

Selection and identification of a bacterial community able to degrade and detoxify *m*-nitrophenol in continuous biofilm reactors



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ABSTRACT

Nitroaromatics are widely used for industrial purposes and constitute a group of compounds of environmental concern because of their persistence and toxic properties. Biological processes used for decontamination of nitroaromatic-polluted sources have then attracted worldwide attention. In the present investigation *m*-nitrophenol (MNP) biodegradation was studied in batch and continuous reactors. A bacterial community able to degrade the compound was first selected from a polluted freshwater stream and the isolates were identified by the analysis of the 16S rRNA gene sequence. The bacterial community was then used in biodegradation assays. Batch experiments were conducted in a 2 L aerobic microfermentor at 28 °C and with agitation (200 rpm). The influence of abiotic factors in the biodegradation process in batch reactors, such as initial concentration of the compound and initial pH of the medium, was also studied. Continuous degradation of MNP was performed in an aerobic up-flow fixed-bed biofilm reactor. The biodegradation process was evaluated by determining MNP and ammonium concentrations and chemical oxygen demand (COD). Detoxification was assessed by *Vibrio fischeri* and *Pseudokirchneriella subcapitata* toxicity tests. Under batch conditions the bacterial community was able to degrade 0.72 mM of MNP in 32 h, with efficiencies higher than 99.9% and 89.0% of MNP and COD removals respectively and with concomitant release of ammonium. When the initial MNP concentration increased to 1.08 and 1.44 mM MNP the biodegradation process was accomplished in 40 and 44 h, respectively. No biodegradation of the compound was observed at higher concentrations. The community was also able to degrade 0.72 mM of the compound at pH 5, 7 and 9. In the continuous process biodegradation efficiency reached 99.5% and 96.8% of MNP and COD removal respectively. The maximum MNP removal rate was 37.9 g m⁻³ day⁻¹. Toxicity was not detected after the biodegradation process.

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1. Introduction

The presence of nitroaromatic compounds in the environment is a consequence of anthropogenic activities (Schenzle et al., 1997), as they are some of the most widely used industrial organic compounds. Among these xenobiotic compounds, nitrophenols are frequently used as raw materials or intermediates in the manufacture explosives, pharmaceuticals, pesticides, dyes, wood preservatives and rubber chemicals (She et al., 2005). Due to their intense yellow color and pH reactivity nitrophenols are often used directly in titrations as indicators. The isomers *p*-Nitrophenol

(PNP) and *o*-nitrophenol (ONP) coupled to others substrates are also used in monitoring enzyme activity. Pharmaceutical and agricultural uses of PNP include the synthesis of acetaminophen and the organophosphorus pesticides parathion and methylparathion (Zylstra et al., 2000). Because of their widespread use these compounds have been detected in aquatic environments, including river waters and industrial effluents (Iwaki and Hasegawa, 2007), and pesticides-treated soils (Marvin-Sikkema and de Bont, 1994). However, the stability, persistence and toxicity that make these compounds valuable to industry also make them hazardous when they are released into the environment (Nishino et al., 2000). Increased public concern about toxicity and the risks associated with nitroaromatic compounds encouraged the development of cost-effective technologies for their remediation (Kulkarni and Chaudhari, 2007). Biological processes used for decontamination of nitroaromatic-polluted sources have then attracted

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worldwide attention (Alexander, 1999). While ONP and PNP biodegradation processes have been exhaustively studied, information about *m*-nitrophenol (MNP) biodegradation is scarce. In fact, several bacterial strains able to degrade aerobically ONP and PNP have been previously reported and the biodegradation pathways characterized, such as *Alcaligenes* sp. NyZ215 (Xiao et al., 2007), *Arthrobacter protophormiae* RKJ100 (Chauan et al., 2000), *Burkholderia cepacia* RKJ200 (Prakash et al., 1996), and *Pseudomonas* sp. (Zhang et al., 2012), among others. Although some MNP-degrading strains such as *P. putida* (Meulenbergh et al., 1996) and *Cupriavidus necator* JMP134 (Schenzle et al., 1997) have been previously reported, there is a lack of information about the use of these microorganisms for the degradation of this compound in liquid effluents. Moreover, considering that the high toxicity of MNP to aquatic organisms such as algae, invertebrates and fishes, has been demonstrated by several studies (WHO, 2000), detoxification of the compound after biodegradation processes should be evaluated. The aims of this investigation are: (a) to select MNP-degrading microorganisms from a polluted source, (b) to study the MNP biodegradation kinetic in batch reactors, (c) to evaluate the influence of abiotic factors in the biodegradation process and (d) to use the selected degrading microorganisms for the degradation and detoxification of MNP in a continuous-flow biofilm reactor.

