WATER RESEARCH 43 (2009) 2977–2988

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Nonylphenol polyethoxylate degradation in aqueous waste by the use of batch and continuous biofilm bioreactors

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ARTICLE INFO

Article history:
Received 17 July 2008
Received in revised form
3 April 2009
Accepted 9 April 2009
Published online 19 April 2009

Keywords:
Nonylphenol ethoxylates
Wastewater
Packed bed bioreactors
Biofilm
FISH

ABSTRACT

An aerobic bacterial consortium (Consortium A) was recently obtained from textile wastewater and was capable of degrading 4-nonylphenol and nonylphenol polyethoxylates (NPnEOs). In the perspective of developing a biotechnological process for the treatment of effluents from activated sludge plants fed with NPnEO contaminated wastewater, the capability of Consortium A of biodegrading an industrial mixture of NPnEOs in the physiological condition of immobilized cells was investigated. Two identically configured packed bed reactors were developed by immobilizing the consortium on silica beads or granular activated carbon. Both reactors were tested in batch and continuous mode by feeding them with water supplemented with NPnEOs. The two reactors were monitored through chemical, microbiological and molecular integrated methodology. Active biofilms were generated on both immobilization supports. Both reactors displayed comparable NPnEO mineralization under batch and continuous conditions. FISH analyses evidenced that the biofilms evolved with time by changing the reactor operation mode and the organic load. Taken together, the data collected in this study provide a preliminary strong indication on the feasibility of Consortium A-based biofilm technology for the decontamination of NPnEO containing effluents.

1. Introduction

Nonylphenol ethoxylates (NPnEOs, where n is the number of ethoxylic units in the molecule) are non-ionic surfactants widely used in several industrial applications, such as textile and leather processing, paper industry, formulation of pesticides, paints and washing cleaners (Johnson et al., 2005). Commercial NPnEOs consist of mixtures of congeners containing 3–20 ethoxylic units and isomers differing in the branching of the nonyl chain. The use of NPnEOs has been banned in Europe for several industrial uses also including textile processing; an exception is made for industries possessing wastewater treatment technologies able to perform a complete removal of the organic fraction of the waste (EU regulation No. 1816, 2004). Therefore, there is a great interest in the development of efficient treatment technologies, also considering that an effective substitute for NPnEOs has not yet been found in textile processing. NPnEO concentration in textile wastes can show great variations; a report from Rozzi et al. (2000) has defined this amount in a range of 10–15 mg/L. Wastewater from industries employing NPnEOs is usually sent to conventional activated sludge treatment plants, where they are partially degraded to short chain NPnEOs and other metabolites such as 4-nonylphenol (4-NP)

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doi:10.1016/j.watres.2009.04.011

(Hayashi et al., 2005). Although threshold levels for these compounds in wastewater treatment plant effluents have not yet been defined in Europe, most textile wasterwater processing plants perform a tertiary treatment aimed both at completely removing the residual organic compounds, including non-ionic surfactants, and at decolorizing the waste (Arslan-Alaton and Seremet, 2004). Ozonation is actually the most effective one, although it is not completely successful on short chain NPnEOs (Ike et al., 2002), costly (Vanhulle et al., 2008) and it often gives rise to uncharacterized and potentially toxic break-down products (Choi and Hong, 2007). Other alternatives such as photocatalytic degradation in the presence of titanium dioxide (Ike et al., 2002), photolysis using a Hg lamp (Ahel et al., 1994) or a UV-solar simulating light (Neamtu and Frimmel, 2006) have been examined but none of them has been found to be as efficient as ozonation, by also producing, in some cases, persistent aromatic intermediates such as benzoquinones (Neamtu and Frimmel, 2006).

Biotechnological processes can represent an effective alternative to chemical–physical treatments for the decontamination of effluents containing NPnEOs and related metabolites (Soares et al., 2006). In particular, packed bed biofilm reactors appear to be an effective technology for wastewater treatment, as they allow to maintain a high biomass concentration and activity during the treatment minimizing, at the same time, start up operation and cell washout (Soares et al., 2003); in addition, they display a high tolerance versus very high and variable organic loads (Annadurai et al., 2002; Soares et al., 2006). Soares et al. (2003) developed a continuous biofilm reactor with a *Sphingomonas* sp. strain able to remove 4-NP from groundwater. The same research group successively set up a packed bed reactor inoculated with a cold-adapted bacterial mixed culture capable of removing 4-NP mixtures at temperatures below 15 °C (Soares et al., 2006); Fujii et al. (2003) developed a laboratory-scale packed bed column reactor inoculated with *S. cloacae* S-3^T capable of removing 4-NP from textile wastewater. In addition, the use of immobilized enzymes such as laccase operating in a continuous fluidized bed reactor has recently been regarded as an effective biotechnological way of eliminating nonylphenol from aqueous waste (Cabana et al., 2007).

To our knowledge, only a few studies have been focused on the development of packed bed biofilm reactors for the clean up of both NPnEO and 4-NP contaminated wastewater. Recently, in our laboratories, a set of parallel batch immobilized cell bioreactors, packed with different materials and inoculated with a *Pseudomonas* sp. strain, have been developed for the treatment of NPnEO artificially contaminated water (Bertin et al., 2007; Di Gioia et al., 2008a). The result obtained evidenced a remarkable decontamination potential and microbial stability along with biodegradation performances higher than those displayed by freely suspended cells of the same strain (Bertin et al., 2007). However, NPnEO biodegradation was not complete and trace amounts of 4-NP persisted in the reactors at the end of the treatment. The research for more efficient biocatalysts has led to the isolation (from a full-scale pre-treatment oxidation tank fed with textile wastewater) of an aerobic bacterial consortium, namely Consortium A, capable of degrading a large variety of NPnEO congeners as well as an industrial mixture of branched side-chain isomers of 4-NP (Di Gioia et al., 2008b). The consortium was composed mainly of *Alfa* and *Gammaproteobacteria*, with a high percentage of members of *Pseudomonas* genus among the latter group (Di Gioia et al., 2008b).

The objective of the research was to evaluate the possibility of employing Consortium A in the development of a continuous biofilm technology for the treatment of effluents deriving from conventional activated sludge processes fed with NPnEO contaminated wastewater. A Consortium A biofilm was developed within two identically configured column bioreactors packed with different materials, i.e. silica beads (SB) and granular activated carbon (GAC), and tested for its capability of decontaminating a synthetic water contaminated with an industrial NPnEO mixture. The bioreactors operated first in batch and then in continuous mode. The composition and stability of the bacterial communities working in the reactors under both batch and continuous conditions at different organic loads were studied via fluorescent in situ hybridization (FISH) with 16S RNA targeted oligonucleotide probes.

