/10 plates were prepared with ASW salts, peptone (1 g L^{-1}), yeast extract (0.5 g L^{-1}) and agar (Scharlau).

In order to isolate the heterotrophic bacteria present in the hypersaline phenol-degrading reactors, liquid samples were serially diluted in sterile ASW salts solution and plated on ASW-LB1/10 agar plates and in the same medium containing a higher concentration of NaCl (final concentration 80 g L^{-1}) to mimic the saline conditions in the biological reactors. All plates were incubated at 25 °C in aerobic conditions.

To check the phenol degrading capability of the obtained isolates, colonies were streaked on ASW agar plates and exposed to phenol vapors. This was accomplished by placing the plates in a closed jar containing a small vial with a concentrate phenol solution (90% in water) (Panreac, Barcelona, Spain). Another set of ASW agar plates were equally inoculated and incubated in a closed jar in the same conditions but without phenol to serve as negative controls.

Aerobic liquid cultures for phenol degradation studies were prepared in 25 mL bottles containing 10 ml of ASW medium and incubated at 25 °C with rotary shaking (200 rpm).

2.5. DNA extraction and PCR amplification of eubacterial 16S rRNA genes

Total DNA from the microbial consortia was extracted from 200 μl of the liquid cell suspension from the phenol-degrading reactor using the PowerSoil DNA isolation kit (MoBio, CA, USA). The purified DNA was stored at −20 °C.

For denaturing gradient gel electrophoresis (DGGE) analysis, the 16S rRNA genes of the microbial community or single isolates were PCR-amplified using the eubacterial primers GC40-63f and 518r (El Fantroussi et al., 1999) as described in Vila et al. (2010). For sequence analysis, the 16S rRNA genes from pure bacterial isolates were PCR amplified by using primers 16F27 and 16R1521 (Weisburg et al. 1991). PCR reactions and temperature cycles were performed as described in Vila et al. (2010). After analysis on 1.5% agarose gel electrophoresis, the PCR products were directly used for DGGE analysis or purified with a Wizard DNA clean-up kit (Promega) and utilized for sequencing reactions.

2.6. Sequencing and sequence analysis

Sequencing reactions were prepared with an ABI Prism BigDye Terminator (3.1) kit using the eubacterial primer 16F27 and following the manufacturer instructions. The DNA sequences obtained were edited, compared and aligned with the BioEdit software (Hall, 1999). The phylogenetic positioning of the bacterial isolates and the determination of percent similarities to closest neighbors were obtained by BLAST online searches at the NCBI web server (Altschul et al., 1997). Relevant partial 16S rRNA gene sequences were deposited at the GenBank database with accession numbers HM467192, HM467193, HM467194, and HM467195.

2.7. DGGE analysis

The PCR-products from the microbial consortia were examined on 1.5% agarose gels and directly used for DGGE analysis on 6% polyacrylamide gels with denaturing gradients ranging from 40% to 60% (100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was performed at a constant voltage of 100 V for 16 h in 1× TAE buffer at 60 °C on a DGGE-machine (C.B.S. Scientific Company, Del Mar, California). The gels were stained for 30 min with 1× SYBR Gold nucleic acid gel stain (Molecular Probes Europe BV, Leiden, The Netherlands) and photographed under UV light, using a ChemiDoc XRS System (Bio-Rad, California) with Quantity One software (Bio-Rad).

2.8. Time-course biodegradation of phenol

Bacterial strain PHE025 was used to inoculate duplicated sets of 25 ml flasks containing 10 mL of ASW medium with phenol (100 mg L^{-1}) as a sole carbon source. Sterile non-inoculated flasks and inoculated flaks without phenol were included as controls. Cultures were incubated at 25 °C under fully aerobic conditions (rotary shaking, 200 rpm). At 0, 14, 23, 30, 41, 55 and 72 h duplicated samples were removed from controls and cultures and used for viable cell counting and phenol quantification (HPLC).