2. Materials and methods

2.1. Selection and identification of MNP-degrading microorganisms

Enrichment was performed in an aerated batch reactor with an effective volume of 1 L, by exposing a water sample from the Reconquista River (located in Buenos Aires, Argentina) to 0.36 mM of MNP as the sole source of carbon and nitrogen. Once a consistent depletion of the compound was achieved, an aliquot of 1 mL of this enrichment was transferred to a 250 mL Erlenmeyer flask containing 100 mL of a synthetic minimal medium (pH 7) described by Korol et al. (1989) with MNP (0.36 mM) as the sole carbon and nitrogen source, and successively subcultured in a rotatory shaker at 200 rpm and 28 °C. The medium was modified by removing the ammonium salt to obtain a mineral medium with no nitrogen source other than the compound under study. The bacterial community obtained by this selection procedure was then employed as inoculum in biodegradation tests in batch and continuous reactors.

Individual strains from the bacterial community were isolated by streaking onto tryptone soy agar medium (Merck, Darmstadt, Germany) supplemented with 0.36 mM of MNP for further identification. In order to identify the individual strains Gram staining and molecular techniques were employed. Molecular techniques were described previously (González et al., 2012). They consisted in the amplification of the 16S ribosomal RNA (rRNA) gene by the polymerase chain reaction (PCR) and the sequencing of the amplified fragments. For amplification the following primers (5′–3′) were used: 16SR: GYTACCTTGTTACGACTT and 16SF: AGAGTTT-GATCMTGGCTCAG. Amplified fragments were purified with the QIAquick PCR Purification Kit (QIAGEN, Duesseldorf, Germany) according to manufacturer's instructions, and sequenced using an ABI Prism DNA 3700 sequencer (Applied Biosystems, California, USA). Finally, nucleotide sequences were compared with databases using the NCBI's Basic Local Alignment Search Tool (BLAST).

2.2. Chemicals

MNP (99% purity) was purchased from Sigma-Aldrich (Steinheim, Germany). All the other chemicals were of analytical reagent grade and purchased from Mallinckrodt Chemical (St. Louis, USA)

and Merck (Darmstadt, Germany). The MNP solution was aseptically prepared by dissolving the necessary amount in sterile 0.1 N NaOH.

2.3. Preparation of stock cultures for biodegradation assays

In order to obtain stock cultures for batch and continuous experiments, the bacterial community was pre-exposed to the compound by inoculating 100 mL of the synthetic minimal medium described in Section 2.1. supplemented with 0.72 mM of MNP as the sole source of carbon and nitrogen. Incubation was carried out in a rotatory shaker at 200 rpm and 28 °C until the compound was not detected in the medium.

2.4. Degradation of different nitroaromatic compounds by the bacterial community

Several nitroaromatic compounds were tested as the sole carbon source for the bacterial community. These experiments were carried out in Erlenmeyer flasks with 100 mL of minimal medium (pH 7) supplemented with 20 mg L⁻¹ of *p*-nitrobenzoate, *o*-nitrophenol, *p*-nitrophenol, 2,4-dinitrophenol or picric acid. An aliquot of 1 mL of the stock culture was used to inoculate the flasks. Incubation was performed in a rotatory shaker at 200 rpm and 28 °C. After 7 days of incubation degradation of each substrate was evaluated.

2.5. Degradation of MNP by the individual strains

The ability of the individual strains to degrade MNP was also studied. The assays were performed in Erlenmeyer flasks with 100 mL of minimal medium (pH 7) supplemented with 0.36 mM of the compound and inoculated with 1 mL of a cell suspension of each individual strain. The flasks were incubated in a rotatory shaker at 200 rpm and 28 °C, for 7 days. At the end of the experiment the remaining concentration of MNP was determined.

2.6. Biodegradation assays in batch reactors

MNP biodegradation kinetic was studied in a New Brunswick Multigen™ microfermentor aerobically operated at 28 °C, with agitation (200 rpm), and an effective volume of 1250 mL of the same medium supplemented with 0.72 mM of MNP as carbon and nitrogen source. An aliquot of 12.5 mL of the stock culture was used to inoculate the system, so as to obtain a cell concentration of 10⁶ cells mL⁻¹. During the incubation period samples of 10 mL were aseptically removed from the microfermentor at appropriate intervals in order to determine the remaining MNP concentration and evaluate microbial growth.

In order to evaluate the influence of abiotic factors on the biodegradation process two experiments were performed, with different concentrations of the compound and pH values. These experiments were carried out in Erlenmeyer flasks with 100 mL of minimal medium (pH 7) supplemented with 0.72, 1.08 or 1.44 mM of MNP, or 100 mL of minimal medium at pH 5, 7 or 9, supplemented with 0.72 mM of MNP. The initial pH of the medium was adjusted to 5 and 9 with concentrated phosphoric acid or 0.1 N NaOH respectively. An aliquot of 1 mL of the stock culture was used to inoculate each Erlenmeyer flask, so as to obtain a cell concentration of 10⁶ cells mL⁻¹. Incubation was performed in a rotatory shaker at 200 rpm and 28 °C. Samples were aseptically removed from the flasks at appropriate intervals in order to determine the remaining MNP concentration and evaluate microbial growth.

