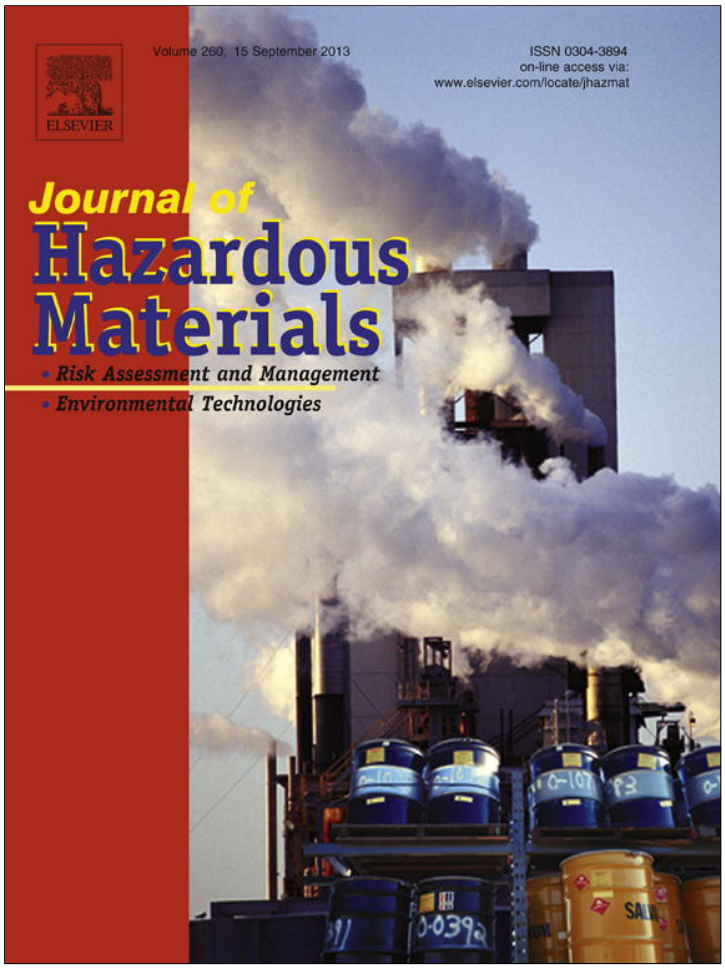

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Biological anoxic treatment of O_2-free VOC emissions from the petrochemical industry: A proof of concept study

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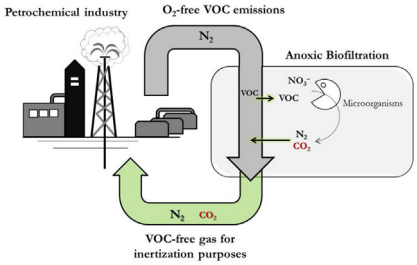
HIGHLIGHTS

- The treatment of O_2-free VOC emissions can be done by means of denitrifying processes.
- Toluene vapors were successfully removed under anoxic denitrifying conditions.
- A high bacterial diversity was observed.
- *Actinobacteria* and *Proteobacteria* were the predominant phyla.
- The nature and number of metabolites accumulated varied with the toluene load

GRAPHICAL ABSTRACT



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ABSTRACT

An innovative biofiltration technology based on anoxic biodegradation was proposed in this work for the treatment of inert VOC-laden emissions from the petrochemical industry. Anoxic biofiltration does not require conventional O_2 supply to mineralize VOCs, which increases process safety and allows for the reuse of the residual gas for inertization purposes in plant. The potential of this technology was evaluated in a biotrickling filter using toluene as a model VOC at loads of 3, 5, 12 and 34 g m^{-3} h^{-1} (corresponding to empty bed residence times of 16, 8, 4 and 1.3 min) with a maximum elimination capacity of ~3 g m^{-3} h^{-1}. However, significant differences in the nature and number of metabolites accumulated at each toluene load tested were observed, o- and p-cresol being detected only at 34 g m^{-3} h^{-1}, while benzyl alcohol, benzaldehyde and phenol were detected at lower loads. A complete toluene removal was maintained after increasing the inlet toluene concentration from 0.5 to 1 g m^{-3} (which entailed a loading rate increase from 3 to 6 g m^{-3} h^{-1}), indicating that the system was limited by mass transfer rather than by biological activity. A high bacterial diversity was observed, the predominant phyla being *Actinobacteria* and *Proteobacteria*.

1. Introduction

Petrochemical industrial facilities are nowadays identified as major emission hotspots of volatile organic compounds (VOCs) worldwide [1,2]. Approximately 20% of the total VOC emissions in the USA are emitted by facilities devoted to oil and gas production, bulk fuel/solvent storage and petroleum refining [3]. Likewise, petroleum refining and bulk storage (including storage for energy production and industrial processes) account for approximately 15% of the total non-methane VOC emissions in Europe [4]. Most VOC emissions from the petrochemical industry are characterized by their O_2-free nature and explosion risk when O_2 is present [5]. Explosion risks in petroleum refining and bulk storage tanks are normally controlled by headspace inertization with N_2 or CO_2, leading to VOC emissions when these inert atmospheres are vented [6]. Despite VOC emissions from the petrochemical industry are commonly treated by means of gas flaring (often with the addition of a support fuel), the release of VOC-laden emissions to the

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atmosphere without any treatment (gas venting) is still common [5,7]. For instance, in 2004 the World Bank estimated that 1×10^{12} m^3 of gas derived from petrochemical activities were flared or simply vented to the atmosphere worldwide [7]. Moreover, it is worth noting that VOC flaring does not constitute an environmentally friendly practice, with complete combustion in conventional flares being rarely achieved (combustion efficiencies as low as 10–15% being reported at wind speeds higher than 20 km h^{-1}) [8]. Therefore, there is a lack of environmentally friendly, cost-efficient and robust technologies for the treatment of such particular VOC emissions.