2. Materials and methods

2.1. Chemicals

Culture media components were purchased from Biolife Italia (Milan, Italy) and from Sigma–Aldrich (Milan, Italy). The commercial mixtures of 4-nonylphenol (4-NP), composed of different nonyl chain isomers, and Igepal CO-520 (industrial mixture of NPnEOs with an average ethoxylation degree of 5) were purchased from Sigma–Aldrich (Milan, Italy). High performance liquid chromatography (HPLC) solvents were provided by Carlo Erba (Milan, Italy). NaOH and HCl solutions were obtained from Merck (Darmstadt, Germany). The two immobilization carriers, granular activated carbon (GAC, commercial name: CP4–60, consisting of cylinders of 3 mm diameter and 10 mm length) and silica beads (SB, commercial name: Celite R-635, consisting of irregular cylinders of about 5 mm diameter and 10 mm length) were supplied by Chemviron Carbon (Feluy, Belgium) and World Minerals (Santa Barbara, CA, USA), respectively.

2.2. Microorganisms and media

The microbial consortium employed as the processes biocatalyst (named Consortium A) was isolated from an oxidation pre-treatment tank receiving wastewater from a textile industry, by employing 4-nonylphenol (4-NP) as the sole carbon and energy source (Di Gioia et al., 2008b). It was grown on minimum mineral medium (MMM, Fava et al., 1996a), supplemented with 100 mg/L of 4-NP. Tryptic soy agar (TSA, Fava et al., 1996a) plates were used for cell concentration determination.

2.3. Packed bed bioreactors

Each bioreactor consisted of a glass column (diameter 5 cm, height 45 cm) with an empty volume of about 700 mL, filled with either SB or GAC as the immobilization supports (Fig. 1A).

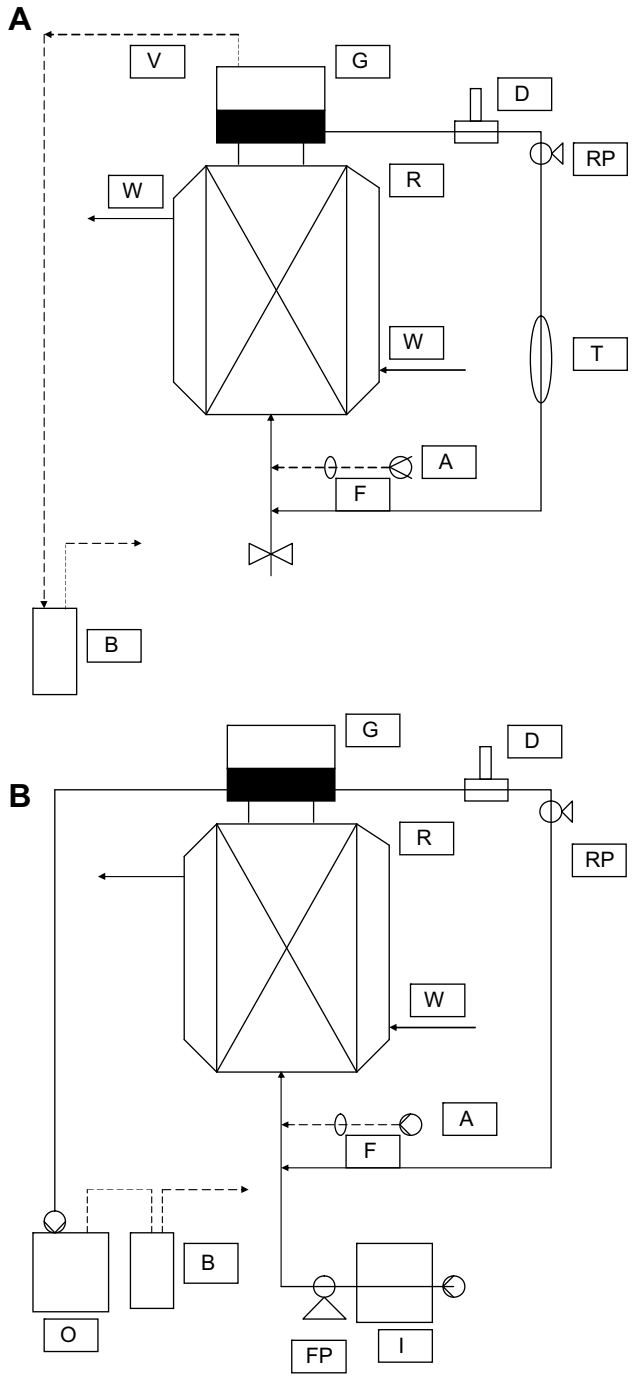

Fig. 1 – Scheme of the developed reactor in the batch mode (A) and in continuous mode (B) configuration (R: bioreactor, G: glass chamber for the insertion of the exhaust air-line, the recycle-line and pH probe, B: air vent-line + Drechsler system for CO_2 capture, D: sampling door, RP: recycling pump, T: feeding trap, F: air filter; A = air-inlet line; V = air vent line; W = water at 30 °C; I = feeding tank; O = effluent tank; FP = feeding pump; dotted lines represent gas lines).

Working volumes of 350 and 330 mL were obtained for the SB reactor (R-SB) and the GAC reactor (R-GAC), respectively. Both bioreactors were equipped with an external jacket in which water at 30 °C was continuously recycled. The inlet line for the feeding of the fresh medium and the air-inlet line were at the bottom of the bioreactor. Air was filter sterilized with a 0.22 μm cellulose acetate filter (Millipore S.p.A., Milan, Italy) and passed through a glass trap filled with soda lime, which adsorbed the carbon dioxide present in the air, before entering the system. The gas outline and a recycle line by which the cultural broth was continuously recycled from the top to the bottom of the vessel were inserted in a glass chamber placed at the top of the bioreactor, which also allowed the introduction of a pH probe. The exhausted air was first forced to bubble in a 500 mL bottle containing distilled water, so to collect the surfactants accidentally exiting the system as foam, and then in a 250 mL Drechsler bottle containing 200 mL of NaOH 0.1 M. This apparatus allowed to evaluate, after titration of the residual NaOH solution with HCl 0.1 M, the CO_2 produced as a result of the biological activity. A sampling door and a feeding trap (when the reactors operated under batch conditions) were positioned along the recycle line; the latter was constituted by a small glass column (volume = 30 mL) filled with acid-washed-glass beads (diameter = 3 mm) on which pre-established amounts of NPnEO mixtures were injected and dispersed. In order to allow continuous mode operations, both the inlet and the outlet-line (by which both the effluent and the exhausted air reached the effluent tank) were added (Fig. 1B), and the feeding trap was eliminated as the surfactant mixture was directly injected in the feeding tank. After having bubbled in the effluent tank, the exhausted air passed in the Drechsler bottle for the produced CO_2 measurement. The bioreactor parts were autoclaved (121 °C for 20 min) before being set up, then an aqueous ethanol solution (70% v/v) containing HCl (1% v/v) was recycled for 2 days in order to sterilize the assembled materials. The reactors were washed with sterile water to remove ethanol residues and then packed with 255 g of SB or 273 g of GAC (dry weights), previously autoclaved (121 °C for 20 min). The capacity of the empty bioreactors, the volume occupied by the supports and by air are reported in Table 1. The biofilms were generated by inoculating both bioreactors with Consortium A and by supplying 4-NP through the feeding trap at the theoretical concentration of 100 mg/L (calculated by considering the surfactant completely soluble in water). The inoculated culture was obtained on MMM plus 100 mg/L 4-NP at 30 °C in shaken flasks (150 rpm) up to the stationary growth phase. The culture was re-circulated in the reactor for 2 weeks in batch conditions in order to allow biofilm formation by the members of the Consortium A; the ratio between the actual working volumes and the recycling flow rates was 0.5 h for both reactors.

