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**Research Article:** A continuous biofilm reactor with a community composed of *Pseudomonas* sp., *Achromobacter* sp., and *Sphingopyxis* sp. reached high efficiency in *p*-chloroaniline (PCA) removal. The absence of organic metabolites from PCA biodegradation and detoxification were confirmed after biodegradation.

**Biodegradation of *p*-Chloroaniline and Ammonium Removal in Continuous Biofilm Reactors**

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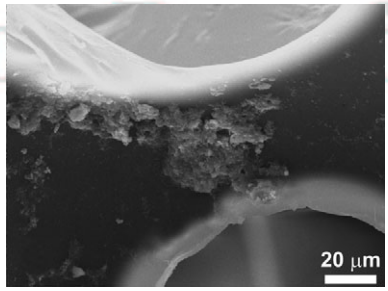

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Research Article

# Biodegradation of *p*-Chloroaniline and Ammonium Removal in Continuous Biofilm Reactors

In this investigation, we isolated from an industrial effluent a bacterial community capable of utilizing *p*-chloroaniline (PCA) as the sole source of carbon and nitrogen. The isolates were identified as *Pseudomonas* sp., *Achromobacter* sp., and *Sphingopyxis* sp. The bacterial community was employed to set up a continuous system for PCA degradation and ammonium removal. The system consists in two sequential aerobic fixed-bed reactors, for PCA biodegradation and nitrification respectively, and an anoxic reactor for denitrification. Biodegradation process was evaluated by chemical analysis of PCA, chloride and ammonium, and bacterial count. Nitrification and denitrification processes were evaluated by chemical analysis of ammonium, nitrite and nitrate, and nitrifying bacteria count. Bioassays of acute toxicity using the standard organisms *Vibrio fischeri*, *Pseudokirchneriella subcapitata*, and *Daphnia magna* were performed in order to assess detoxification. An average efficiency of 88.5%, expressed as PCA removal, was achieved in the biodegradation process. The maximum PCA removal rate reached 11.7 g/m$^3$/day. PCA degradation was associated with the release of chloride and ammonium. Ammonium was completely removed by the nitrification/denitrification process. Toxicity was not detected after the biodegradation process.

**Keywords:** Aquatic environment; Chlorinated aromatic amines; Detoxification; Herbicides; Microorganisms

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## 1 Introduction

Chlorinated aromatic amines are metabolites from the microbial transformation of acylanilide, phenylcarbamate, and phenylurea herbicides [1]. They are also important intermediates in the manufacture of several organic chemicals and polymers, including polyurethanes, rubber, azo dyes, pharmaceutical products, photographic chemicals, varnishes, and herbicides [2]. Thus, contamination of the aquatic environment by these compounds can be the result of the release of several effluents from the chemical industry into surface water streams. *p*-Chloroaniline (PCA), also designated as 4-chloroaniline, with molecular formula $C_6H_4ClNH_2$, is persistent and toxic to fishes (www.pbtprofiler.net/ChemDetails.asp?I=0) and other aquatic organisms, such as daphnids and green algae (www.who.int/ipcs/publications/cicad/en/cicad48.pdf). This compound is also dangerous to human health, as it has been classified as possibly carcinogenic to humans (group 2B) by the International Agency for Research on Cancer (http://monographs.iarc.fr/ENG/Monographs/vol57/mono57.pdf). It is considered as a priority pollutant by the Agency for Toxic Substances and Disease Registry (ATSDR) (www.atsdr.cdc.gov/SPL/index.html). In aquatic environments, the major way to remove chloroanilines is through biodegradation. Then, the selection of chloroanilines-degrading microorganisms from natural sources and its use in biodegradation processes is a feasible alternative to conventional activated sludge systems and physicochemical processes for the removal of these compounds from polluted sites and liquid effluents. Diverse microorganisms have shown the ability to degrade chloroanilines [3–6]. However, only few microorganisms with the ability to use PCA as the sole source of carbon and nitrogen have been reported previously [7, 8]. On the other hand, the conventional activated sludge systems that have been used for chloroanilines removal from wastewaters for many years fail to effectively remove these xenobiotic compounds because of their toxicity to the metabolizing bacteria [9]. Thus, new bioreactor designs are necessary to treat liquid effluents with high concentrations of chloroanilines. For this purpose, the performance of different bioreactors has been studied, such as membrane bioreactors [10], biofilm reactors [11], sequencing airlift bioreactors [12], and sequencing batch reactors [13]. However, the information about the removal of the metabolites released during biodegradation of chloroanilines and the assessment of toxicity levels after biodegradation process is scarce. The purposes of this study are: (i) the selection of PCA-degrading microorganisms from natural waters, (ii) the use of these microorganisms for the degradation of PCA in a continuous flow biofilm reactor, (iii) the removal of the released ammonium by a nitrification/denitrification step, and (iv) the assessment of toxicity before and after the biodegradation process using the standard organisms *Vibrio fischeri*, *Pseudokirchneriella subcapitata*, and *Daphnia magna*.