Despite biotechnologies constitute a promising off-gas treatment alternative, the O_2-free nature and the explosion risks associated with the presence of O_2 have strongly limited the use of conventional aerobic biofiltration as an end-of-pipe technology for the control of VOC emissions from the petrochemical industry. Nevertheless, although anaerobic biocatalytic processes have not been explored so far in biological gas treatment systems, there is enough empirical evidence to support the fact that VOC biodegradation can also be achieved in the absence of O_2. In this regard, VOCs such as benzene, toluene, ethylbenzene and xylene have been successfully removed under anoxic and iron reducing environments, and particularly high VOC biodegradation rates were recorded under anoxic denitrifying conditions [9,10]. Likewise, anoxic methane mineralization via denitrification has also been reported [11,12]. However, these preliminary studies conducted batchwise in closed bottles were focused on the treatment of pollutants in wastewater, and the feasibility of using denitrification as a core metabolic process for off-gas VOC biodegradation under continuous operation in bioreactors must still be evaluated.

In this work, the potential of an innovative biofiltration technology based on the anoxic biodegradation of VOCs was evaluated. In contrast to the traditional aerobic biofiltration, anoxic biofiltration does not require O_2 to mineralize VOCs, and generates N_2 and CO_2 as innocuous by-products. Thus, besides increasing process safety, this biotechnology would allow the reuse of the residual gas for inertization purposes in petrochemical processes (e.g. blanketing of storage tanks). For this proof of concept, toluene was used as model VOC and the anoxic biofiltration system was implemented in a biotrickling filter.

2. Materials and methods

2.1. Chemicals and mineral salt medium

All chemicals for mineral salt medium (MSM) preparation were purchased from PANREAC (Barcelona, Spain) with a purity of at least 99%. Toluene (99.0% purity) was obtained from Sigma–Aldrich (Madrid, Spain). The MSM was composed of (g L^{-1}): $Na_2HPO_4{\cdot}12H_2O$, 6.15; KH_2PO_4, 1.52; $MgSO_4{\cdot}7H_2O$, 0.2; $CaCl_2$, 0.038; and 10 mL L^{-1} of a trace element solution containing (g L^{-1}): EDTA, 0.5; $FeSO_4{\cdot}7H_2O$, 0.2; $ZnSO_4{\cdot}7H_2O$, 0.01; $MnCl_2{\cdot}4H_2O$, 0.003; H_3BO_3, 0.03; $CoCl_2{\cdot}6H_2O$, 0.02; $CuCl_2{\cdot}2H_2O$, 0.001; $NiCl_2{\cdot}6H_2O$, 0.002; $NaMoO_4{\cdot}2H_2O$, 0.003. A $NaNO_3$ stock solution of 100 g L^{-1} was prepared to supplement the MSM with NO_3^- as electron acceptor for toluene oxidation and as nitrogen source for microbial growth. The final pH of the medium was 7.

2.2. Enrichment of toluene denitrifying microorganisms

Enrichment cultures were performed batchwise in 1.25-L serum bottles to isolate microorganisms able to degrade toluene under anoxic denitrifying conditions from activated sludge (Valladolid wastewater treatment plant, Spain). The bottles, containing 94 mL of MSM, 1 mL of $NaNO_3$ stock solution and 5 mL of fresh activated sludge, were gas-tight closed with butyl septa and plastic caps. Then, the O_2 contained in the headspace was flushed with helium for 5 min and once the bottles were deoxygenated 20 μL of toluene were added. The bottles were incubated at 25 °C and 300 rpm. Toluene and CO_2 concentrations in the headspace, as well as NO_3^- and NO_2^- concentrations in the liquid phase, were periodically measured. Ten cycles of toluene/NO_3^- additions were performed, with a reproducible toluene mineralization being observed from the 5th cycle onwards (overall enrichment period of 30 days). The enriched consortium served as inoculum for the biotrickling filter.

2.3. Toluene biodegradation in a biotrickling filter

Continuous toluene biodegradation under anoxic denitrifying conditions was performed in a biotrickling filter reactor (BTF). The BTF consisted of a cylindrical jacketed PVC column (0.07 m inner diameter, 0.6 m height) with a working packed bed volume of 1.5 L of Kaldnes K1 rings (Evolution Aqua Ltd., UK). The packing material was characterized by: ring diameter 1 cm, density 0.17 g mL^{-1}, void fraction 83%, and water-holding capacity (volume basis) 11% [13]. The bioreactor was operated in countercurrent flow mode at 25 °C throughout the entire experiment.

The O_2-free toluene emission was obtained by mixing a pure and humidified N_2 stream (99.0% purity, Abello Linde, Spain) with a toluene-saturated N_2 stream regulated by a mass flow controller (Aalborg, Denmark). The BTF was operated at 0.5 g toluene m^{-3} and empty bed residence times (EBRT) of 1.3, 4, 8 and 16 min. The nutrients solution (0.5 L of MSM) was continuously agitated at 200 rpm and 25 °C in an external 0.6-L holding tank and recycled at 0.63 m h^{-1}. NO_3^- was supplied by periodically injecting 10 mL of a 100 gNO_3^- L^{-1} stock solution to the holding tank. The NO_3^- supply frequency varied according to the experimental stage in order to avoid NO_3^- limitation in the recycling liquid medium. In addition, to avoid nutrient limitation 25 mL of liquid culture broth were replaced by fresh MSM each 3 days. The pH of the liquid recycling media was daily adjusted to 7. A complete replacement of the recycling nutrient solution by fresh mineral salt medium was conducted at day 22 to assess the role of potentially accumulated metabolites on the toluene removal performance.

Toluene and CO_2 concentrations in gas phase as well as NO_3^- and NO_2^- concentrations in the liquid phase were periodically monitored. On-line measurements of pH and temperature were acquired each 4 h with calibrated probes via a multiparameter analyzer C-3020 (Consort, Belgium) connected to a computer. The pressure drop (ΔP) across the packed column of the BTF was determined with a water U-tube manometer. A detailed diagram of the experimental set-up is shown in Fig. 1.