Table 1 – Working parameters of the two packed bed reactors developed.

	Empty volume (mL)	Packing volume (mL)	Air volume (mL)	Working volume (mL)	Recycling flow rate (mL/h)
R-SB	730	360	20	350	700
R-GAC	715	365	20	330	660

2.4. Biodegradation experiments in batch mode

The degradation capability of the biofilm developed was initially studied in batch conditions. The first experiment was performed by supplying 4-NP as the sole carbon and energy source through the feeding trap, in order to have an initial theoretical concentration of 60 mg/L in the bioreactors (calculated as if 4-NP was completely soluble in the water phase). This experiment was performed for 7 days. During the experiment, two 1 mL-samples were timely withdrawn from the sampling door on the recycle line, acidified to pH 3 with TCA 2M, and batch extracted as described below. Another broth aliquot was withdrawn to monitor cell concentration, through plate counting on TSA medium. At the end of the experiment, the bioreactor medium, the content of the air-bottle traps and the glass beads in the feeding trap were removed, subjected to extraction with diethyl ether and the solvent fraction was analyzed via HPLC-DAD in order to evaluate 4-NP and related metabolites concentration in each of the compartments; moreover, the NaOH solution employed to neutralize CO_2 was titrated with HCl 0.1 M to calculate the CO_2 amount produced by the two reactors. Calculation for 4-NP considers the production of 15 moles of CO_2/mole. Two successive batch experiments were then performed with Igepal CO-520 as the sole carbon and energy source. The substrate was applied through the feeding trap at an initial theoretical concentration of 60 mg/L (calculated as if Igepal CO-520 was completely soluble in the water phase); the experiment was performed for 10 days. Samplings to determine pollutant concentrations, CO_2 production and cell concentration were periodically withdrawn as described above. CO_2 produced has been used to calculate the amount of mineralized pollutant, considering an average ethoxylation degree of five ethoxylic units for Igepal CO-520, as specified by the producer, which gives rise to 25 moles of CO_2/mole of Igepal CO-520. At the end of each experiment, the bioreactors were washed with sterile saline solution (9 g/L of NaCl) and then refilled with fresh MMM medium. The performances of the system were evaluated in terms of pollutant overall removal and mineralization, calculated according to the following equations:

$$\text{Overall removal } (\%) = \left[(\mathrm{Xi} - \mathrm{Xr}_{(\mathrm{ft})}) - (\mathrm{Xr}_{(\mathrm{w})} + \mathrm{Xr}_{(\mathrm{at})})/(\mathrm{Xi} - \mathrm{Xr}_{(\mathrm{ft})})\right] \times 100$$

$$\text{Mineralization } (\%) = \left[\mathrm{Xm}/(\mathrm{Xi} - \mathrm{Xr}_{(\mathrm{ft})})\right] \times 100$$

$$\text{Mineralization of the removed material } (\%) = \left[\mathrm{Xm}/(\mathrm{Xi} - \mathrm{Xr}_{(\mathrm{ft})} - \mathrm{Xr}_{(\mathrm{w})} - \mathrm{Xr}_{(\mathrm{at})})\right] \times 100$$

where

Xi = pollutant initially introduced in the feeding trap, $Xr_{(ft)}$ = pollutant recovered at the end of the experiment adsorbed onto the glass beads in the feeding trap, $Xr_{(w)}$ = pollutant recovered at the end of the experiment in the exhausted broth, $Xr_{(at)}$ = pollutant recovered at the end of the experiment in the air-trap, Xm = pollutant mineralized on the basis of CO_2 produced.

2.5. Biodegradation experiments in continuous mode

The bioreactors were forced to operate in continuous mode: they were fed with synthetic minimal medium containing Igepal CO-520 as the only carbon and energy source provided by a reservoir in which the NPnEO mixture was supplied at the theoretical concentration of 15 mg/L. The actual concentration of Igepal CO-520 in the inlet of the bioreactors determined through repeated quantitative HPLC-DAD analyses of samples withdrawn from the feeding tank was 12.22 ± 0.78. After the reactors were fed in continuous mode 1 week long with Igepal CO-520 at 1.4 mg/L × h, three sequential experiments (15 days each) were performed by feeding the reactors at different organic loads (1.4, 3.3 and 4.9 mg/L × h), which were obtained by increasing the dilution rate; the recycling ratio (defined as the ratio between the recycling flow rate and the sum of the feeding and recycling flow rates) was maintained constant (0.95 in both bioreactors) by increasing proportionally the flow rate of the recycling line. The working conditions employed in such experiments are presented in Table 2. Viable cell concentration, pollutant concentration in the recycle line and CO_2 production were periodically determined as described above. In addition, the effluent was periodically sampled, extracted with solvent and the organic phase obtained subjected to HPLC analysis for Igepal CO-520 and related metabolites quantification.

At the end of all the experiments, the bioreactors were opened and samples of carriers were taken from different portions of the reactor packed beds (at the bottom and at the top of the reactors), in order to evaluate the amount of Igepal CO-520 adsorbed onto GAC and SB and the amount of immobilized biomass per dry weight of carrier. The immobilized biomass was quantified by applying the procedure reported by Bertin et al. (2001), based on protein concentration measurements.

2.6. FISH

Samples of the recycle broth at the end of the batch experiments and at the 11th day of each of the three continuous experiments were immediately fixed in 4% paraformaldehyde

Table 2 – Working conditions under continuous mode of treatment.

	Feeding flow rate R-SB (mL/h)	Feeding flow rate R-GAC (mL/h)	Organic load (both reactors) (mg/L × h)	Hydraulic retention time (both reactors) (h)	Recycling flow rate R-SB (mL/h)	Recycling flow rate R-GAC (mL/h)	Recycling ratio (both reactors)
1st experiment (15 days)	32	30	1.4	11.0	608	570	0.95
2nd experiment (14 days)	76	72	3.3	4.6	1444	1368	0.95
3rd experiment (14 days)	115	108	4.9	3.1	2185	2052	0.95

and then subjected to FISH analyses as described in Salvadori et al. (2006). They were then hybridized with the ribosomal RNA targeted fluorescent probes already used to characterize the microbial composition of Consortium A (Di Gioia et al., 2008b; Salvadori et al., 2006). In particular, probes specific for bacteria, EUB338 (Amann et al., 1995), for *Alfaproteobacteria*, ALF968 (Neef et al., 1999), for *Betaproteobacteria*, BET42a (Manz et al., 1992), for *Gammaproteobacteria*, i.e. a mixture of GAM42a (Manz et al., 1992), GAM42b (Salvadori et al., 2006) and GAM42_C1033 (Yeates et al., 2003) defined as GAM (Di Gioia et al., 2008b), for the *Pseudomonas* genus, PSM G (Braun-Howland et al., 1993), for *P. aeruginosa*, Pseaer A (Hogardt et al., 2000) and for *Stenotrophomonas* spp., Steni (Salvadori et al., 2006) were used in FISH analyses. Cell concentrations were calculated as the percentage of: (i) 4′,6-diamidino-2-phenylindole (DAPI) stained cells, in the case EUB positive cells; (ii) EUB positive cells, in the case of ALF968, BET42A and GAM probes; or (iii) GAM positive cells, in the case of PSM G, Pseaer A and Steni probes.