Two replicates of each biodegradation assay were carried out. Average values of MNP concentration and bacterial count were

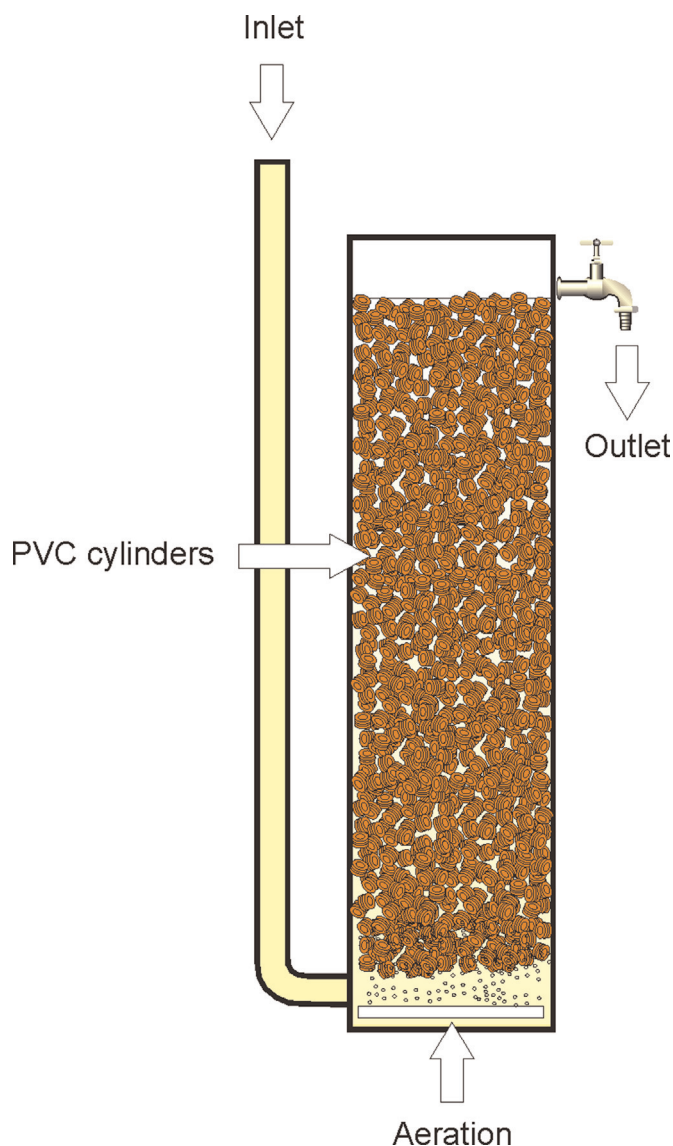


Fig. 1. Continuous up-flow fixed-bed biofilm reactor employed for *m*-nitrophenol degradation by the bacterial community.

plotted in function of time. The bacterial growth curve was analyzed by non-linear regression and fitted to an exponential growth model, using the GraphPad Prism software (version 5.0).

Uninoculated batch experiments were also conducted in order to exclude the possibility of abiotic losses of the compound during incubation.

2.7. Biodegradation assay in an aerobic continuous-flow reactor

Continuous biodegradation assay was performed in an aerated up-flow fixed-bed reactor constructed from acrylic (50 cm in height \times 15 cm in internal diameter), with an effective volume of 7.7 L (Fig 1). The reactor was filled with PVC cylinders (15 mm of length \times 5 mm of internal diameter) which were employed as a support for immobilizing the bacterial biofilm (Qureschi et al., 2001).

Before start-up, the reactor was operated in fed-batch mode during two months in order to promote the adherence of MNP-degrading microorganisms to the PVC cylinders. The feeding of the reactor was prepared by dissolving the compound in free chlorine tap water supplemented with 32 mg L^{-1} of K_2HPO_4 . The reactor was inoculated with 100 mL of the stock culture of MNP-degrading

microorganisms.

Once the PVC cylinders were colonized by the biofilm, the reactor was continuously operated with an inlet concentration of MNP from 0.29 to 0.94 mM under non-sterile environmental conditions, at room temperature. The influent flow rate was 2.5 L day^{-1} and the hydraulic retention time (HRT) was 7.8 h.

In order to estimate the abiotic loss of the compound, a control reactor was also constructed. It was filled with uninoculated PVC cylinders, and was fed with the same synthetic effluent as that of the inoculated reactor.