2.4. Biodegradation performance

Toluene biodegradation performance was evaluated in terms of elimination capacity (EC, g m^{-3} h^{-1}) and removal efficiency (RE, %) as defined by Muñoz et al. [14]. The carbon mineralization efficiency (CME, %) was defined as:

$$\mathrm{CME} = \frac{\mathrm{C-CO_2\ \ produced}}{\mathrm{C-Toluene\ \ degraded}} \times 100 \quad (1)$$

2.5. Molecular biology analysis

Biomass samples at day 0 (inoculum of the BTF) and day 60 (end of BTF operation) were collected and stored immediately at −20 °C to evaluate the richness and composition of the bacterial community. Genomic DNA was extracted according to Lebrero et al. [15]. The PCR mixture (50 μL) was composed of 25 μL of BIOMIX

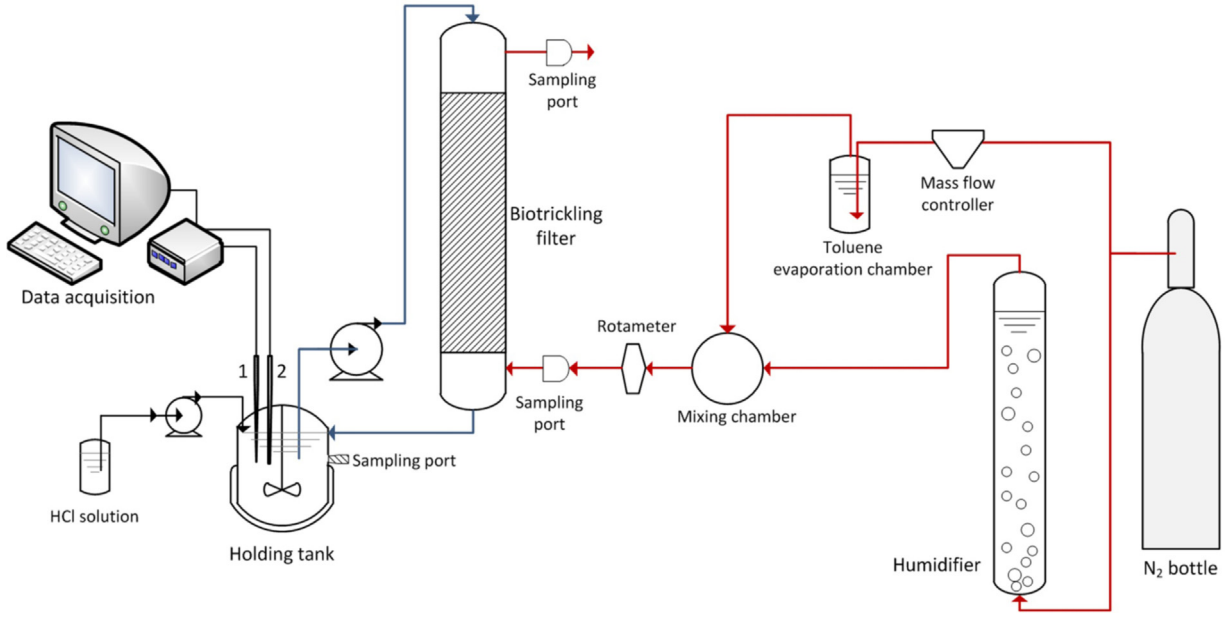

Fig. 1. Schematic representation of the experimental set-up, where 1 and 2 are the pH and temperature probes, respectively.

ready-to-use 2× reaction mix (Bioline, Ecogen), containing reaction buffer, magnesium, deoxynucleotide triphosphates (dNTPs), Taq polymerase and additives, 1 or 2 μL of the extracted DNA, PCR primers 968-F-GC and 1401-R (10 μM) (Sigma–Aldrich, St. Louis, MO, USA) for bacterial 16S rRNA gene amplification, and Milli-Q water up to a final volume of 50 μL. The PCR thermo-cycling program used was previously described in Lebrero et al. [15].

DGGE analysis of the amplicons was performed with a D-Code Universal Mutation Detection System (Bio Rad Laboratories), using 8% (w/v) polyacrilamide gels with an urea/formamide denaturing gradient of 45 to 65%. DGGE running conditions were applied according to Roest et al. [16]. The gels were stained with SYBR Green I nucleic acid gel stain (Sigma–Aldrich, St.Louis, MO, USA) for 1 h. The obtained DGGE patterns were processed using the GelCompar IITM software (Applied Maths BVBA, Sint-Martens-Latem, Belgium). After image normalization, bands were defined for each sample using the band search algorithm within the program. Similarity indices of the compared profiles were calculated from the densitometric curves of the scanned DGGE profiles by using the Pearson product-moment correlation coefficient [17]. The peak heights in the densitometric curves were also used to determine the Shannon–Wiener diversity index (H), which takes into account both the relative number of the DGGE bands (richness) and their relative intensities (evenness) according to:

$$H = -\sum [P_i \ln(P_i)] \quad (2)$$

where H is the diversity index and P_i is the importance probability of the bands in a lane ($P_i = n_i/n$, where n_i is the height of an individual peak and n is the sum of all peak heights in the densitometric curves). Some bands were excised from the DGGE gel in order to identify the microorganisms present into the reactor according to Lebrero et al. [18]. The taxonomic position of the sequenced DGGE bands was obtained using the RDP classifier tool (50% confidence level) [19]. The closest matches to each band were obtained using the BLAST search tool at the NCBI (National Centre for Biotechnology Information) [20]. Sequences were deposited in GenBank database under accession numbers KC508671 to KC508686.

2.6. Analytical methods

Toluene gas concentration was measured using a Varian 3900 gas chromatograph (Palo Alto, USA) equipped with a flame ionization detector and a SupelcoWax (15 m × 0.25 mm × 0.25 μm) capillary column. The injector, detector and oven temperatures were set at 200 °C, 200 °C and 140 °C, respectively. Helium was used as the carrier gas at 2 mL min^{-1}. CO_2 concentration was determined in a Bruker 430 gas chromatograph (Palo Alto, USA) coupled with a thermal conductivity detector and equipped with a CP-Molsieve 5A (15 m × 0.53 μm × 15 μm) and a CP-PoraBOND Q (25 m × 0.53 μm × 10 μm) columns. The oven, injector and detector temperatures were maintained at 40 °C, 150 °C and 175 °C, respectively. Helium was used as the carrier gas at 13.7 mL min^{-1}.