3. Results and discussion

3.1. Wastewater characterisation

The wastewater tested in this study comes from a polymerization plant. As shown in Table 1, this wastewater was mainly characterised by a phenol concentration in the range of 8–16 mg L^{-1}, an extremely high salinity (around 95 g dissolved TS L^{-1}; 160 mS cm^{-1}), a low TOC concentration and a pH near 8.0 (the wastewater had a pH control before the treatment). In order to characterise the TOC present in this industrial wastewater, polyphenols, polyanilines and polyformaldehydes concentrations were also analysed, but none of these species were detected in the waste-

Table 1
Characteristics of the industrial wasterwater of this study.

Parameter	Units	Wastewater	Treated effluent
pH	–	7.8–8.3	8.4
Phenol concentration	mg phenol L^{-1}	8.3–15.6	<0.5
Inorganic carbon (IC)	mg C L^{-1}	n.d.	n.d.
Total organic carbon (TOC)	mg C L^{-1}	94.4–143.4	76.4–106.4
Polyphenols	mg L^{-1}	n.d.	n.d.
Polyanilines	mg L^{-1}	n.d.	n.d.
Polyformaldehydes	mg L^{-1}	n.d.	n.d.
Alkalinity	mmole L^{-1}	1.4	1.4
Conductivity	mS cm^{-1}	149–163	149–163
Total solids (TS)	g TS L^{-1}	94.2–96.1	92.8–95.9
Total suspended solids (TSS)	g TSS L^{-1}	<0.03	n.d.
Volatile TS (VTS)	g VTS L^{-1}	0.42–0.87	0.35–0.75

n.d.: not detected

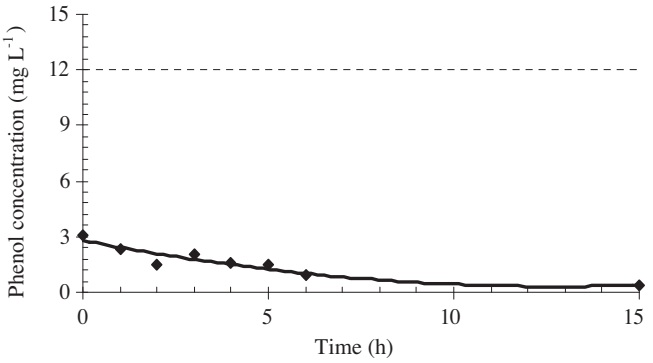

Fig. 2. Phenol biological removal rate in the MBR. Phenol concentration in the mixed liquor (■) and phenol concentration in the influent (——).

water (detection threshold 200 ppb). The content of inorganic carbon was practically inexistent and, therefore, the buffer capacity of this wastewater was very limited. Since the industrial wastewater had a lack of nutrients for the development of microorganisms, NH_4NO_3 and KH_2PO_4 were added to the wastewater to achieve a C:N:P ratio of 100:5:1 in order to ensure a balanced biomass growth.

3.2. MBR operation at lab-scale conditions

The industrial wastewater was initially treated by a continuous biological reactor with attached biomass where the phenol loading rate was progressively increased and less than 1 mg phenol L^{-1} was detected in the effluent for Hydraulic Retention Times (HRT) above 1 day. For higher phenol loading rates, a detachment of biofilms and a wash-out of the biomass present in the digester were observed. Therefore, it was decided to test the performance of a Membrane Biological Reactor (MBR) at lab-scale conditions in order to propose a more compact treatment capable to work at lower HRTs and to prevent the possible wash-out of biomass.

3.2.1. Inoculation of the MBR

The MBR was inoculated with the effluent VSS from the continuous reactor with attached biomass. The effluent of this reactor was stored during 2 months. It was assumed that phenol degradation kinetics from biomass present in this effluent was not strongly affected by the starvation period, as demonstrated by Buitrón and Ortiz (1997). Then, the effluent was passed through a ultrafiltration flat membrane in order to accumulate the VSS in the retained water. At the end of this period, 350 mg VSS L^{-1} were reached in the MBR.