2.8. Analytical methods

To determine the remaining MNP concentration, samples from batch and continuous reactors were centrifuged at 4000 rpm during 10 min and analyzed by UV spectrophotometry (Metrolab UV 1700 Spectrophotometer), by measuring the absorbance at 290 nm. The absorbance values were then extrapolated on a calibration curve. To corroborate the complete degradation of the compound under study, samples from the batch and the continuous reactors were also analyzed by reverse-phase high performance liquid chromatography (RP-HPLC). Analyses were conducted on a Waters Alliance 2695 chromatograph, equipped with a Waters 996 photodiode array detector. A LiChroCART[®] (125 \times 4 mm) cartridge packed with LiChrospher[®] 60 RP-select B (5 μm) was used, coupled to a precolumn LiChrospher[®] (4 \times 4 mm) 60 RP-select B, at 35 $^\circ\text{C}$. Water plus 1% acetic acid was used as mobile phase A, while water:methanol (50:50, v/v) plus 1% acetic acid was used as mobile phase B, at a flow of 1.0 mL min^{-1} . The concentration of ammonium was determined by the phenate method (APHA, 2012) and the concentration of nitrite by the colorimetric method of diazotization (APHA, 1998). Chemical oxygen demand was determined by the closed reflux method according to APHA (2012). Determination of cell viability in batch and continuous reactors was performed by the plate count method in triptone soy agar plates (APHA, 2012) and the biofilm attached to the support material was observed by scanning electronic microscopy (SEM). Samples for SEM analysis were taken from the reactor and fixed according to the technique described by Varesche et al. (1997). Fixation was carried out at 4 $^\circ\text{C}$ for 10 h using 0.1 M phosphate buffer (pH 7.3) added with 1% glutaraldehyde. After fixation, samples were rinsed three times in 0.1 M phosphate buffer (pH 7.3) and dehydrated gradually by successive immersions in ethanol solutions of increasing concentration (50%, 70%, 80%, 90% and 95%). The samples were then washed three times in 100% ethanol and dried for 2 h at 30 $^\circ\text{C}$. Finally samples were metalized with a 40:60 gold-palladium mixture and attached to the supports of a Philips XL 30 scanning electron microscope for their subsequent observation.

2.9. Toxicity tests

Bioassays of acute toxicity were performed to assess detoxification. Toxicity was evaluated in samples taken at the influent and effluent of the continuous reactor. The luminescent bacterium *Vibrio fischeri* and the green algae *Pseudokirchneriella subcapitata* were used as test organisms.

2.9.1. Microtox[®] test

The short term toxicity test Microtox[®] was conducted in a Microtox[®] Model 500 Toxicity Analyzer (Azur Environmental, Carlsbad, CA, USA), by exposing for 15 min the luminescent bacterium *Vibrio fischeri* to different sample dilutions, at $15 \pm 1 \text{ }^\circ\text{C}$, according to ISO 11348-3 (1998). The test organism *V. fischeri* (strain NRRLB-11177) was purchased as a lyophilized form from Strategic Diagnostic Inc. (Carlsbad, CA, USA). After the exposure

period, acute toxicity was estimated by determining effective concentration 50 (EC_{50}), concentration which produces a 50% reduction in bacterial luminescence relative to the test control. The EC_{50} values were calculated using the Microtox Omni[®] software. It should be noted that the sensitivity of the test organism was evaluated before the test by using phenol as the reference toxic compound.

2.9.2. Alga test

P. subcapitata growth inhibition tests were performed in 96-well microplates, according to the technique described by Blaise (1991) at 24 ± 2 °C, under continuous cool-white illumination. Four replicates for control and different sample dilutions with an initial cell density of 5×10^4 cells mL⁻¹ were used. Algal cell concentration was estimated by measuring the absorbance at 650 nm after 96 h incubation. The effective concentrations inducing 50% effect (EC_{50}) were calculated using the sigmoidal equation in the Origin Program (version 7.5). The program plots the percentage inhibition of the algal growth in each sample dilution with respect to a control. The reference chemical potassium dichromate was employed as a positive control to ensure the sensitivity of the organisms.

3. Results and discussion

3.1. Selection and identification of MNP-degrading microorganisms

A microbial community able to degrade MNP was isolated from the Reconquista River, a severely polluted freshwater stream in the province of Buenos Aires, Argentina. The community is composed of three Gram negative, non-fermentative bacterial strains (strains MNP1, MNP2 and MNP3), identified as *Achromobacter* sp., *Pseudomonas* spp. and *Variovorax* spp. by the analysis of the 16S rRNA gene sequence, with an identity of 99%, 99%, and 100% respectively. Bacteria belonging to the genera *Pseudomonas*, *Rhodococcus* and *Cupriavidus* have been reported previously as MNP-degraders (Meulenberg et al., 1996; Navrátilová et al., 2005; Kristanti et al., 2012; Chi et al., 2013). However, this is the first report about a *Variovorax* strain able to degrade this compound.