Metabolite characterization was performed in an Agilent 6890 N GC-MS equipped with a DB-WAX column (30 m × 0.250 mm × 0.25 μm) (J&W Scientific®, CA, USA). The injector temperature was set at 200 °C, while the oven temperature was kept at 40 °C for 4 min and then increased at 10 °C min^{-1} up to 200 °C. Source and MS quadrupole temperatures were set at 230 and 150 °C, respectively.

NO_3^- and NO_2^- concentrations in the liquid phase were analyzed via HPLC-IC using a Waters 515 HPLC pump coupled with a conductivity detector (Waters 432) and equipped with an IC-PAK Anion HC column (4.6 × 150 mm) and an IC-Pak Anion Guard-Pak (Waters). Samples were eluted isocratically at 2 mL min^{-1} (at room temperature) with a solution of distilled water/acetonitrile/n-butanol/buffer at 84/12/2/2% v/v.

3. Results and discussion

3.1. Toluene biodegradation performance

Process operation at a toluene load of 34 g m^{-3} h^{-1}, corresponding to an EBRT of 1.3 min, resulted in a poor toluene abatement performance, the BTF reaching an average RE of 8% for 23 days (Fig. 2a). The load was then decreased to 12 g m^{-3} h^{-1} (EBRT of 4 min), which improved the RE up to 32%. Further decreases in the toluene load down to 6 and 3 g m^{-3} h^{-1} (EBRT of 8 and

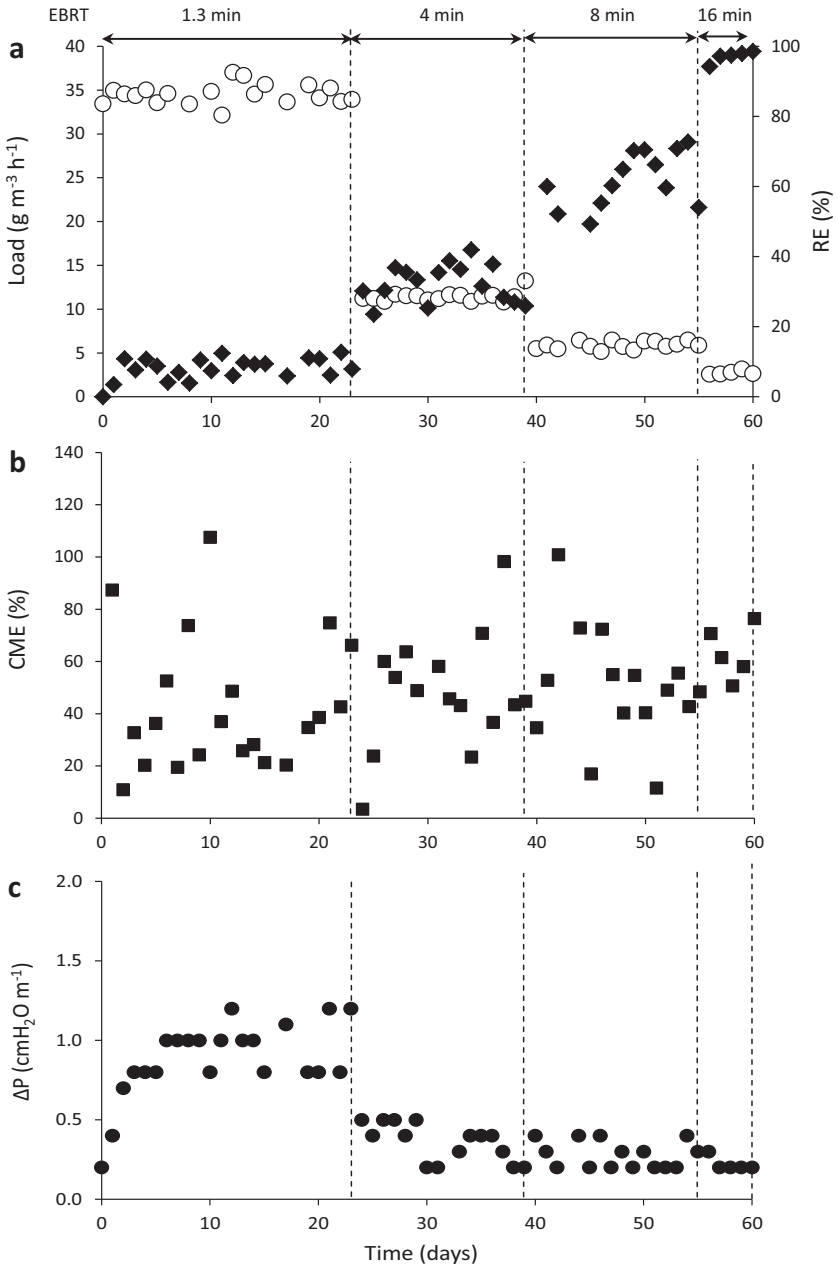

Fig. 2. Time course of (a) toluene load (○) and RE (♦), (b) carbon mineralization efficiency, and (c) pressure drop.

16 min, respectively) resulted in REs of 62% and 100%, respectively. Unsteady toluene mineralization was recorded at the loads tested (Fig. 2b). The CME fluctuated from 5 to 110%, which suggested that toluene biodegradation metabolites accumulated and were further mineralized intermittently. A stabilization of the CME at 63% was observed when the toluene load was decreased to 3 g m^{-3} h^{-1} (EBRT = 16 min). As a rough estimation considering these fluctuations, the average CMEs observed at EBRTs of 1.3, 4 and 8 min were 43, 48 and 50%, respectively. On the other hand, a maximum average pressure drop of ~1 cm H_2O $m_{packing}^{-1}$ was observed at the highest toluene load studied (corresponding to the lowest EBRT), while the pressure drop remained roughly constant at an average value of 0.27 cm H_2O $m_{packing}^{-1}$ at EBRTs higher than 1.3 min (Fig. 2c).