2.7. Main properties of the cell immobilization supports

The adsorption properties of SB and GAC with respect to Igepal CO-520 NPnEOs were determined in duplicate by placing about 10 g of each support (previously sterilized in autoclave) in 100 mL sterile bottles containing 20 mL of sterile MMM additioned with Igepal CO-520 at the theoretical concentration of 10, 25, 50, 100 and 200 mg/L. Blank tests were performed with a parallel set of identical support-free bottles. All bottles were allowed to reach equilibrium conditions by shaking them on a rotary shaker at 30 °C and 150 rpm for 2 days. Igepal CO-520 adsorption data were calculated as follows: the medium was separated from the support and extracted twice with an equal volume of diethyl ether. After solvent addition, the two-phase system was subjected to a 10 min treatment in an ultrasonic bath. The organic phase obtained was collected, evaporated, re-suspended in HPLC-grade methanol and analyzed via HPLC-diode array detector (DAD) (see below). The blanks were extracted through the procedure described below and the HPLC results obtained from their analyses were used for determining the extraction yield. Igepal CO-520 adsorbed to the supports was calculated as the difference of the introduced amount and the amount present in the broth. Either ''Freundlich'' or linear sorption kinetics for Igepal CO-520 on the two supports were then studied to evaluate which one fitted better the experimental data.

For the determination of adsorbed Igepal CO-520, support samples (about 1 g) were washed with distilled water to remove the unadsorbed material, subjected to solvent extraction with 10 mL of diethyl ether and treated in an ultrasonic bath for 10 min. The extraction was repeated twice, the organic phases were collected, evaporated, then re-suspended in HPLC-grade methanol and analyzed via HPLC-DAD (see below). The final Igepal CO-520 recovery percentage was 80%.

2.8. Solvent extraction procedures and analytical methods

The pollutant present in the recycle broth was extracted by double batch extraction with diethyl ether: 800 μL of diethyl ether were added to 1 mL of broth, centrifuged (5 min, 10,000 rpm), the organic phase was withdrawn and the aqueous phase was again extracted with the same method. The collected organic phases were evaporated overnight at 35 °C, resuspended in HPLC grade hexane (or methanol for 4-NP) and analyzed via HPLC-DAD. Igepal CO-520 (and related metabolites) present in the effluent tank of the continuous experiments, as well as the pollutant in the exhausted broth and in the air trap in the batch experiments, were extracted through double batch extraction with diethyl ether; a 250 mL sample was extracted with an equal volume of diethyl ether, then the bottle was sonicated for 5 min, then the organic phase was separated in a funnel, while the aqueous one was extracted again; finally the organic phase was evaporated at 35 °C overnight, resuspended in HPLC grade hexane (methanol for 4-NP) and HPLC analyzed. The Igepal CO-520 and 4-NP concentrations evaluated were corrected considering the recovery efficiencies, which were 85% for the former and 80% for the latter. The recovery efficiency were calculated on MMM solutions supplemented with a known amount of Igepal CO-520.

Quantitative and qualitative analysis of Igepal CO-520 was done via HPLC-DAD (Beckman Coulter, USA) as detailed in Di Gioia et al. (2008a); quantitative analyses of Igepal CO-520 were performed by summing the area of the seven peaks ascribed to the different congeners present in this mixture (i.e. congeners ranging from NP4EO to NP10EO) as described in Di Gioia et al. (2008a). Quantitative and qualitative analysis of 4-NP was done via HPLC-DAD reverse phase system (Beckman Coulter, USA), equipped with a Ultrasphere C18 4.6 mm × 250 mm column (Beckman Coulter, USA); column temperature was 35 °C, flow rate was 1 mL/min and injection volume was 20 μL. The eluents employed were water additioned with 1% v/v acetic acid (A) and methanol additioned with 1% v/v acetic acid (B); the eluent was composed by solutions A and B in the ratio 80:20 v/v; the elution was isocratic and lasted 20 min. pH was measured by using a selective probe (81-04 model, ATI-Orion, Boston, USA).

3. Results

3.1. Adsorption of Igepal CO-520 on the cell immobilization supports

The adsorption capability of Igepal CO-520 on SB and GAC was characterized in a range of concentrations, which include those used both in the batch and in the continuous experiments.

Freundlich ($q = k \times C^n$, representing q the Igepal CO-520 adsorbed per unit mass of solid material, mg/g, and C the Igepal CO-520 concentration at the equilibrium, mg/L) and linear adsorption isotherms ($q = k \times C$) were employed to fit the experimental data: a higher regression coefficient (R^2) was obtained by applying the latter one (Fig. 2). Therefore, linear adsorption isotherm was found to best describe Igepal CO-520 adsorption on both supports with a slope which was greater for SB with respect to GAC. Adsorption coefficients were quite low; but in substantial agreement with those obtained by Yu et al. (2008a,b).

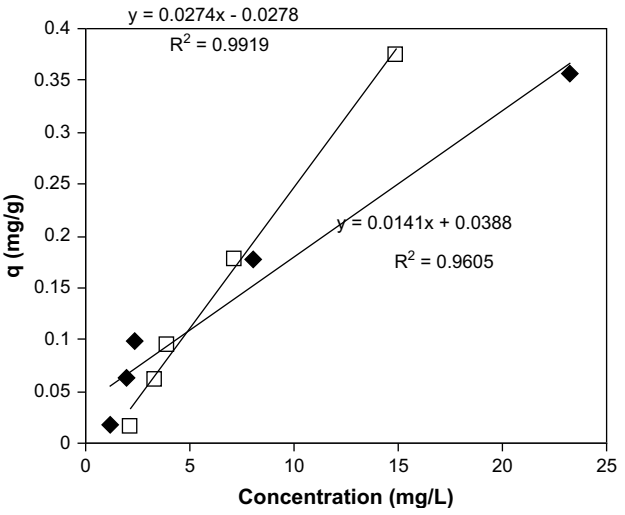

Fig. 2 – Igepal CO520 adsorption on GAC (◆) and on SB (□). (q = Igepal CO-520 adsorbed per unit mass of solid material).