3.2.2. Start-up of the MBR

As an initial approach to determine the maximum phenol degradation capacity of the concentrated biomass in the MBR, the biological phenol removal rate was assessed as follows: 1.5 L of wastewater (15 mg phenol L^{-1}) was added on 3.5 L of mixed liquor (this would represent an exchange volume of 30% in the MBR) and phenol concentration was measured every hour. The phenol addition was discontinuous, reaching an initial phenol concentration around 3.0–4.5 mg phenol L^{-1}. It was considered that this phenol concentration did not imply an inhibition of biomass activity, based on data from literature (Woolard and Irvine, 1995; Peyton et al., 2002). Fig. 2 shows the descent of phenol concentration during this batch assay, which demonstrates the phenol degradation capacity of the biomass. Moreover, the pH value (around 8.5) did not experience a quantifiable variation due to the relatively low concentration of phenol degraded.

3.2.3. Phenol degradation in the MBR

Once the biological phenol removal capacity inside the MBR was assessed, the reactor was run as a Sequencing Batch Reactor (SBR) at an HRT of 3.4 days following an operating MBR cycle of 6 h. The feeding of wastewater was performed during the first 3 h of the cycle length. During the following 1.25 h, the system was left under aeration conditions to completely remove residual phenol and the remaining 1.75 h were spent to perform the draw of permeate. Aeration was maintained along the operating cycle, since it is essential during the aerobic degradation of phenol and prevents membrane fouling (Cornel and Krause, 2006). Besides, the permeation pump worked intermittently (as recommended by the supplier of the membrane) to avoid membrane fouling. Backwashing was not performed in this study since it could lead to negative effects for the flat membrane. Moreover, the biomass present and developed in the MBR was non-flocculating, which is in accordance with the results reported by Ahn et al. (2008).

In this work, several modes of operation (or periods) were carried out in the MBR in which the wastewater loading rate was progressively increased. Fig. 3 shows the evolution of the phenol concentration in the influent and effluent of the MBR as the HRT was reduced. As observed, phenol removal efficiency was higher than 96% although the industrial wastewater loading rate was increased and drastic changes in the flow rate were performed: during period 5, the HRT was increased to 3.6 days, but in the subsequent period the HRT of the system was drastically reduced to 1.3 days without phenol accumulation. Consequently, during the whole operation period, the effluent quality (see Table 1) was maintained relatively constant.

As shown in Table 1, phenol concentration in the effluent wastewater was always below 0.5 mg L^{-1}. The total organic carbon (TOC) of the wastewater was around 90–140, and therefore was not only related to phenol. A TOC removal efficiency of 19–26% was observed during the operation of the MBR. The conductivity and the total dissolved solids of the influent and the treated wastewater were nearly the same, which demonstrates that salinity was not retained in the reactor. Moreover, suspended solids in the effluent were practically inexistent (<0.04 g L^{-1}) due to the membrane filtration.

The main operational parameters of the MBR during Period 10 are summarized in Table 2. In this period a specific phenol loading rate of 35 g phenol $(m^3\ day)^{-1}$ was efficiently treated (>98% removal efficiency). From Table 2 is also important to highlight that in the influent wastewater, the ratio VSS/SS was below 1.3%, while inside the digester, this ratio was always above 4.3%. This fact demonstrates that the VSS concentration present in the reactor was not related to an accumulation of solids coming from the feed, but to the growth of a microbial consortium capable to degrade the