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**Abbreviations:** **PCA**, *p*-chloroaniline; **SEM**, scanning electronic microscopy.

## 2 Materials and methods

### 2.1 Selection and identification of *p*-chloroaniline-degrading microorganisms

The selection of PCA-degrading microorganisms was performed by exposing and sub-culturing a sample of an industrial effluent from a pharmaceutical company to 0.39 mM of PCA as the sole carbon and nitrogen source in a synthetic minimal medium, until a consistent depletion of the compound was observed. The composition of the minimal medium employed in this study was previously described by Korol et al. [14]. Characterization and identification of the PCA-degrading microorganisms were carried out by Gram-staining and 16S rRNA gene sequencing. The technique for the 16S rRNA gene sequencing was described in a previous investigation [15].

### 2.2 Enrichment of ammonia oxidizing bacteria

An enrichment of ammonia oxidizing bacteria was carried out in order to obtain the inoculum for nitrification process. The enrichment was performed in batch culture and aerobic conditions, in a culture medium described by Marín et al. [16]. A commercial organic amendment purchased from Terrafertil (Buenos Aires, Argentina) was used as a source of nitrifying bacteria. The organic amendment consisted in a mixture of organic compost and loamy soil. The characteristics of this amendment were: 40% of moisture, 20% of organic matter, C/N ratio 7.7, and pH 6.2.

### 2.3 Chemicals

PCA was purchased from Sigma–Aldrich (purity $\geq$ 98%). All the other chemicals used in this study were purchased from Mallinckrodt Chemicals (St. Louis, USA) and Merck (Darmstadt, Germany), and all of them were of analytical reagent grade. The solution of PCA was prepared aseptically by dissolving the compound in sterile distilled water.

### 2.4 *p*-Chloroaniline biodegradation in a continuous flow biofilm reactor

The biodegradation of PCA was carried out in an aerobic down-flow fixed-bed reactor previously described by Gallego et al. [17]. The reactor was constructed from a PVC conduit, and its dimensions were: 100 cm of height $\times$ 10 cm of internal diameter (Fig. 1). The support material employed for the immobilization of the PCA-degrading microorganisms was polyurethane foam. The material was cut in cubes of 2 cm side. Before the reactor started to operate with a continuous flow, the bacterial community was inoculated into a fed-batch reactor filled with the polyurethane foam cubes. The feeding of this reactor consisted in synthetic minimal medium supplemented with 0.39 mM PCA. The reactor worked in fed-batch mode during 1 month of operation. Only when a consistent depletion of the compound was achieved and the biofilm successfully colonized the support material, the cubes were transferred into the continuous flow reactor.

The operating conditions of the continuous flow reactor were the following: without sterility and room temperature. The feeding consisted in a synthetic effluent composed of free chlorine tap water supplemented with dipotassium phosphate and PCA (from 0.16 to 0.78 mM). The synthetic effluent percolates through the support material by effect of the gravitational force. The influent flow rate was 1 L/day and the hydraulic retention time was 5 min.

To exclude the possibility of abiotic losses of PCA and its adsorption on polyurethane foam, a control reactor was also constructed. It was filled with uninoculated polyurethane foam cubes, and was fed with the same synthetic effluent that the inoculated reactor.