3.2. *Biodegradation of 4-nonylphenol and Igepal CO-520 under batch mode*

The biodegradation capability of the two biofilm reactors was preliminary checked with 4-NP supplied at the initial theoretical concentration of 60 mg/L and then studied through two sequential batch experiments with Igepal CO-520 also applied at the theoretical concentration of 60 mg/L. The presented results and mass balances do not consider the amount of 4-NP potentially adsorbed on the two immobilization supports, as samples of immobilization supports were collected from each reactor and subjected to solvent extraction only at the end of all the experiments. pH measured in the recycle broth of both reactors was around 7 in both batch experiments. During the first study (i.e. with 4-NP), which was 7 days long, the concentration of freely suspended cells in the recycle medium was in the range 5.0–6.0 log (CFU/mL) in both bioreactors (data not shown). 4-NP concentrations lower than 1 mg/L were measured in the recycle of both bioreactors throughout the experiment where no other metabolites with aromatic moieties were evidenced and 4-NP concentrations much lower than the theoretical one (60 mg/L) were supplied to the reactors due to the low water solubility of 4-NP. The overall mass balance of this experiment, determined evaluating 4-NP amounts present in the exhausted broth, feeding trap and air trap at the end of the experiment, is presented in Table 3. The data show that a high amount of the introduced 4-NP was retained in the feeding trap, in particular in the R-SB reactor, and therefore was not transferred to the bioreactors. 4-NP removal percentage, referred to the amount that entered the reactors, were 94 and 99%, for R-SB and R-GAC, respectively, whereas the mineralization percentage of the biodegraded 4-NP were 93 and 83% for the same reactors.

The batch experiments with Igepal CO-520 were run for 10 days each one. The reported results are the average (±standard deviation) of the two sequential experiments performed. Free cell concentration, measured in the recycle line, was almost constant throughout the time and higher in R-GAC [in the range 6.0–7.0 log (CFU/mL)] than in R-SB [in the range 6.0–6.3 log (CFU/mL)] (data not shown). Igepal CO-520 was detected in the recycle broth in the range 14–20 mg/L for both bioreactors (Fig. 3A), i.e. at a concentration lower than that theoretically provided, but, once again, in agreement with the water solubility of NPnEO mixtures (Ahel and Giger, 1993). The relative amount of each NPnEO congener composing Igepal CO-520 was found not to change during the experiment course (data not shown). 4-NP was transiently accumulated in both bioreactors, but it was no more present at the end of the experiment (Fig. 3B). Regarding the final mass balance (Table 3), the overall Igepal CO-520 removal was about 80% in both bioreactors, while the mineralization of the depleted pollutants was 77 and 71% for R-SB and R-GAC, respectively, the remaining 2% (in R-SB) and 10% (in R-GAC) of Igepal CO-520 supplied can probably be ascribed to the adsorption on supports.

3.3. *Experiments in continuous mode*

After the batch experiments, both bioreactors were continuously fed with the synthetic wastewater containing Igepal CO-520 as the only carbon and energy source at 1.4 mg/L × h. After 1 week, three sequential 15 days experiments were performed at different and increasing organic loads (1.4, 3.3 and 4.9 mg/L × h) in order to study the performances and robustness of

Table 3 – Removal percentages of 4-NP and Igepal CO-520 with respect to the amount of overall pollutants transferred in the reactors through the feeding traps (initial theoretical 4-NP and Igepal CO-520 concentrations: 60 mg/L).

	Amount added to feeding trap (mg)	Amount recovered from			Removal % with respect to the amount transferred in the reactors	Mineralized amount (mg)	Mineralization % with respect to the amount transferred in the reactors	Mineralization % with respect to the amount removed
		Exhausted broth (mg)	Feeding trap (mg)	Air trap (mg)				
Experiment with 4-NP								
R-SB	21.00	0.00	11.12	0.57	94.23	9.23	93.42	99.14
R-GAC	19.80	0.08	4.42	0.06	99.09	12.76	82.96	83.73
Experiment with Igepal CO-520								
R-SB	21.00 ± 0.01	1.87 ± 0.13	0.03 ± 0.00	2.44 ± 0.15	79.50 ± 9.47	16.19 ± 1.70	77.21 ± 8.10	97.18 ± 10.2
R-GAC	19.80 ± 0.01	1.49 ± 0.15	0.03 ± 0.00	2.29 ± 0.12	80.80 ± 9.65	14.00 ± 1.20	70.81 ± 6.10	87.55 ± 7.51

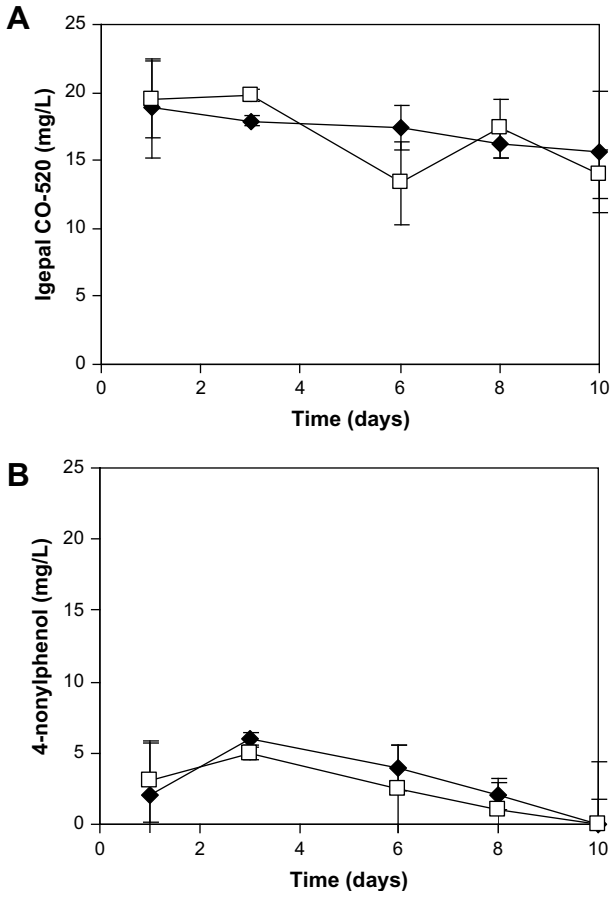

Fig. 3 – Concentration of Igepal CO-520 (A) and of 4-nonylphenol (B) as a function of the time in the R-SB (□) and in the R-GAC (◆) during the batch experiment with Igepal CO-520 applied at the original theoretical concentration of 60 mg/L.