Conventional aerobic BTFs can achieve toluene elimination capacities of ~30 g m^{-3} h^{-1} at EBRTs of 1–3 min (corresponding to REs of 50–90% and loads of 30–64 g m^{-3} h^{-1}) [21,22]. In recent studies, toluene elimination capacities of 152–373 g m^{-3} h^{-1} were recorded in aerobic BTFs operated at EBRTs of 1.5–3.3 min (corresponding to REs of ~100%) [23,24], which are significantly higher than those reached in the present study. These reports indicate that aerobic BTFs indeed support superior toluene removal compared with the anoxic system here investigated. However, these aerobic BTFs presented pressure drops ranging from 0.6 to 15 cm H_2O $m_{packing}^{-1}$, which are values 2.2–55 higher than those recorded in the anoxic system. Hence, a proportional power consumption saving can be expected in the anoxic system. In this context, the use of NO_3^- as nitrogen source has been reported as an efficient strategy to control the pressure drop in both biotrickling filters and biofilters due to the lower biomass production associated to this nitrogen source [25,26]. In addition, considering the average CMEs here observed, the performance of the anoxic BTF in terms of carbon mineralization was comparable with previous reports on aerobic toluene biofiltration. For instance, Garcia-Peña et al. [27] reported

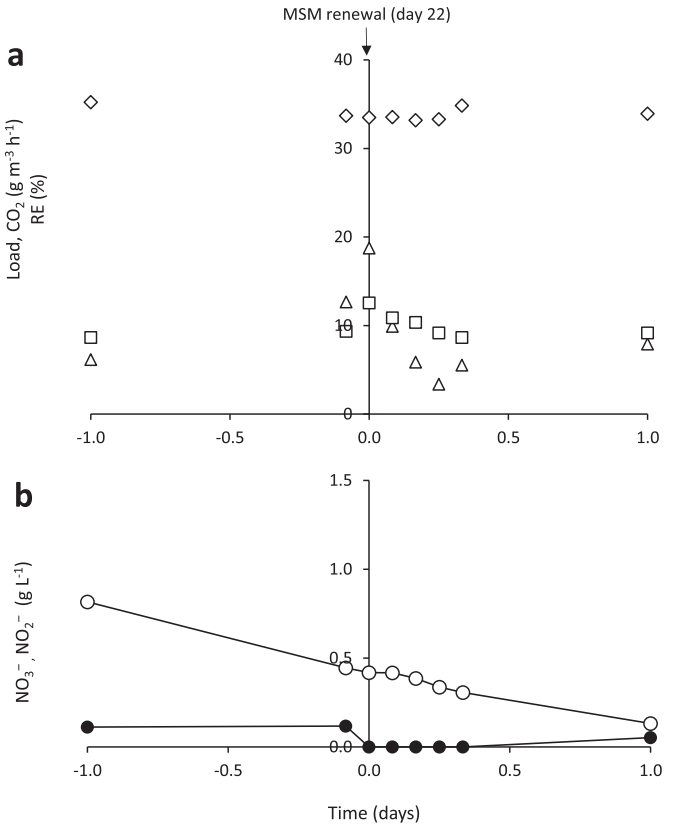

Fig. 3. Influence of complete MSM renewal at day 22 on (a) toluene removal performance: (◊) load, (Δ) RE, (□) CO_2 production, and (b) electron acceptor uptake: (○) NO_3^-, (●) NO_2^-.

an average toluene CME of 48% in an aerobic biofilter, while Cox and Deshusses [22] recorded average CME values ranging from 50% to 75% in an aerobic biotrickling filter.

3.2. Metabolites accumulated

The fluctuating CME values suggested that metabolites were periodically accumulated and mineralized (Fig. 2b). Indeed, o-cresol and p-cresol were identified in the liquid cultivation broth during process operation at a load of 34 g m^{-3} h^{-1}. Particularly, o-cresol, p-cresol, 2-butenoic acid and acetic acid were identified at day 21 (Fig. 3). A complete replacement of the recycling nutrient solution by fresh mineral salt medium was conducted at day 22 to assess the role of accumulated metabolites on the toluene removal performance. Immediately after the complete liquid phase renewal, the RE increased from 13% to 19% concomitant with a slight increase in CO_2 production. However, the RE gradually decreased to ~5% within the next 8 h, o-cresol, 2-butenoic acid and acetic acid being detected again, which revealed a fast metabolite production. The RE and CO_2 production achieved steady state values 24 h after liquid phase renewal. These results indicated that an increase in the culture broth renewal frequency by fresh MSM will not result in a significant improvement of the toluene removal performance due to the fast metabolite production at the highest load.

Further decreases of the toluene load by increasing the EBRT resulted in significant differences on the type and number of metabolites produced (Fig. 4). In this regard, it is worth noting that o- and p-cresol were only detected at a load of 34 g m^{-3} h^{-1} (EBRT = 1.3 min), while at loads of 12, 6 and 3 g m^{-3} h^{-1} (corresponding to EBRTs of 4, 8 and 16 min, respectively) compounds such as benzyl alcohol, benzaldehyde and phenol were identified.

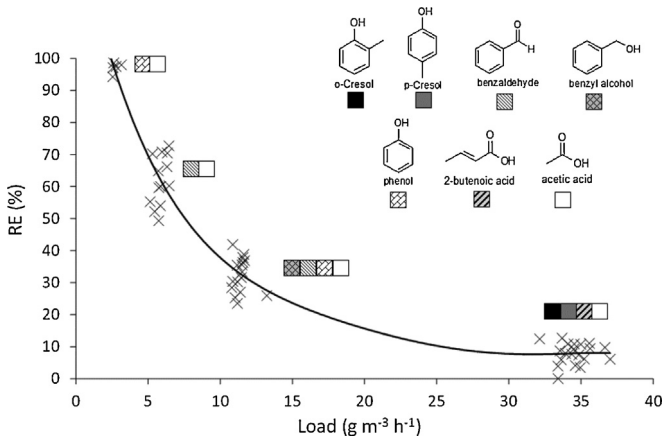

Fig. 4. Influence of toluene load on RE and metabolites produced. The symbols ■, ▩, ▨, ▩, ▨, ▨ and □ stand for each metabolite detected at the toluene loads tested.