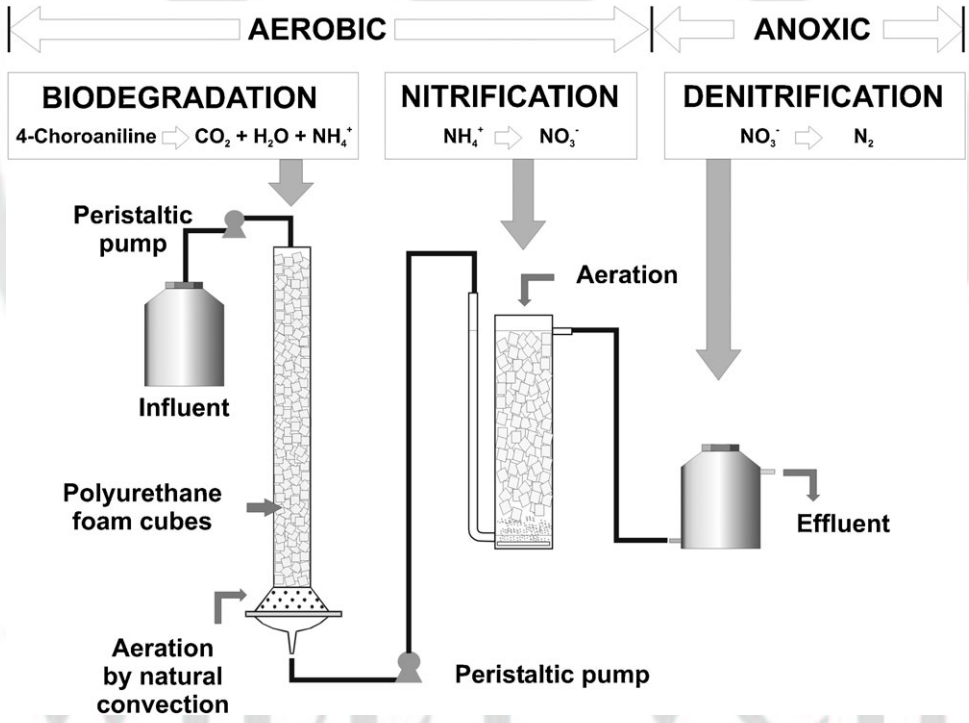


**Figure 1.** Experimental continuous aerobic-anoxic system.

## 2.5 Nitrification/denitrification process for ammonium removal

In order to remove the ammonium released during PCA biodegradation, a nitrification/denitrification process was coupled to the aerobic biodegradation. Nitrification was performed in an aerobic up-flow fixed-bed reactor, with the same support material than that of the biodegradation reactor (Fig. 1). The nitrifying inoculum was obtained from the enrichment of ammonia oxidizing bacteria (Section 2.2). The reactor was continuously fed with the effluent from the biodegradation process.

Then, a denitrification process was carried out in order to remove the nitrate produced in the nitrification reactor. This process was performed in an anoxic reactor. The anoxic conditions were obtained by filling up and sealing a 22-L cylindrical tank (Fig. 1). The reactor was continuously fed with the effluent from the nitrification reactor. Water samples collected from a pollution-free freshwater stream were used as inoculum and sodium acetate (100 mg/L) was added as carbon source. Operating conditions in both nitrification and denitrification reactors were the same as the biodegradation reactor.

## 2.6 Analytical methods

The concentration of PCA was determined by UV spectrophotometry. Samples were previously centrifuged for 10 min at 4000 rpm and filtered through a membrane filter with a 0.45-μm pore size. Analyses were carried out on a Metrolab UV 1700 spectrophotometer, by measuring the absorbance at 290 nm.

PCA concentration was also determined by GC–MS in order to corroborate the complete degradation of the compound. Analyses were conducted on an Agilent 6890 gas chromatograph with a selective mass detector. An INNOWAX capillary column of 50 m length and 0.2 mm internal diameter was used, with Helium as carrier gas. Samples of 1 μL were injected in the column at 240°C and the temperature gradient was the following: the initial temperature was 180°C. This temperature remained constant for 2 min; then, the temperature increased at a rate of 20°C/min to 240°C. Chloride concentration was also measured before and after the biodegradation process, according to APHA [18]. In order to evaluate ammonium removal, ammonium nitrite and nitrate were determined before and after PCA biodegradation, and after nitrification and denitrification processes, according to APHA [18]. Cell viability in the biodegradation reactor was evaluated in both the liquid effluent and the support material by the plate count method in triptone soy agar plates [18]. Bacteria from the support material were previously removed by vortex agitation in a saline solution of 0.85% NaCl. Colonization of polyurethane foam by the biofilm was observed by scanning electronic microscopy (SEM). Samples for SEM analysis were previously fixed according to the technique described by Varesche et al. [19]. After fixation, samples were metalized and attached to the supports of a Philips XI30 microscope for their subsequent observation. Nitrifying bacteria count in the nitrification reactor was performed by the most probable number method, according to APHA [18].