the system. In the first experiment, the total amount of Igepal CO-520 provided was 140.77 and 132.98 mg for the R-SB and the R-GAC, respectively. Cell concentration in the recycle broth was found to be nearly constant in the R-SB, whereas it was fluctuating during the 1st week of the experiment in the R-GAC (Fig. 4A). The Igepal concentration stabilized around a constant value only after the 11th day of the experiment in both reactors (Fig. 4A). As in the batch experiment, the relative amount of each Igepal CO-520 congener did not change during the continuous experiments (data not shown). pH was 8.00 ± 0.78 and 7.33 ± 0.52 in R-SB and R-GAC, respectively, and no aromatic metabolites accumulated in the reactors, throughout the experiment. The whole mass balance calculated at the end of this experiment indicates that about 48% of Igepal CO-520 entering the systems was removed in both reactors and that, on the basis of CO_2 evaluation, almost all the depleted surfactant was mineralized in the same bioreactors (Table 4). In the second experiment, cell concentration in the recycle broth was found to be nearly constant throughout the experiment in both reactors (Fig. 4A). The Igepal concentration detected in the reactor effluents was found to be constant after the 19th day (corresponding to the 4th day of the second experiment), with an average value of 1.31 ± 0.24 and 1.27 ± 0.14 mg/L, in the R-SB and the R-GAC, respectively (Fig. 4B). pH was almost constant in both bioreactors (average value of 7.18 ± 0.10 in R-SB and 6.93 ± 0.10 in R-GAC). The production of CO_2 was stable throughout the experiment in both bioreactors (data not shown). As in the previous experiment, no metabolites with aromatic moiety were evidenced in the reactors. Considering all these data, it can be assumed that the steady state conditions were reached in this experiment. Almost 70% of Igepal CO-520 entering both reactors was removed whereas about 65% of this amount was found to be mineralized (Table 4). In the third experiment, the Igepal concentration in the recycle line was in the range 1–3 mg/L in both bioreactors until the 41st day of treatment (corresponding to the 11th day of the third experiment), then it started to increase, reaching about 8 mg/L in the R-SB and 12 mg/L in the R-GAC at the 44th day of treatment. CO_2 production occurred throughout the test, but it decreased during the last days of treatment (data not shown), concomitantly with the increase of Igepal concentration in the recycle medium (Fig. 4B). However, total cell concentration in the recycle line did not show the same fluctuation being almost constant in both bioreactors (Fig. 4A), with an average value of 7.1 ± 0.09 and 7.0 ± 0.11 log (CFU/mL) in the R-SB and in the R-GAC, respectively. pH values were 7.2 ± 0.01 and 7.13 ± 0.02 for the R-SB and the R-GAC, respectively. In this experiment the overall Igepal CO-520 removal were 56.0 and 55.1%, in the R-SB and in the R-GAC, respectively, and the corresponding mineralization percentages were 38.5 and 38.3%.

At the end of the continuous experiments, both immobilization supports and in particular silica beads appeared to have suffered mechanical crumbling. Indeed, small quantity of thin powder derived from SB support was found to be present in the reactor body or at the bottom of the glass chamber positioned in the upper part of the reactor. After the last continuous experiment, the bioreactors were opened and samples of each immobilization support were collected at different positions in the reactors in order to evaluate the amount of both Igepal adsorbed and of biomass immobilized on them. The adsorbed Igepal CO-520 amount was 79.69 ± 1.36 and 75.67 ± 3.05 mg in the R-SB and in the R-GAC fixed bed, respectively, corresponding to 0.310 mg of Igepal CO-520/g of dry SB (0.12 mg/g on the top part of the reactor and 0.51 mg/g on the bottom one) and to 0.288 mg of Igepal CO-520/g of dry GAC (0.17 mg/g on the top part of the reactor and 0.39 mg/g on the bottom one). These experimental data are in substantial agreement with those calculated by the adsorption isotherm (i.e. 0.19 and 0.20 mg/g for SB and GAC, respectively), which were determined in supports not covered by the biofilm. In the whole experiment 925 and 871 mg of Igepal CO-520 were supplied in R-SB and R-GAC respectively; at the end of the experiment 842 and 793 mg of Igepal were found in the exhausted broth, mineralized or adsorbed on the support, whereas the remaining amounts (respectively 10 and 9% of the total entering amount) was probably used by biofilm for its generation (Corvini et al., 2004). The average value of biomass immobilized on SB and GAC samples, collected in two different sections of the bioreactor, was (expressed as mg of dried biomass/g of dried support) 1.49 ± 0.13 and 0.38 ± 0.04 mg/g, in R-SB and R-GAC,

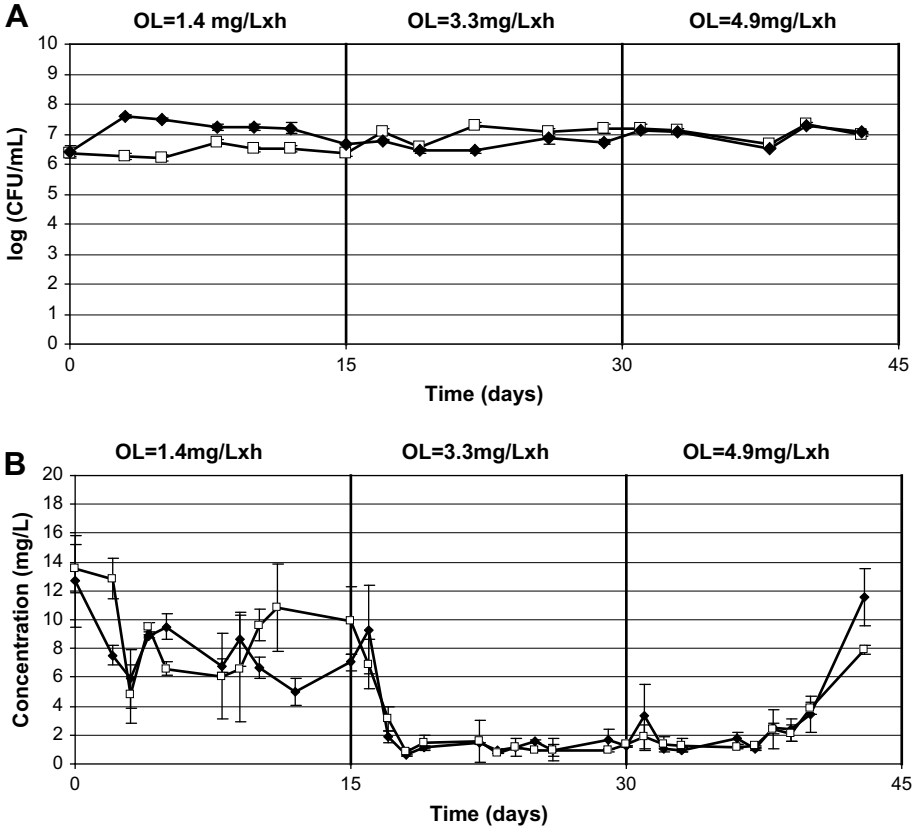

Fig. 4 – Cell concentration (A) and Igepal CO-520 concentration (B) as a function of the time in the R-SB (□) and in the R-GAC (◆) during the three continuous experiments.

respectively. Considering the dry weight of the support which constituted the SB and GAC fixed beds (255 and 273 g, respectively), the average total immobilized biomass available in the R-SB and the R-GAC was 379.95 and 103.74 mg, respectively. Thus the specific activity was 1.437 and 5.078 mg of Igepal overall removed for mg of biomass for R-SB and R-GAC respectively, suggesting that biomass immobilized on GAC could be more specialized in the removal of NPnEOs than that on R-SB.