Moreover, acetic acid, a metabolite associated with advanced toluene mineralization under anoxic conditions [28,29], was identified in the liquid phase regardless of the toluene load, confirming (together with the CO_2 production) that toluene mineralization was achieved under all the experimental conditions. Interestingly, 1,2,3-trimethylbenzene was detected at toluene load of 12 g m^{-3} h^{-1}, however, as far as the authors know this metabolite has not been reported as a by-product of anoxic toluene biodegradation. Thus, the production of this metabolite under anoxic denitrifying conditions must be further confirmed.

Several authors agree on the fact that o-cresol is a particularly recalcitrant compound compared with other metabolites derived from toluene biodegradation under anoxic denitrifying conditions such as p-cresol, m-cresol, phenol, benzaldehyde or benzyl alcohol [30–32]. Therefore, operational conditions promoting the generation of o-cresol must be avoided. In this regard, process operation at toluene loads below 34 g m^{-3} h^{-1} avoided the production of cresols and reduced the number of potentially toxic metabolites.

3.3. Limiting step of the process

Despite the fact that the nature of the accumulated metabolites did vary significantly with toluene load, an overall EC of ~3 g m^{-3} h^{-1} was recorded regardless of the load imposed (Fig. 4). These results suggested that toluene transfer from the gas phase to the microorganisms, rather than microbial activity was the limiting step of the process. In order to confirm this hypothesis, the toluene load was increased from 3 to 6 g m^{-3} h^{-1} for 6 h by increasing the toluene inlet concentration while maintaining an EBRT of 16 min. Fig. 5 shows the last 4 days of BTF operation at 3 g m^{-3} h^{-1} (days 57–60) and the step increase in toluene load at day 61. The RE remained at 100% concomitantly with an increase in CO_2 production after the step increase in toluene concentration from 0.5 to 1 g m^{-3}, which confirmed the toluene mass transfer limitation and the capacity of the microbial community to mineralize higher toluene loads.

3.4. Characteristics of the denitrification process

Data on continuous toluene removal under anoxic denitrifying conditions is scarce in the literature and therefore, several NO_3^- concentrations were tested within the first days of BTF operation. NO_3^- concentrations from ~7 to 0.15 g L^{-1} supported roughly the same toluene removal performance for the first 21 days of operation (Figs. 2a and 6). Hence, a potential microbial inhibition by NO_3^- at the tested concentrations was ruled out. From day 23

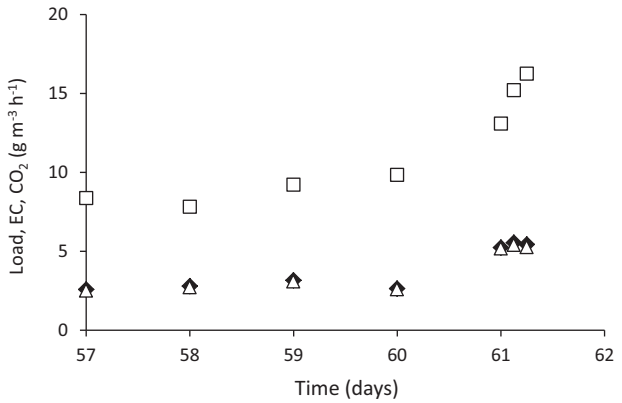

Fig. 5. Time course of toluene load (♦), EC (Δ) and CO_2 production (□) during the increase in toluene load at an EBRT of 16 min.

on, NO_3^- pulses of 1.5–3.6 g L^{-1} were added to the BTF and a stable NO_3^- uptake was achieved. The detection of NO_2^- in the liquid phase confirmed the reduction of NO_3^- coupled to the toluene oxidation, dynamic NO_2^- accumulation and depletion being observed during the whole experimental period, with a maximum NO_2^- concentration of 0.68 g L^{-1} recorded at day 34. The fact that the toluene removal performance remained constant regardless of the NO_2^- concentrations confirmed that the microorganisms were not inhibited by NO_2^- accumulation in the liquid phase. However, NO_2^- accumulation during toluene removal under denitrifying conditions seems to be inherent to the process. Thus, Peña-Calva et al. [33] and Martínez et al. [34] observed that the higher the toluene concentration in liquid phase, the higher the NO_2^- accumulated as a result of the likely inhibitory effect of toluene on the nitrite oxide reductase (Nir) of the denitrification pathway.

The coexistence of NO_3^- and NO_2^- as electron acceptors in the recycling culture broth, along with toluene and its degradation metabolites as electron donors, created a rather complex and dynamic environment inside the reactor, not easily assessable. Nevertheless, the results here obtained confirmed that toluene vapors can be continuously mineralized under anoxic denitrifying conditions, broadening the applicability of BTFs as a technology for the treatment of O_2-free VOC emissions from the petrochemical industry. However, a significant research effort is still necessary to optimize the process (especially in terms of VOC mass transfer) before considering this technology as alternative to aerobic processes.

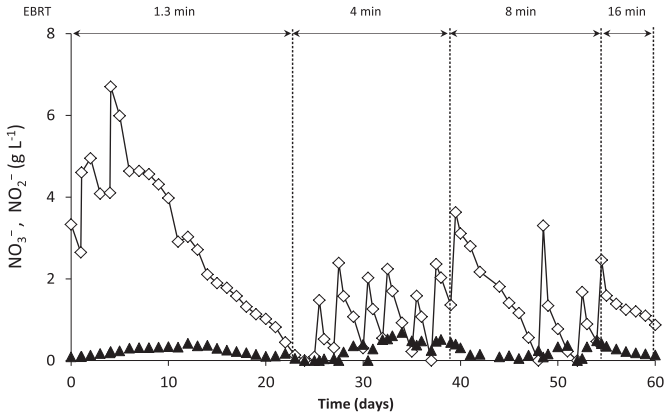

Fig. 6. Time course of (◊) NO_3^- and (▲) NO_2^- concentrations during the anoxic toluene removal.