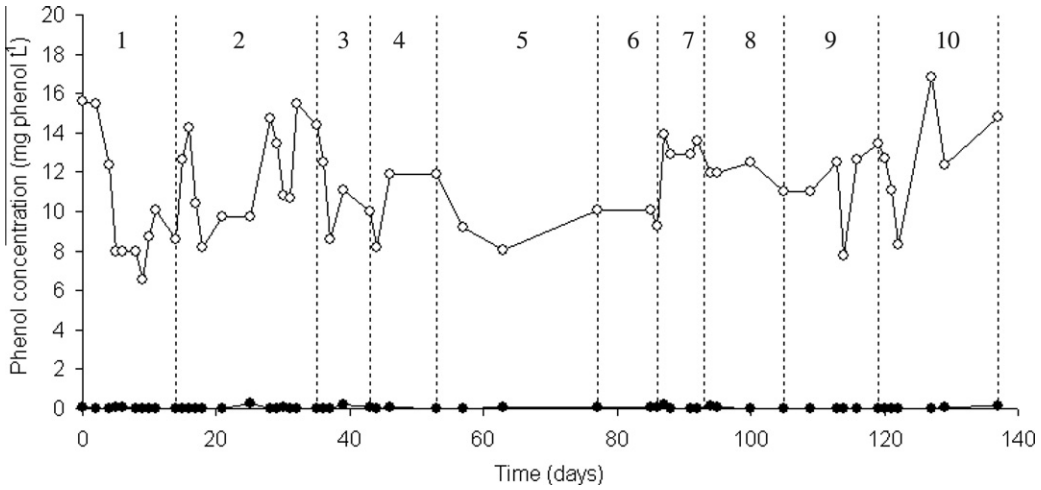

Fig. 3. MBR operation for the treatment of industrial wastewater containing phenol (○ influent phenol concentration; ● effluent phenol concentration). (Periods: 1 (HRT = 3.6 days), 2 (HRT = 1.9 days), 3 (HRT = 1.3 days), 4 (HRT = 1.9 days), 5 (HRT = 3.6 days), 6 (HRT = 1.3 days), 7 (HRT = 1.1 days), 8 (HRT = 0.75 days), 9 (HRT = 0.5 days), 10 (HRT = 0.45 days).

Table 2
Operational parameters of the MBR (Period 10).

Operational parameters of the MBR	Units	Value
Cycle temporization		
Cycle length	h	6
Feeding	h	3
Permeation (intermittent operation)	h	1.75
VSS	mg VSS L^{-1}	480
% VSS/SS	%	>4.5
Solid retention time (SRT)	days	>100
Hydraulic retention time (HRT)	days	0.45
pH	–	8.4
Conductivity	mS cm^{-1}	149–163
Specific phenol loading rate	g phenol $(m^3$ reactor day$)^{-1}$	35
Phenol removal efficiency	%	>98

phenols present in the wastewater. For microbiological analysis, samples inside the lab-scale MBR were extracted at day 116.

3.3. MBR operation at pilot-plant conditions

The MBR pilot plant described above was set up to test the biological degradation of phenol at a bigger scale. The MBR pilot plant was inoculated with suspended biomass coming from an industrial WWTP where biodegradable COD was treated at a relatively high conductivity (~60 mS cm^{-1}). For the start up of the reactor, several batch tests were carried out in which industrial wastewater was added to the reactor and phenol concentration was followed. Based on the registered phenol removal rate, a continuous feeding to the MBR was performed to reach an HRT of 2.8 days. Progressively, this HRT was decreased until a value of 0.7 days was achieved.

Fig. 4 shows the monitoring of phenol removal efficiency, HRT, conductivity (influent and effluent), pH (influent and effluent) and VSS inside the reaction tank along the whole experimental period. Phenol removal efficiency was always above 99.5%, except for days 37–40, where a problem related to the pH control system appeared due to a decalibration of the pH probe. Since minimum pH values of 5.5 were reached, a decrease of phenol degradation and a consequent accumulation of this pollutant were registered. However, when the pH probe was recalibrated, high biological phenol removal rates were recovered very fast, as stated in Fig. 4.