After several subcultures in MM supplemented with the compound, the same three strains were isolated on several occasions and identification was confirmed by repeating the 16S rRNA gene sequencing. This result demonstrates that the bacterial community is stable over time.

The ability of the individual strains to use MNP as the sole source of carbon and nitrogen was also studied. *Pseudomonas* sp. and *Variovorax* sp. were able to degrade MNP as individual strains, while *Achromobacter* sp. did not show this capability. However biodegradation efficiency of the community was higher than that of the individual strains (data not shown). It has also been suggested that from an applied perspective, using a microbial consortium rather than a pure culture in biodegradation processes is more advantageous as it provides the metabolic diversity and robustness needed for field applications (Tyagi et al., 2011). For these reasons biodegradation assays in batch and continuous reactors were performed with the whole community. The community was also able to degrade the nitroaromatic compound *p*-nitrobenzoate, but did not degrade *o*-nitrophenol, *p*-nitrophenol, 2,4-dinitrophenol nor picric acid.

3.2. Biodegradation assays in batch reactors

MNP degradation kinetic by the bacterial community was studied in batch reactors. Fig. 2 shows that the community was able to degrade 0.72 mM of MNP in 32 h. In order to confirm the

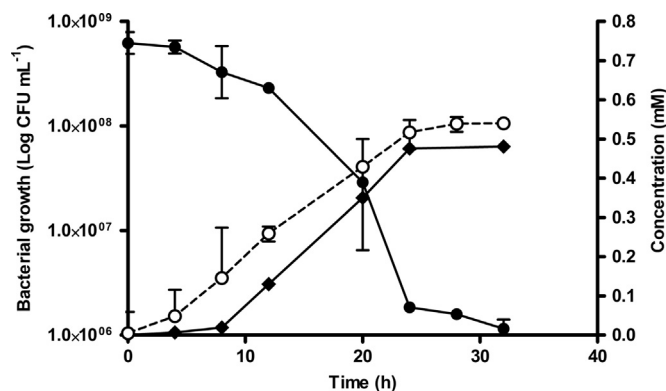


Fig. 2. Biodegradation kinetic of 0.72 mM of *m*-nitrophenol at pH 7 in batch reactor. (—●—) *m*-Nitrophenol concentration; (---●---) ammonium concentration; (---○---) bacterial growth.

complete degradation of MNP, samples taken at the beginning and the end of the biodegradation process were analyzed by RP-HPLC. *m*-Nitrophenol was not detected after the biodegradation process. MNP and COD removals were higher than 99.9% and 89.0%, respectively (Table 1). The concomitant release of ammonium was observed as a result of MNP degradation, while nitrite was not detected as a biodegradation product. Similar results were obtained by Zhao and Ward (2001), who observed a release of ammonium from MNP degradation by *P. putida*, but no production of nitrite. It has been reported that aerobic biodegradation pathways of nitrophenols include those involving either initial reduction of the nitro group prior to removal from the aromatic ring or direct removal as nitrite via oxygenolytic cleavage (Zylstra et al., 2000). Two major pathways have been proposed for bacterial degradation of PNP: In the first one, PNP is converted to 1,4-benzoquinone (BQ) by the enzyme 4-nitrophenol-4-monooxygenase with the release of nitrite ion. Then BQ is reduced to hydroquinone (HQ), which is further cleaved. An analogous pathway was described for the biodegradation of ONP. In the second pathway, PNP is initially hydroxylated to nitrocatechol (NC), which is further monooxygenated to 1,2,4-benzenetriol (BT) with release of nitrite ion (Arora et al., 2014). While degradation of the isomers *o*- and *p*-nitrophenol occurs by direct removal of nitrite, the biodegradation pathway of MNP involves the initial reduction of the nitro group and the subsequent release as ammonium. Two metabolic pathways have been described. In both of these pathways the initial reaction is a partial reduction of the nitro group to a hydroxylamino moiety. Then, *m*-hydroxylaminophenol is converted to aminohydroquinone (Schenzle et al., 1997) or to 1,2,4-benzenetriol with the release of ammonium (Meulenberg et al., 1996). The later pathway was also described for the nitroaromatic compound *p*-nitrobenzoate (Groenewegen et al., 1992). This could be the reason why the community was able to degrade *p*-nitrobenzoate but not *o*- and *p*-nitrophenol. It should be noted that the concentration of the released ammonium was not stoichiometric (66.9% of the initial MNP concentration) due to the fact that the community not only employs the compound as a carbon source, but also as nitrogen source.

Considering the variability of liquid effluents, it is essential to study the behavior of MNP-degraders under several environmental conditions. For this purpose, in the present investigation experiments with different concentrations of the compound and pH values were performed. In the assay with initial MNP concentrations of 1.08 and 1.44 mM biodegradation time increased to 40 and 44 h respectively (Fig. 3). Similar results were obtained by Gemini et al. (2006) for the biodegradation of increasing concentrations of *p*-nitrophenol. No lag phase was observed at MNP concentrations of 0.72 and 1.08 mM, and a lag phase of 8 h

Table 1Efficiency of *m*-nitrophenol degradation by the bacterial community in batch and continuous reactors.