## 2.7 Toxicity assays

Bioassays of acute toxicity were performed to assess detoxification. Toxicity was evaluated before and after the biodegradation process. Three different species were used as test organisms: the luminescent bacterium *V. fischeri*, the green alga *P. subcapitata*, and the crustacea *D. magna*.

### 2.7.1 Microtox® test

The Microtox® test was conducted on a Microtox® Model 500 toxicity analyzer (Azur Environmental, Carlsbad, CA, USA). The luminescent bacterium *V. fischeri* (strain NRRLB-11177) employed as test organism was purchased as a lyophilized form from Strategic Diagnostics (Carlsbad, CA, USA). We previously evaluated the sensitivity of the test organisms with phenol as the reference toxic compound. Then, the bacteria were exposed to different sample dilutions for 15 min at 15 ± 1°C. The acute toxicity, expressed as effective concentration 50 ($EC_{50}$), was estimated by measuring the inhibition of the luminescence of *V. fischeri* with respect to the control, according to ISO 11348-3 [20]. We used the Microtox Omni® software to calculate the $EC_{50}$ values.

### 2.7.2 Alga test

*P. subcapitata* growth inhibition tests were performed in 96-well microplates, according to the technique describe by Blaise [21] at 24 ± 2°C, under continuous cool-white illumination. Four replicates for control and different sample concentrations with an initial cell density of $5 \times 10^4$ cells/mL were used. Algal cells 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 Origen Program (version 7.5). This software plots the percentage inhibition of the algal growth in each sample with respect to the control. The reference chemical potassium dichromate was employed as a positive control to ensure the sensitivity of the organisms.

### 2.7.3 *Daphnia magna* test

Acute toxicity was assessed by measuring the inhibition of mobility of the crustacean *D. magna*, according to ISO 6341 [22]. Before the assays, we evaluated the sensitivity of the organisms by using potassium dichromate as the reference toxic compound.

The test was carried out in static conditions, at 20 ± 0.5°C, without illumination, by exposing the organisms to different sample dilutions for 48 h. The concentration, which produces immobility in 50% of the organisms of the tested population relative to the test control ($EC_{50}$) was estimated by the use of the Spearman–Karber method.

# 3 Results and discussion

## 3.1 Selection and identification of *p*-chloroaniline-degrading microorganisms

After 1 month of incubation in minimal medium supplemented with 0.39 mM of PCA, we isolated from an industrial effluent from the synthesis of the pharmaceutical compound diclofenac, a native microbial community with the ability to degrade this compound. The microbial community is composed of three gram-negative, non-fermentative bacterial strains. The analysis of the 16S rRNA gene sequence showed an identity of 99% with the genera *Pseudomonas* sp., *Achromobacter* sp., and *Sphingopyxis* sp. Bacterial strains belonging to the genera *Pseudomonas* and *Achromobacter* have been reported previously as chloroanilines-degrading microorganisms [8, 23].

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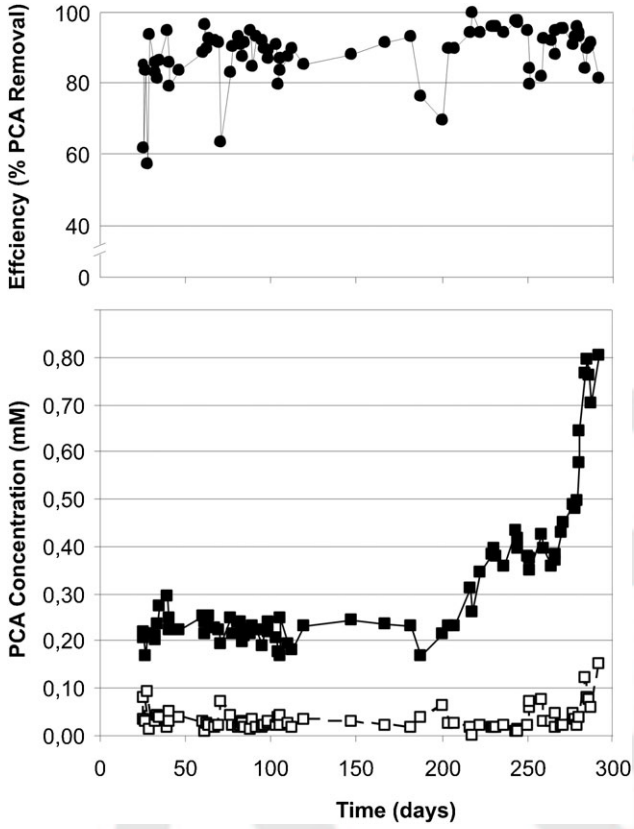