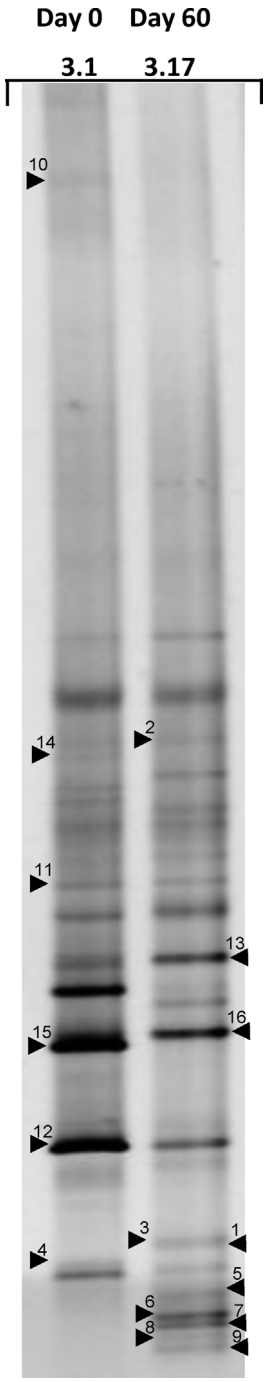

Fig. 7. Bacterial DGGE profiles of the microbial consortium inoculated in the biotrickling filter (day 0) and the microbial consortium established at the end of the reactor operation (day 60). The Shannon–Wiener diversity indices are indicated in the upper part of the gel. The sequenced bands are indicated by "►" and the corresponding number of each band.

3.5. Microbiology of the anoxic toluene mineralization

The Shannon-Wiener diversity index takes into account both the number (richness) and the evenness of the species (by evaluating and comparing the intensity of the bands), allowing to obtain semi-quantitative results from the DGGE analysis. Typical values ranging from 1.5 to 3.5 correspond to low and high species evenness and richness, respectively [35]. In our study, the Shannon-Wiener diversity indices at day 0 and day 60 were 3.10 and 3.17, respectively, which indicates the high species evenness

Table 1
RDP classification of the sequenced DGGE bands and corresponding matches (BLASTN) using the NCBI database with indication of the similarity percentages and sources of origin.

Taxonomic placement (50% confidence level)	DGGE band	Day 0	Day 60	Closest relatives in Blast Name (accession number)	Similarity (%)	Source of origin
Phylum *Actinobacteria*						
Class *Actinobacteria*						
Subclass *Actinobacteridae*						
Order *Actinomycetales*						
	1		X	*Rhodococcus sp.* (AJ007003)	94	Compost biofilter treating toluene vapors
Suborder *Corynebacterineae*						
Family *Nocardiaceae*						
Genus *Rhodococcus*						
	2	X	X	*Rhodococcus sp.* (AJ007003)	100	Compost biofilter treating toluene vapors
				Rhodococcus pyridinivorans (FR745423)	99	Sewage treatment plant
				Rhodococcus sp. (EF450777)	99	Petroleum contaminated soil
	3		X	*Rhodococcus sp.* (HM008918)	99	Activated sludge at chemical factory sewage treatment plant
				Rhodococcus ruber (JX050136)	97	Diesel contaminated soil
				Rhodococcus sp. (EU382217)	97	Activated sludge from a denitrification bioreactor
	4	X		*Rhodococcus sp.* (EU871647)	98	Sewage treatment plant sludge
				Rhodococcus pyridinivorans (FR745423)	98	Sewage treatment plant sludge
				Rhodococcus sp. (GU085235)	98	Soil near an oil refinery
	5		X	Uncultured bacterium (JX627818)	98	Membrane bioreactor treating acetone, toluene, limonene and hexane
				Rhodococcus sp. (GU196259)	98	Sludge from a sequencing batch reactor treating nitrogen-containing aromatic wastewater
				Rhodococcus sp. (EU382217)	98	Activated sludge from a denitrification bioreactor
	6		X	*Rhodococcus sp.* (FJ169467)	99	Refinery wastewater
				Rhodococcus sp. (EU382217)	98	Activated sludge from a denitrification bioreactor
	7		X	*Rhodococcus sp.* (GU085235)	99	Soil near an oil refinery
				Rhodococcus sp. (EF450777)	99	Petroleum contaminated soil
	8		X	*Rhodococcus ruber* (JX050136)	98	Diesel contaminated soil
				Rhodococcus sp. (GU196259)	98	Sludge from a sequencing batch reactor treating nitrogen-containing aromatic wastewater
				Rhodococcus sp. (EU382217)	98	Activated sludge from a denitrification bioreactor
				Rhodococcus sp. (AM113710)	98	Hydrocarbon contaminated soil
	9		X	*Rhodococcus ruber* (HQ864591)	98	Gasoline contaminated groundwater
				Rhodococcus sp. (GU196259)	98	Sludge from a sequencing batch reactor treating nitrogen-containing aromatic wastewater
				Rhodococcus sp. (EU382217)	98	Activated sludge from a denitrification bioreactor
Phylum Proteobacteria						
Class *Betaproteobacteria*						
Order *Burkholderiales*						
	10	X		Uncultured bacterium (JX310928)	94	Enriched and biostimulated culture from PAH-contaminated soil
	11	X	X	*Burkholderia sp.* (JN254804)	94	Soil
Family *Burkholderiaceae*						
Genus *Burkholderia*						
	12	X	X	*Burkholderia vietnamiensis* (FN556562)	99	Petroleum fuel samples
				Burkholderia sp. (AJ555477)	99	Industrial biofilter
				Burkholderia sp. (JN171680)	99	Phenolic compounds-contaminating sediment sample
Order *Rhocyclales*						
Family *Rhodocyclaceae*						
Genus *Thauera*						
	13	X	X	*Thauera aminoaromatica* (JX974344)	97	Solid-phase denitrification reactor
				Uncultured bacterium (HQ010714)	97	Anaerobic/aerobic/anoxic multistage sequencing batch reactor
				Uncultured bacterium (FJ167501)	97	Denitrifying bioreactor with p-cresol and sulfide as electron donors
Class *Gammaproteobacteria*						
Order *Pseudomonadales*						
Family *Pseudomonadaceae*						
Genus *Pseudomonas*						
	14	X		*Pseudomonas sp.* (AB773822)	99	Soil polluted with phenol
				Pseudomonas sp. (KC013932)	99	Activated sludge
				Pseudomonas plecoglossicida (JQ976892)	99	Oil-contaminated soil