Parameter	Batch reactor			Continuous reactor		
	Initial	Final	% Removal	Influent	Effluent	% Removal ⁺
MNP concentration (mM)	0.75	ND*	> 99.9	0.41	< 0.01	99.5
COD (mg L ⁻¹)	183.2	20.0	89.1	92.6	< 3.0	> 96.8

* ND: Not detected.

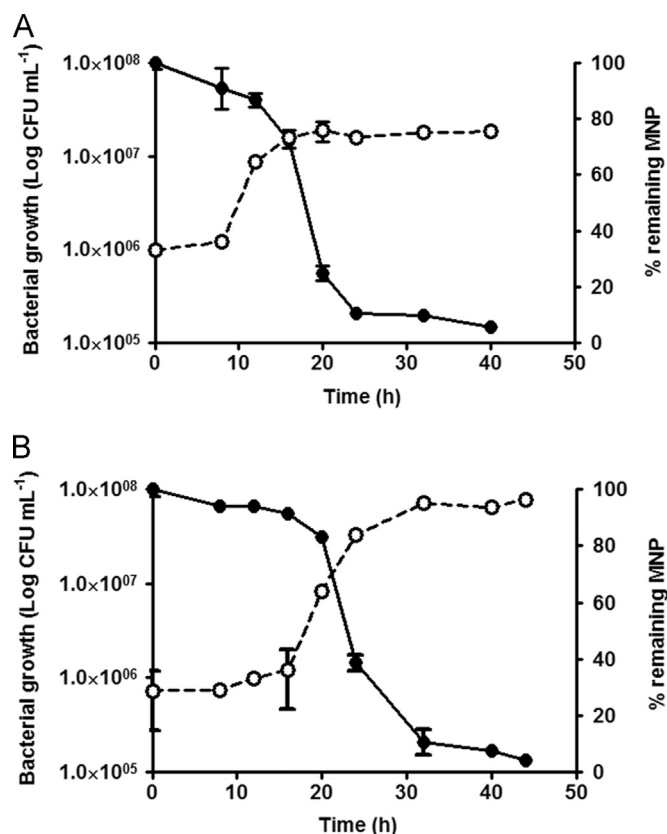
⁺ Maximum efficiency.

Fig. 3. *m*-Nitrophenol biodegradation kinetics at different initial concentrations of the compound in batch reactors. (A) 1.08 mM; (B) 1.44 mM. (—●—) *m*-Nitrophenol concentration; (---○---) bacterial growth.

appeared with the higher concentration of the compound (Fig. 3). Sahoo et al. (2011) observed during *p*-nitrophenol (PNP) degradation by *Arthrobacter chlorophenolicus* that the presence and length of the lag phase depend on the initial concentration of PNP. This effect was also described by Navrátilová et al. (2005) during the degradation of increasing concentrations of 4-nitrocatechol by *Rhodococcus wratislaviensis*. These results show that the initial concentration of the compound affects the biodegradation kinetic.

Modifications in the pH of the culture medium also affected MNP degradation by the bacterial community. Fig. 4 shows that at pH 5 the biodegradation of 0.72 mM of MNP was completed in 48 h. It was mentioned by Cabral et al. (2003) that the lower the pH value, greater the toxicity of weak acids. Kulkarni and Chaudhari (2006) observed that acidic pH inhibits PNP degradation by *P. putida*. The authors suggested that this effect could be attributed to the growth inhibition of the strain and to the toxicity of the compound, which increase at acidic pH. The MNP-degrading community however was able to grow at the three pH values in the absence of *m*-nitrophenol (data not shown). This suggests that MNP degradation time increased at pH 5 because of the toxicity of the compound at this pH value.