Volatile suspended solids concentration inside the reactor were stabilized around 2.5 g VSS L^{-1} (purge of biomass was not performed since it was considered that this VSS value was low and not harmful for membrane). Conductivity was the same at the influent and the effluent, which indicates that no salts were accumulated in the system. Moreover, transmembrane pressure (TMP) was always below 0.4 bars and, therefore, membrane cleaning was not necessary in the period tested. For microbiological analysis, samples inside the aeration tank were extracted at day 34.

3.4. Isolation and identification of bacterial strains from the MBRs

Viable counting and isolation of bacterial strains from the pilot plant reactor were carried out using two types of solid media containing different salt concentration. After 48 h of incubation, ASW LB1/10 plates gave counts of 1.0×10^7 cfu mL^{-1}, while plates containing the same medium with extra NaCl (80 g L^{-1}) produced 2×10^3 cfu mL^{-1}. Colonies representing different morphologies were purified and used for specific 16SrRNA gene amplification and sequencing. Sequence analysis showed that all of the isolates (12) recovered from the high salt plates were very similar, being closely related (99% sequence similarity) to species of *Halomonas,* an halophilic bacteria classified within the *gammaproteobacteria*. However, the little differences (0.4%) observed in the partial 16 S rRNA gene sequences between some of these isolates indicated that the reactor's bacterial community contained at least two different populations of *Halomonas,* one represented by isolates PHE004 and PHE008, and the other by the rest of the obtained isolates (see Supplementary material 1).

Six of the isolates recovered from low-salt plates showed identical 16S rRNA gene sequences to *Halomonas* strains previously obtained in the high salt plates (see Supplementary material 1). Four additional isolates had the same 16S rRNA gene sequence, matching (99%) species of the genus *Marinobacter* (see Supplementary material 2). Interestingly, in a preliminary microbiological study of the laboratory-scale reactor all the obtained isolates had also been identified as *Marinobacter* (isolates PHEL01, 02 and 03), with sequences indicating that they were all strains belonging to the same or closely related species of this genus (see Supplementary material 2).

In order to establish their role in phenol degradation, each of the isolates from the pilot plant reactor were inoculated on ASW plates and exposed to phenol vapors as sole carbon source. Only the isolates identified as *Marinobacter* sp. showed significant growth in respect to the control plates without carbon source.