**Figure 2.** PCA biodegradation in the aerobic fixed-bed biofilm reactor. (a) PCA concentration in the influent (–■–) and effluent (–□–) of the reactor; (b) efficiency of PCA biodegradation.

Although *Sphingopyxis* strains have shown the ability to degrade trichlorophenol and microcystin [24, 25] this is the first report about a strain of this genus associated to PCA biodegradation. None of the individual strains were able to degrade the compound; however the whole community was capable of using PCA as the sole source of carbon and nitrogen. There are many reports about chloroanilines biodegradation by pure or mixed bacterial cultures [1, 3, 26–28]. However, most of them require a cometabolic substrate, like aniline. Only few studies resulted in the isolation of bacterial strains with the ability for PCA biodegradation without any additional substrate [7, 8].

## 3.2 *p*-Chloroaniline biodegradation in a continuous flow biofilm reactor

Liquid effluents from several industries are usually refractory to conventional biological treatments, because the presence of persistent and toxic compounds inhibits the growth of microorganisms. Taking into account that successful biodegradation of slowly degradable pollutants, such as chloroanilines and others chlorinated aromatic compounds, depends on the survival and activity of specialized metabolizing bacteria [29], biofilm reactors have shown to be an alternative for the removal of these compounds, due to their high biomass concentration and because biofilms reduce the sensitivity of degrading-microorganisms to toxicity compared with suspended sludge processes [30]. Fixed-bed biofilm reactors are extensively used for the removal of organic pollutants because of their simple mechanical configuration, low energy requirements and operating costs [31]. A wide variety of support materials has been employed in this kind of reactors, such as high density polyethylene [32], polyvinyl chloride [17, 33], and polyurethane foam [19, 34].

We studied the biodegradation of PCA by an indigenous bacterial community in a continuous fixed-bed biofilm reactor, with polyurethane foam as a support material. After the reactor started to operate, it took 25 days to reach minimum concentrations of PCA in the treated effluent (data not shown). The average efficiency of the biodegradation process was 88.5%, expressed as compound removal. In spite of variations in the PCA concentration of the influent, the efficiency remained approximately constant (Fig. 2). The maximum PCA removal rate reached 11.7 g/m$^3$/day. PCA degradation was associated with the release of chloride and ammonium (Fig. 3). Neither abiotic losses of PCA nor adsorption of the compound on polyurethane foam were observed in the control reactor.

It has been reported that mineralization of chloroanilines is often characterized by the transient accumulation of the corresponding chlorocatechols. Radianingtyas et al. [11] studied the biodegradation of PCA in a biofilm reactor. Under certain operating conditions they