3.4. *FISH*

FISH monitoring was performed on samples collected from the recycle line of each reactor at the end of the Igepal CO-520 batch experiment and during the three continuous mode experiments in order to evaluate the microbial composition of reactor in terms of both freely suspended and immobilized biomass under the different operation modes, assuming that the composition of the suspended cells reflects that of the immobilized biomass (Lazarova et al., 1998). The cells hybridizing to a specific probe (i.e. ALF968, BET42A, GAM, PSM G, Pseaer A and Steni) were quantified referring to a broader specificity probe, as indicated in Table 5; in addition, the table reports the microbial composition of Consortium A employed to inoculate the reactors as recently reported (Di Gioia et al., 2008b). FISH analyses performed on the recycled media at the end of the batch experiments evidenced in both reactors a net increase of *Betaproteobacteria* with respect to the composition of the inoculated consortium and a decrease of *Gammaproteobacteria*. The alfa, beta and gamma classes were almost equally represented after the batch experiment with Igepal CO-520 in the R-SB, whereas a predominance of *Alfa* and *Betaproteobacteria* with respect to *Gammaproteobacteria* was observed in the R-GAC. Major shifts in microbial population were observed when the reactors were operated in the continuous mode where, generally, bacteria positive to the ALF968 probe were less represented in both reactors at all organic loads assayed, although they were 45% of total bacteria in the inoculum and almost 30% in the reactors working in batch conditions. On the contrary, bacteria positive to the GAM probe were the most represented ones, in particular in the SB reactor where their amount was higher than 60% of total bacteria in all continuous experiments (Table 5). In addition to this general trend, differences were observed between the two reactors as a function of the organic load. In the SB reactor, *Alfaproteobacteria* were just above the limit of detection in the first continuous experiments, whereas their percentage increased by increasing the organic load up to 10–12%. On the contrary, *Betaproteobacteria* did not show significant changes by increasing the organic load. *Gammaproteobacteria* percentages were very high in all the experiments and an increase of *Gammaproteobacteria* positive to the Steni probe was evidenced by increasing the organic load (in the experiment at the higher organic load, they were more than 90% of total members of the *gamma* class). In the reactor packed with GAC, a slight increase of

Table 4 – Mass balance of the three continuous experiments performed (OL = organic load).

Reactor	Experiments	Volume of liquid treated (L)	Total Igepal entering (mg)	Igepal present in the effluent (i.e. not removed) (mg)	Igepal overall removed (mg)	Removal %	Igepal mineralized (mg)	Mineralization %	Mineralization of the removed amount %	Igepal adsorbed (mg)
R-SB (15 days)	1 (OL = 1.4 mg/L × h)	11.52	140.77	72.94	67.83	48.2	65.47	46.5	96.5	ND
R-GAC (15 days)		10.80	131.98	67.81	64.17	48.6	62.66	47.5	97.6	ND
R-SB (14 days)	2 (OL = 3.3 mg/L × h)	25.54	312.10	98.20	213.90	68.5	134.82	43.2	63.0	ND
R-GAC (14 days)		24.19	295.60	77.20	218.40	73.9	140.8	47.6	64.5	ND
R-SB (14 days)	3 (OL = 4.9 mg/L × h)	38.64	472.18	207.92	264.26	56.0	181.98	38.5	68.9	79.69
R-GAC (14 days)		36.29	443.46	199.2	244.26	55.1	169.67	38.3	69.5	75.67

Alfaproteobacteria and *Betaproteobacteria* percentages was observed by increasing the organic load, whereas *Gammaproteobacteria* were present at percentages higher than 60% in the first two continuous experiments and then slightly decreased in the third one. Among *Gammaproteobacteria*, *Pseudomonas* members were very abundant in the experiments at the lowest and the mid organic loads, but then they were totally overwhelmed by *Stenotrophomonas* at the highest organic load (Table 5). However, the sum of the percentages of *Alfa*, *Beta* and *Gammaproteobacteria* was close to 100 in all the experiments.

4. Discussion

Consortium A is an aerobic bacterial mixed culture, recently isolated from textile wastewater in the presence of 4-NP (Di Gioia et al., 2008b), capable of efficiently degrading 4-NP and, to a less extent, NPnEOs. The goal of this work was to investigate the NPnEO biodegradation potential of this consortium in aerobic packed bed biofilm reactors, in the perspective to develop an intensified and robust biofilm technology for the post-treatment of the effluents from activated sludge plants fed with NPnEO contaminated wastewater, containing not degraded NPnEOs, low ethoxylated NPnEOs and 4-NP. As it is reported that the immobilization support can influence the overall biodegradation potential of a immobilized culture, in particular when a mixed culture is used (Fava et al., 1996b; Bertin et al., 2004), biofilms of Consortium A were developed on two cell immobilization supports (GAC and SB) inside two identically configured packed bed bioreactors. The performances of the two reactors have been studied and compared through a sequence of batch and continuous experiments.

The employed consortium was able to colonize both supports, giving rise to efficiently degrading biofilms whose microbial components were present throughout the experiments. The degradation capability of the generated biofilms was initially checked in the presence of 4-NP, i.e. the substrate on which Consortium A was isolated, and the results obtained (complete mineralization of 4-NP entering the R-SB and of about 80% of that entering the R-GAC) clearly evidenced the efficiency of the consortium in the immobilized cell mode. Experiments were then performed with Igepal-CO 520 both in batch and in continuous mode. The developed biofilms degraded and mineralized Igepal CO-520 very efficiently, with complete degradation of 4-NP produced as intermediate metabolite (Table 3, Fig. 3B), and more extensively than when Consortium A was employed as suspended cells (Di Gioia et al., 2008b). The degradation capabilities confirmed the greater activity displayed by immobilized cell systems with respect to the freely suspended ones already outlined by several studies in the literature (Fava et al., 1996b; De Filippi and Lupton, 1998; Bertin et al., 2001, 2004; Annadurai et al., 2002). Furthermore, the obtained results allowed us to conclude that the biofilm systems developed had a greater bioremediation potential with respect to the identically configurated batch biofilm reactors previously set up with the pure culture *Pseudomonas* sp. BCb 12/3 (Bertin et al., 2007).

The similar degradation and mineralization percentages observed in the two reactors (Table 3) did not allow to attribute

Table 5 – Results of FISH experiments performed on samples of recycled medium at the end of the batch experiment performed with Igepal CO-520 as the carbon source and at the 11th day of each continuous experiment (OL = organic load).