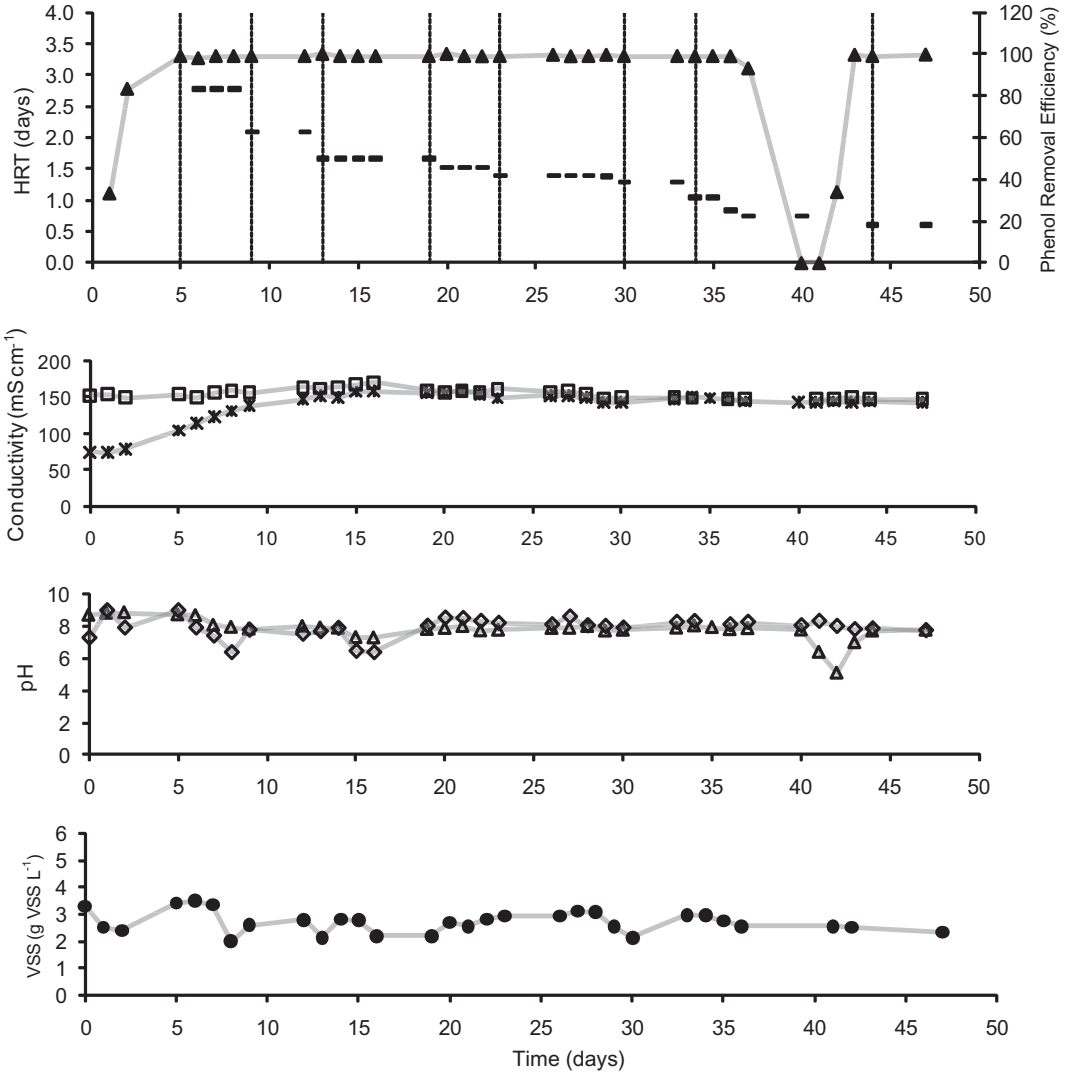

Fig. 4. MBR operation for the treatment of industrial wastewater containing phenol at pilot plant conditions (phenol removal efficiency (▲); HRT (▬); influent conductivity (□) effluent conductivity (Ж); influent pH (◇) effluent pH (Δ); VSS concentration in the reactor (●)).

One of them, PHE025, was selected for further experimentation. These results suggested that *Marinobacter* plays a major role in phenol removal from the pilot plant reactor. The fact that the tested *Halomonas* isolates did not show significant growth on phenol in the conditions of study leaves unanswered the question of the reason for its relative abundance in the reactor. *Marinobacter* is a member of the *gammaproteobacteria*, family *Alteromonadaceae*. The type species of the genus (*M. hydrocarbonoclasticus* SP.17) is a halophile, inhabitant of sea waters (Gauthier et al., 1992). Certain strains of this bacterium have been found associated to degradation of aromatic hydrocarbons (Nicholson and Fathepure, 2004; Vila et al., 2010; Berlendis et al., 2010).

3.5. Analysis of the microbial community structure of the pilot plant reactor

PCR amplification of the 16S rRNA genes of the whole bacterial community in the pilot plant reactor and subsequent DGGE analysis rendered a few major bands, thus suggesting a relatively low diverse population consistent with the selective conditions of the system (see Fig. 5). The PCR–DGGE analysis of pure cultures of the *Marinobacter* isolates PHE025 and PHE026, and the *Halomonas* isolates PHE021 and PHE022 produced two bands (B2 and B3) with similar migration, that coincided with one of the major bands in the whole microbial community DGGE fingerprint. This fits well with the results of the culture-dependent analysis which suggested that *Halomonas* sp. strains and *Marinobacter* sp. strains are major constituents. The DGGE profile of the whole community presented a second intense band with slower mobility (B1), suggesting the presence of an abundant microbial population that had not been recovered with the culture conditions used in this work and, therefore, was not identified. Other minor DGGE bands obtained for the whole microbial community could also correspond to unknown microbial members that did not appear in the agar plates. Hence, the possibility that other microbial populations other than *Marinobacter* sp. could be involved in the degradation of phenol in the pilot plant reactor can not be ruled out.