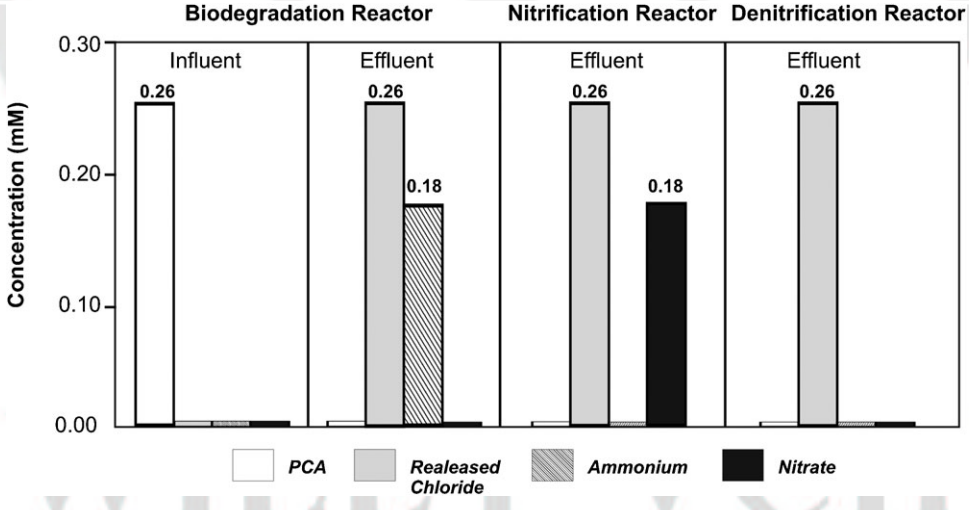


**Figure 3.** Chloride, ammonium, and nitrate concentrations at the different stages of the biodegradation process.

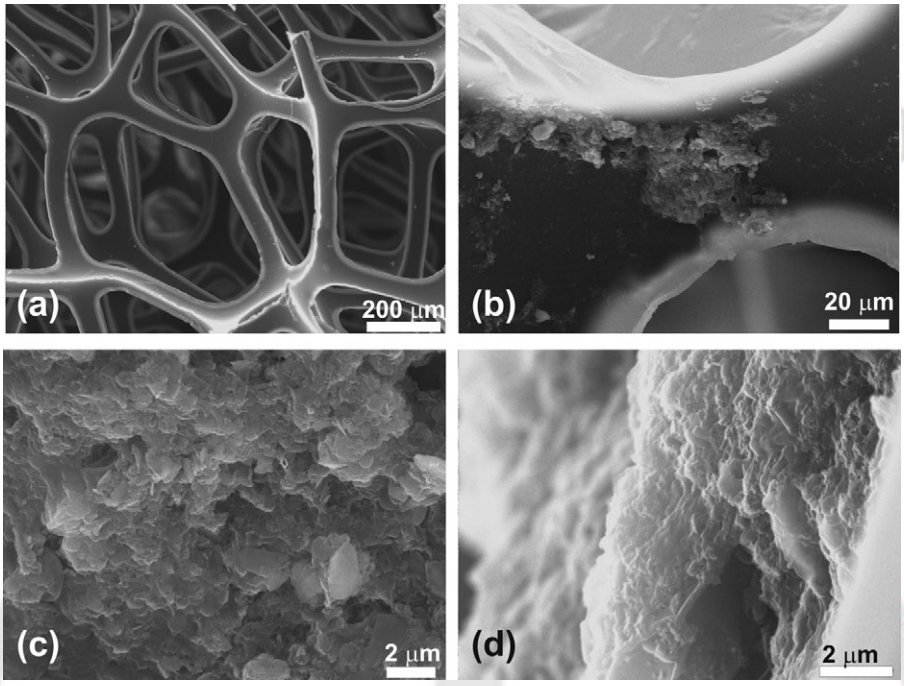


**Figure 4.** Scanning electron microscopy of polyurethane foam cubes from the biodegradation reactor. (a) Before inoculation (100×); (b), (c), and (d) after inoculation (800×, 6500×, and 10 000×, respectively).

observed an accumulation of 4-chlorocatechol that correlated directly with an increase in toxicity. Thus, the complete degradation of the compound is necessary in order to avoid the accumulation of toxic metabolites and to achieve detoxification. We corroborated by GC–MS the complete degradation of the compound. Moreover, the full-scan MS allows us to confirm the absence of organic metabolites from PCA biodegradation.

Cell viability in the reactor was determined in both the reactor effluent and the support material. The heterotrophic bacteria count in the effluent of the reactor reached $1.9 \times 10^5$ CFU/mL. The biomass in the support material was estimated in $1.6 \times 10^8$ CFU/g. Figure 4 shows the scanning electron microscopy of polyurethane foam cubes before and after inoculation. It is remarkable that during operation period it was not necessary to reinoculate the reactor. Moreover, the interruption of the feeding during a period of 20 days did not affect the viability of the biofilm. After influent restarting at a concentration of 0.39 mM the reactor took 48 h to achieve the minimum concentrations in the effluent (data not shown).

## 3.3 Nitrification/denitrification process for ammonium removal

As a result of PCA biodegradation an almost stoichiometric release of ammonium was observed in the biodegradation reactor (Fig. 3). This compound is also an environmental toxic agent. It exhibits acute toxicity to aquatic life [35]. Thus, a cost-effective and efficient nitrogen removal is necessary in order to protect the aquatic environment.