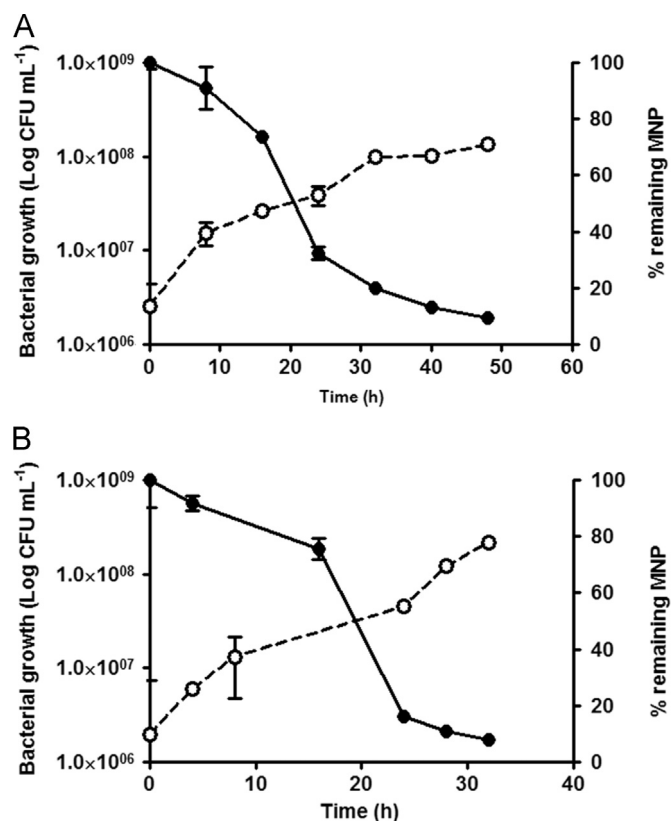


Fig. 4. *m*-Nitrophenol biodegradation kinetics at different pH values of the medium in batch reactors. (A) pH 5; (B) pH 9. (—●—) Influent; (---○---) effluent.

3.3. Biodegradation assays in a continuous biofilm reactor

Because of the serious concern caused by the environmental impact of nitroaromatic compounds, much attention has been given to their degradation in soils, waters and wastewaters. However, the most extensively studied nitroaromatics are 4-nitrophenol, 2,4-dinitrophenol, nitrobenzene and TNT (Marvin-Sikkema and de Bont, 1994). Moreover, most of the previous research on MNP biodegradation focuses mainly on the elucidation of the degradation pathways by the MNP-degrading microorganisms (Schenzle et al., 1997, 1999; Zhao and Ward, 2001). Therefore, there is a lack of information about the use of degrading microorganisms for the treatment of liquid effluents that contain *m*-nitrophenol. In this investigation the selected MNP-degrading microorganisms were employed to simulate an aerobic treatment for the removal of MNP from a synthetic effluent.

Toxic shock is usually observed in wastewater treatment plants as a consequence of the presence of hazardous organic pollutants (Wagner and Loy, 2002). For this reason, fixed-bed biofilm reactors are extensively used for the removal of organic pollutants from wastewaters (Guo et al., 2010). These reactors offer some advantages for the removal of this kind of compounds, such as a high

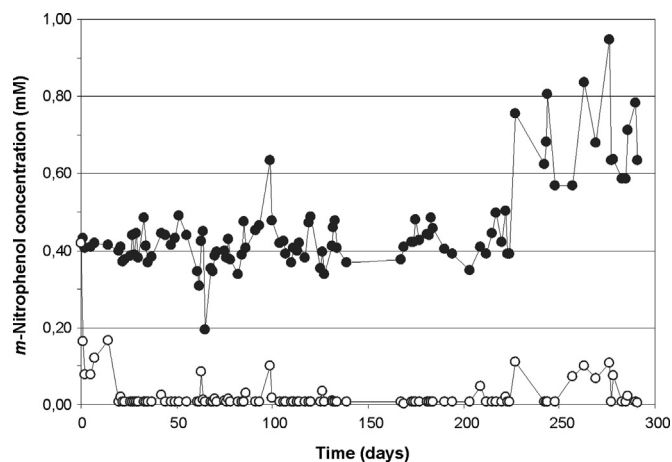


Fig. 5. *m*-Nitrophenol concentration at the influent and effluent of the continuous reactor. (—●—) Influent; (---○---) effluent.

biomass concentration and the protective effect of the biofilm that prevent the inhibition of degrading microorganisms (Perron and Welander, 2004). In the present investigation the use of a continuous up-flow fixed-bed biofilm reactor successfully prevented this toxic effect, since no inhibition of degrading microorganisms was observed. Fig. 5 shows the MNP concentrations at the influent and effluent of the reactor. After 20 days of continuous operation the biodegradation process reached more than 95% of MNP removal and the process efficiency remained approximately constant throughout the operating period of 290 days. Maximum efficiency of the biodegradation process was 99.5% and 96.8% of MNP and COD removal respectively and took place at an inlet MNP concentration of 0.41 mM (Table 1). The maximum MNP removal rate was $37.9 \text{ gm}^{-3} \text{ day}^{-1}$. Chromatography results of samples taken from the influent and effluent of the continuous reactor showed the complete disappearance of the compound. No abiotic losses of MNP were observed in the control reactor (data not shown). In addition, the results of heterotrophic bacteria counts carried out in both the support material and the liquid effluent of the reactor in order to determine the viability of the MNP-

degrading community showed a high biomass concentration, mostly in the support material ($4.4 \times 10^8 \text{ CFU g}^{-1}$) than in the liquid effluent ($2.0 \times 10^6 \text{ CFU mL}^{-1}$). The strains from the bacterial community predominated in the bacterial counts and no other MNP-degraders could be isolated from the samples of the biofilm reactor, suggesting that the community was the only responsible of MNP biodegradation despite the non-sterile conditions of operation. The biofilm in the support material was observed by scanning electron microscopy (Fig. 6).