Table 1 (*Continued*)

Taxonomic placement (50% confidence level)	DGGE band	Day 0	Day 60	Closest relatives in Blast Name (accession number)	Similarity (%)	Source of origin
	15	X		Uncultured *Pseudomonas sp.* (JF277943)	100	Biofilm grown on polyethylene terephthalate in flow cell with water from storm water canal
				Pseudomonas sp. (AB773822)	99	Phenol polluted soil
				Pseudomonas sp. (JX994141)	99	Plants growing on PAHs-contaminated site
Order *Xanthomonadales* Family *Xanthomonadaceae*						
	16	X	X	Uncultured bacterium (JN087931)	99	Nitrifying bioreactor under inorganic carbon limitation
				Uncultured bacterium (AB576911)	99	High-rate denitrifying bioreactor
				Uncultured bacterium (JN684004)	97	Activated sludge from wastewater containing high petroleum hydrocarbon content
				Uncultured bacterium (JQ038782)	97	Biotrickling filter treating methyl mercaptan, toluene, alpha-pinene and hexane

and richness. The relatively low toluene concentration in the gas phase ($0.5\,g\,m^{-3}$) likely supported the maintenance of a highly diverse bacterial community, which is in agreement with the recent findings of Estrada et al. [36] during the enrichment of aerobic toluene degrading microorganisms.

In order to elucidate the structure of the bacterial communities inside the biotrickling filter, the initial and final bacterial populations were analyzed using the Pearson similarity correlation coefficient. A similarity value of 58% was obtained between day 0 and day 60 samples, highlighting the fact that changes between the inoculated consortium and the final bacterial community after 60 days of reactor operation indeed occurred. Such differences were likely driven by the more stringent conditions established in the biotrickling filter (e.g. continuous toluene supply, dynamic metabolite production and biodegradation) compared to those prevailing in the batch cultures used for inoculum enrichment. Thus, further sequencing of the DGGE bands was carried out to elucidate these specific changes in the bacterial community.

Sixteen bands from the DGGE gel were sequenced (Fig. 7), two different phyla being retrieved according to the RDP classifier tool (bootstrap value of 50%) in the RDP database: *Actinobacteria* (9 bands) and *Proteobacteria* (7 bands). The closest matches for each band (BLASTN) according to the NCBI database are shown in Table 1 along with its similarity percentages and sources of origin. In many cases, the matches coincided with microorganisms isolated from media containing toluene, gasoline, petroleum and polycyclic aromatic hydrocarbons (PAH), and even from denitrifying bioreactors as in the case of *Rhodococcus sp.* (EU382217), *Thauera aminoaromatica* (JX974344) and the uncultured bacteria FJ167501 and AB576911.

Microorganisms in the *Actinobacteria* phylum (DGGE fragments 1–9) include aromatic and aliphatic hydrocarbon-degrading bacteria. The DGGE fragments 2–9 showed a high similarity with *Rhodococcus*, a genus associated with the biodegradation of a wide variety of environmental pollutants such as aromatic compounds and halogenated hydrocarbons [37]. The DGGE fragments 2 and 4 showed, respectively, a 99% and 98% similarity with *Rhodococcus pyridinivorans*, whose ability to degrade benzene, toluene, ethylbenzene and xylene has also been reported in the literature [38,39]. On the other hand, members of the *Proteobacteria* phylum have been also found in biological gas treatment systems [40]. The fragments 10–12 belonged to the *Betaproteobacteria* class and were affiliated with the *Burkholderiales* order. Fragment 12 showed a 99% similarity with *Burkholderia vietnamiensis* (FN556562), which was detected during toluene vapors degradation in a membrane bioreactor [41]. Fragment 13 was affiliated to *Thauera*, a denitrifying bacterium with the ability to grow on toluene under both aerobic and anaerobic conditions [42]. Fragment 16 belonged to the *Gammaproteobacteria* class and was affiliated with the *Xanthomonadaceae* family. Microorganisms classified into the *Xanthomonadaceae* family such as *Pseudoxanthomonas* are well known degraders of benzene, toluene, ethylbenzene, and o-, m-, and p-xylene [43]. Interestingly, the DGGE fragments 14 and 15, affiliated with the *Pseudomonas* genus, were found only in the inoculum, suggesting that the conditions prevailing in the BTF particularly favored the growth of microorganisms belonging to the *Rhodococcus* genus, which are able to tolerate and grow on phenol, o-, m- and p-cresol under denitrifying conditions [44].

4. Conclusions

To the best of our knowledge, the present work constitutes the first bioreactor study exploring the continuous VOC removal under anoxic denitrifying conditions. This study demonstrated the feasibility of using anoxic biotrickling filtration as an alternative treatment for VOC emissions from the petrochemical industry, which are characterized by their O_2-free nature and explosion risks when O_2 is present. The particular features of this process make it suitable as end-of-pipe technology. However, more research on VOC mass transfer enhancement is still required for process optimization. Finally, the high bacterial diversity found in the anoxic BTF, together with the ubiquity of most bacteria identified, confirmed the easy implementation of this technology.

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