Probes	Consortium A*	Batch experiment with Igepal CO-520		Continuous mode 1st experiment OL = 1.4 mg/L × h		Continuous mode 2nd experiment OL = 3.3 mg/L × h		Continuous mode 3rd experiment OL = 4.9 mg/L × h	
		R-SB	R-GAC	R-SB	R-GAC	R-SB	R-GAC	R-SB	R-GAC
ALF968/EUB	45.4 ± 3.2	32.3 ± 2.4	35.5 ± 7.2	0.3 ± 0.2	6.2 ± 3.4	12.1 ± 3.9	9.1 ± 0.7	10.8 ± 1.7	16.5 ± 5.6
BET42A/EUB	6.3 ± 1.0	25.2 ± 7.2	48.0 ± 2.8	13.0 ± 1.2	28.4 ± 0.5	15.7 ± 1.9	31.8 ± 5.2	16.7 ± 4.6	35.6 ± 1.43
GAM/EUB	44.7 ± 2.5	30.0 ± 4.2	11.4 ± 1.8	74.8 ± 1.0	65.3 ± 11.3	65.6 ± 4.1	61.3 ± 5.0	71.7 ± 4.9	50.4 ± 3.6
PSM G/GAM	98.2 ± 10.0	75.9 ± 0.6	84.5 ± 5.0	3.5 ± 0.1	60.3 ± 4.2	2.7 ± 1.9	56.0 ± 0.0	1.4 ± 0.8	0.6 ± 0.1
Pseaer A/GAM	11.7 ± 1.0	6.5 ± 1.0	5.5 ± 0.8	<0.2	3.8 ± 0.4	0.8 ± 0.4	8.8 ± 0.8	<0.2	0.2 ± 0.21
Steni/GAM	1.8 ± 0.5	<0.2	<0.2	75 ± 8.7	27.82 ± 4.8	83.8 ± 0.7	29.3 ± 0.0	93.9 ± 4.2	87.6 ± 12.1

* Results published in Di Gioia et al. (2008b).

to the two different supports a specific influence in the degradation process, differently from previously developed systems where a clear effect of the support on the reactor performances was observed (Fava et al., 1996b; Bertin et al., 2004).

Consortium A composition was found to greatly change upon immobilization (Table 5). However, the most important microbial shift observed, i.e. a strong increase of *Betaproteobacteria* at the end of the batch experiment with Igepal CO-520 with respect to the inoculated consortium, can be correlated not only to the different physiological state of the cells but also to the change in the carbon source provided. In fact, a significant enhancement in *Betaproteobacteria* had already been correlated to the supply of selected NPnEO congeners (Lozada et al., 2004), and has allowed the authors to conclude that *Betaproteobacteria* play an important role in NPnEO degradation, although bacterial genera belonging to this group are not often isolated from NPnEO contaminated environments (Lozada et al., 2006). Conversely, an important reduction of *Gammaproteobacteria*, which include bacterial genera usually involved in short chain NPnEO degradation (Di Gioia et al., 2004; Soares et al., 2003; Salvadori et al., 2006), was observed in the R-GAC with respect to the inoculated consortium, whereas the same phenomenon was poorly observed in the R-SB and this might have contributed to the slightly lower mineralization percentages observed in the R-GAC with respect to the R-SB (about 87 versus 97%, respectively).

The two reactors also behaved quite interestingly under continuous mode of operation, as evidenced by the Igepal CO-520 removed amounts and, in particular, by the mineralization percentages of the depleted pollutant (Table 4). The mineralization percentages decreased by increasing the organic load from 1.4 to 3.3 mg/L × h, although they remained at about 65% of the removed amount, and did not change significantly by further increasing the organic load (Table 4). The overall removal and degradation percentages obtained are remarkable, even though the mass balance data presented in Table 4 are not including NPnEO amount adsorbed by the immobilization supports and the amount of Igepal CO-520 that was integrated in the biomass, which was elsewhere estimated to be about 15% of the overall carbon supply (Corvini et al., 2004). In addition, the high bioremediation performance of the system developed is confirmed by the experimental evidence that no toxic metabolites were found to persist in the reactor effluents. However, the experiment performed at the highest organic load (4.9 mg/L × h) (Table 4) showed an increase of NPnEO concentration in the effluents and a decrease of CO_2 production starting from the 11th day of experiment in both reactors. The concentration of heterotrophic culturable bacteria occurring in the reactor recycle line was almost constant throughout the experiment (Fig. 4A); therefore, the reduced NPnEO mineralization efficiencies of the reactors cannot be ascribed to cell wash-out but to changes in the microbial composition of the biofilms probably induced by the high organic load during this experiment. Great variations in the composition of the microbial population in the reactors were in fact detected by FISH when the configuration was switched from batch to the continuous mode (Table 4). In particular, a clear dominance of *Gammaproteobacteria*, which reaches values close to 60–70% of total bacteria (Table 5), was generally observed, in parallel with a noteworthy decrease of *Alfaproteobacteria*, which were very abundant in the originally 4-NP enriched consortium, in agreement with their large diffusion in 4-NP contaminated environments (Corvini et al., 2004, 2005, 2006). Therefore, the change in the reactor configuration from batch to continuous mode of operation greatly influence the microbial population evolution, as already observed in the literature (Soares et al., 2006). Although *Alfa-*, *Beta-* and *Gammaproteobacteria* percentages did not change noticeably by increasing the organic load operated in the reactors, conversely, remarkable changes were evidenced inside the *Gammaproteobacteria* class: at the highest organic load *Pseudomonas* members were very low and the majority of *Gammaproteobacteria* were found to be positive to *Stenotrophomonas* probe. The increase of *Stenotrophomonas* strains is in agreement with their involvement in the degradation of low ethoxylated nonylphenols and in particular of short chain NPnEOs both in activated sludge plants and in engineered bioremediation process (Di Gioia et al., 2004; Soares et al., 2003; Salvadori et al., 2006), but the extremely high concentration of strains belonging to this genus to the detriment of other strains with a broader degradation spectrum such as *Pseudomonas* may have been unfavourable to the reactor performances under continuous Igepal CO-520 supply and may account for the reduced performances in term of NPnEO depletion and CO_2 production observed at the highest organic load (Fig. 4B). Furthermore, the toxicity of

phenolic derived compounds on microorganisms operating in aerobic wastewater treatment plants, among which *Pseudomonas*, at high organic load is well known in the literature (Marrot et al., 2006); therefore microorganism inhibition by the high organic load reached in the last experiment is also to be considered as a possible cause of the reduced reactor performances.

5. Conclusion

- Consortium A was found to be able to colonize both SB and GAC, giving rise to biologically active biofilms whose microbial members were present throughout the experiments although at overall and relative concentrations changing in the different operative conditions. This may reflect consortium adaptation to variations both in the supplied carbon source (i.e. from 4-NP to NPnEO) and in the reactor operation mode (i.e. from batch to continuous mode).
- The two lab-scale biofilm systems developed in this study showed very promising NPnEO degradation and mineralization rates and yields both under batch and continuous mode of operation.
- In the continuous mode, a remarkable stability of the developed biofilms was observed for feeding loads in the range 1.4–3.3 mg/L × h, which are of relevance for the tertiary treatment of effluents of conventional activated sludge treatment plant fed with NPnEO contaminated wastewater.
- The possibility of decontaminating NPnEO containing effluents with continuous biofilm reactors based on Consortium A immobilized on SB or GAC was therefore preliminary demonstrated in this study.

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