The high saline conditions of the pilot reactor are consistent with the presence of strains of *Marinobacter* and *Halomonas* as major components of the microbial community. These conditions and the presence of phenol are most likely restrictive enough to force the selection of *Marinobacter* strains, and also explain the fact that other less salt-tolerant microbial populations which could be found associated to phenol degradation in low saline

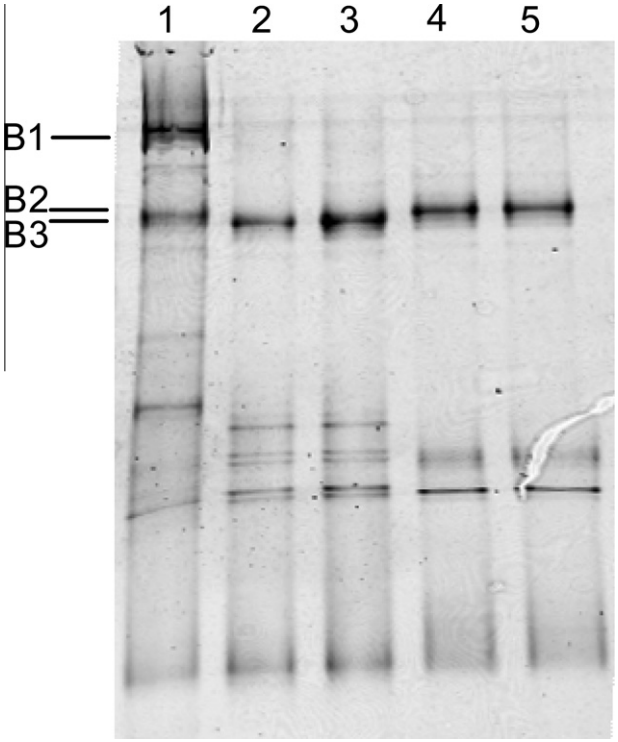

Fig. 5. DGGE analysis of partial PCR-amplified 16S rRNA gene from DNA isolated from whole bacterial population and pure bacterial strains. ((1) Whole bacterial population from slurry of phenol-degrading pilot plant reactor; (2) *Halomonas* sp. strain PHE021; (3) *Halomonas* sp. strain PHE022; (4) *Marinobacter* sp. strain PHE025; and (5) *Marinobacter* sp. strain PHE026). (B1, major uncharacterised bacterial species, B2 and B3, bands representing *Marinobacter* sp. and *Halomonas* sp. bacterial members of the population).

environments (Watanabe et al., 1998) are not found in the present study.

3.6. Growth-linked degradation of phenol by Marinobacter sp. strain PHE025

The range of phenol concentrations tolerated by strain PHE025 was tested in flasks with ASW medium and 25, 50, 100, 200, 500 or 1000 μg mL^{-1} of phenol, inoculated (100 μl) with a fresh suspension of a single colony of *Marinobacter* PHE025 in 1 mL of ASW. A non-inoculated equal set of bottles were included as controls. After 48 h of incubation, growth, evidenciated by an increase of turbidity, was only present in the cultures containing 100 and 200 μg mL^{-1} of phenol, thus indicating that higher concentrations were toxic for this bacterium. Viable counting after 4 days of incubation confirmed that maximum growth was obtained with 100 μg mL^{-1} of phenol (data not shown).