We studied the removal of ammonium by a sequential nitrification/denitrification process coupled to the biodegradation of PCA. It has been reported that aniline inhibits the activity of ammonia-oxidizing bacteria. Gheewala and Annachhatre [36] observed that nitrification can proceed only when aniline concentration drops below 3–4 mg/L. Boon et al. [37] observed this inhibition effect too, with the chlorinated compound 3-chloroaniline in an activated-sludge reactor. They also suggested that bio-augmentation processes accelerate the degradation of 3-chloroaniline and allow a faster recovery of the nitrifying community from the toxic shock. Although the effect of PCA on nitrification is not known, it could be expected that this compound also inhibits nitrification as their isomer 3-chloroaniline. Even though PCA biodegradation and nitrification are both aerobic processes, these were carried out in separated reactors in order to prevent such inhibitory effect of PCA on nitrification. The results demonstrated the total transformation of ammonium into nitrate. Then, elimination of nitrate was achieved by the subsequent denitrification process (Fig. 3).

It is remarkable that nitrite was not detected at the effluent of the nitrification reactor. This result is consistent with nitrifying bacteria counts in the support material, since the most probable number of nitrite oxidizing bacteria was considerably higher than that of the ammonia oxidizing bacteria ($5.3 \times 10^4$ and $6.1 \times 10^2$/g, respectively). It has also been reported that the reaction of ammonia oxidation to nitrite is a velocity-limiting step; in contrast, nitrite is oxidized rapidly to nitrate, so nitrite is seldom accumulated in nitrifying reactors [38].

## 3.4 Toxicity assays

In a biodegradation test, chemical analysis alone does not provide sufficient information about the toxicity of a sample [39]. To exclude the possibility of the appearance of toxic metabolites, it is desirable to complement the chemical analysis with toxicity bioassays [40].

Bioassays of acute toxicity were conducted in order to confirm the total elimination of toxicity from the synthetic effluent as a result of

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**Table 1.** Effective concentration 50 at the influent and effluent of the biodegradation reactor for the three test organisms used

| | $EC_{50}$ (% v/v) | |
|---|---|---|
| | Influent | Effluent |
| *V. fischeri* | 14.4 (11.9–17.3) | ND |
| *P. subcapitata* | 22.0 (17.2–27.4) | ND |
| *D. magna* | 16.0 (12.0–20.4) | ND |

ND, toxicity was not detected.

the biodegradation process. Table 1 shows that toxicity levels in the influent of the biodegradation reactor were high for all the organisms tested, whereas no toxicity was detected after biodegradation process. Although the concentration of the ammonium released from PCA biodegradation has not shown toxicity for the tested organisms, removal of ammonium is still necessary in order to satisfy the stringent discharge standards. It should be noted that the Argentinian Act on Hazardous Wastes (Act 24051/92) establishes a guidance level of 1.37 mg/L for ammonium in surface waters, in order to protect the aquatic wildlife.

The results obtained in these toxicity tests in addition to the chromatography data corroborated not only the disappearance of PCA but also the elimination of toxicity as a result of PCA biodegradation.

## 4 Concluding remarks

In this investigation, we isolated from an industrial effluent a bacterial community capable of utilizing *p*-chloroaniline (PCA) as the sole source of carbon and nitrogen. The community is composed of three bacterial strains identified as *Pseudomonas* sp., *Achromobacter* sp., and *Sphingopyxis* sp. None of them showed the ability to degrade de compound as individual strains, however, we successfully attained to set up a continuous biofilm reactor in which the whole community reached high efficiency in the removal of PCA. The absence of organic metabolites from PCA biodegradation and detoxification were confirmed after the biodegradation process. The released ammonium was also removed by a nitrification/denitrification process. The ability of this indigenous bacterial community could be employed to optimize the treatment of liquid effluents that contain PCA. Also, the application of these sequential biological processes in biofilm reactors is a very promising approach to industrial applications. We previously used a similar reactor design to the biodegradation of several xenobiotic compounds, such as the herbicide 2,4-dichlorophenoxyacetic acid and cresols [15, 17]. This demonstrates the versatility of the reactor and therefore its applicability for the removal of a wide variety of persistent and toxic compounds from liquid effluents.

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*The authors have declared no conflict of interest.*

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