The good performance of continuous biofilm reactors for the treatment of liquid effluents that contain toxic compounds have also been demonstrated by Ray et al. (1999), who studied PNP biodegradation in a continuous fixed bed biofilm reactor. The reactor could remove up to $6428.6 \text{ gm}^{-3} \text{ day}^{-1}$ of the compound, which was almost completely degraded. It was also reported by Bajaj et al. (2008) a removal rate of $2920 \text{ g phenol m}^{-3} \text{ day}^{-1}$ in a similar reactor treating a synthetic effluent containing the compound.

3.4. Toxicity tests

The use of toxicity tests enables a more accurate evaluation of a biodegradation process because these bioassays allow to exclude the possibility of formation of toxic metabolites and to evaluate the effects of the treated effluents on different aquatic organisms. For this reason bioassays of acute toxicity were performed in samples taken from the influent and effluent of the aerobic continuous reactor in order to ensure detoxification after the biodegradation of MNP. Results showed high toxicity levels for *P. subcapitata* in the samples taken from the influent of the reactor ($\text{EC}_{50-96 \text{ h}}=9.41\% \text{ v/v}$), while the toxicity was considerably lower to *V. fischeri* ($\text{EC}_{50-15 \text{ min}}=18.20\% \text{ v/v}$) Apart from this difference in the sensitivity of the test organisms to *m*-nitrophenol, toxicity was not detected in the effluent of the reactor for any of the test organisms.

Most of the research on biodegradation processes evaluates the efficiency of these processes only based on determinations of the compounds under study by diverse analytical techniques. In this investigation not only the removal of MNP was determined, but also the absence of toxic metabolites was confirmed by toxicity

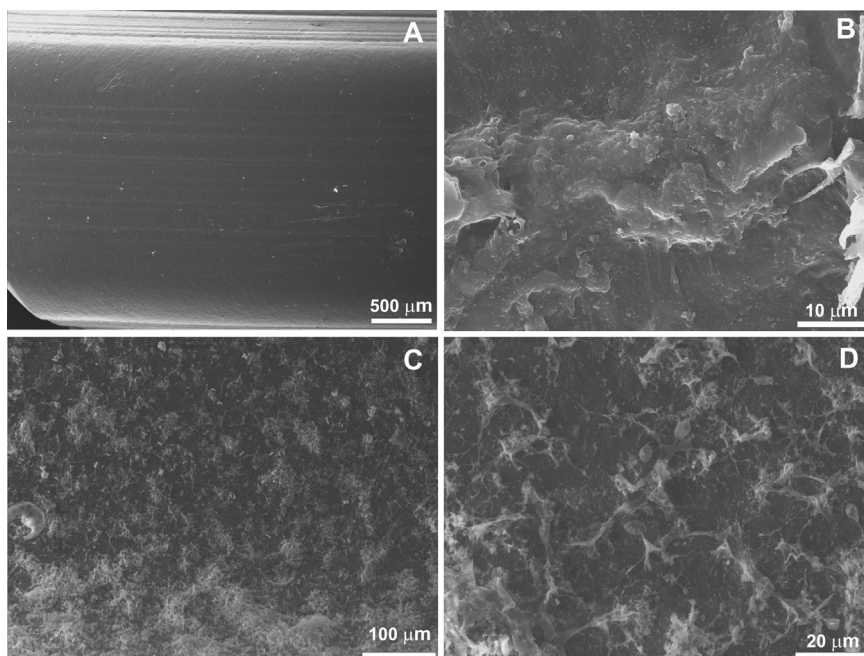


Fig. 6. Scanning electron microscopy of the support material from the continuous reactor. (A) Before inoculation, (B, C and D) during operating period of the reactor.

bioassays, a very important aspect rarely considered in biodegradation studies.

4. Conclusions

In this investigation a bacterial community able to use *m*-nitrophenol as the sole carbon and nitrogen source with concomitant release of ammonium was isolated from a polluted freshwater stream. The community efficiently degraded the compound under diverse environmental conditions, such as different concentrations of MNP and pH values. These results are relevant for industrial applications taking into account the variability of liquid effluents. MNP-degrading microorganisms were then employed to set up a continuous biofilm reactor with high efficiency in *m*-nitrophenol degradation. Not only MNP removal but also detoxification of the compound was demonstrated as a result of *m*-nitrophenol biodegradation. It is remarkable that evaluation of toxicity is essential in the characterization of hazardous effluents, in order to protect the aquatic environment. Therefore it is necessary to include the ecotoxicological approach to biodegradation studies.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ecoenv.2015.07.029>.

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