Utilization of phenol as sole source of carbon and energy by *Marinobacter* sp. strain PHE025 was confirmed by demonstrating progressive removal of this compound from ASW medium (HPLC) with a concomitant increase in the number of cells (see Fig. 6). A set of flasks containing 10 mL ASW media with phenol (100 μg mL^{-1}) were inoculated with a bacterial suspension of strain PHE025. At several time intervals, duplicated cultures and controls were taken from the incubator and used for viable cell counting and for phenol concentration measurement. The reduction of the phenol concentration in cultures was initiated after 24 h and this compound was completely removed at 72 h. This was accompanied by a 5 log increase in cell numbers (from 10^4 to 10^7 cfu ml^{-1}). Control cultures without phenol also showed a substantial increase in viable cell counting, however, as shown in Fig. 6, this growth, occurred during the first 24 h of incubation

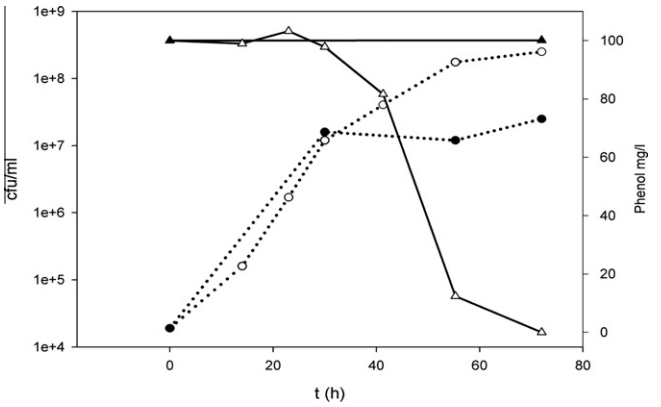

Fig. 6. Time course of phenol degradation (Δ) and growth of *Marinobacter* sp. strain PHE025 in ASW media with phenol as sole added carbon and energy source (○). Growth in the same media without phenol added (●) and concentration of phenol in a control experiment in ASW medium plus phenol but without bacterial inoculum (▲) were also analysed.

when phenol was not being utilized. In fact, this initial growth not linked to carbon source utilization is also present in the phenol cultures and could be explained on the basis of possible reserve substances in the bacterial cells of the initial inoculum.

As a whole, the preponderance of *Halomonas* and *Marinobacter* in the hypersaline, phenol-degrading reactor fit well with the halophilic nature of these bacteria and with previous reports of degradation of aromatic compounds by species of both of these genera (Muñoz et al., 2001; Alva and Peyton, 2003; Nicholson and Fathepure, 2004; García et al., 2004) and by halophilic bacteria in general (García et al., 2005). Although previous reports of other authors have described the capacity of *Halomonas* strains isolated from industrial saline wastewaters to grow on phenol as sole carbon and energy source (Hinteregger and Streichsbier, 1997), our results show instead that *Marinobacter* is a major player in the degradation of phenol in the hypersaline, phenol-containing industrial reactor studied here. Additionally, although strains of *Marinobacter* with the capacity to grow on aromatic compounds (namely, BTEX compounds) have been reported (Berlendis et al., 2010), to our knowledge this is the first report of *Marinobacter* isolates able to grow on phenol as sole carbon and energy source.

Our results indicate that phenol removal in the MBRs of study is associated to a low diversity microbial community. The microbiological identification, isolation in pure culture and maintenance of the main players of phenol removal at high salinity leads to the possibility of developing formulated inocula to shorten start-up periods of industrial wastewater treatment plants.

4. Conclusions

Phenol present in an industrial hypersaline wastewater (8–15 mg L^{-1}) was efficiently removed (>98.5%) by two Membrane Biological Reactors (MBR), reaching low hydraulic retention times (<0.5 days) and high flexibility of operation.

A number of *Marinobacter* and *Halomonas* strains have been found to be major constituents of bacterial populations in the MBRs tested.

Several strains of *Marinobacter* sp. able to grow with phenol as sole carbon source were isolated from the reactors and maintained as pure cultures.

Growth-linked phenol degradation by a representative isolate, *Marinobacter* sp. strain PHE025, was confirmed by monitoring an increase in cell numbers related to phenol removal.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.11.123.

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