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Toluene degradation capabilities of strains isolated from a peat biofilter used for the treatment of a complex mixture of VOCs

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Biofiltration is an efficient biotechnological process widely used for the treatment of industrial waste gases. In order to optimize this process, several studies have focused on operating parameters and have demonstrated their direct impacts on the pollutant removal efficiency.
But little is known concerning the structure and activity of microflora colonizing these complex ecosystems. Research into the microbial ecology, that focuses on characterizing microbial communities by their structure (i.e. their diversity, stability, spatial and temporal dynamics) and their interactions with the environment, is required to reach a better understanding of biological mechanisms occurring in biofilters and furthermore to optimize the management of this biological treatment system.
The present work comes within the context of a larger study which goal is to investigate the relationships between the pollutants removal and the structure of microbial communities in biofilter.
A microbiological study and a measurement of biodegradation activities were simultaneously carried out on two identical peat-packed columns, seeded with two different inocula, treating polluted air containing a complex mixture of oxygenated (alcohol, esters, ketones), aromatic and chlorinated compounds. It has been showed a stratification of degradation activities in function of depth. Oxygenated compounds were removed at the top of the column and aromatics at the bottom. Moreover, this distribution of biodegradation activities correlates with the spatialization of microbial density and diversity.
Furthermore, to better understand the relationship between structural and functional diversity in biofilters, it is of great interest to investigate the functional bacterial groups involved in pollutant biodegradation.
In this preliminary work, we explore the VOCs degradation capabilities of culturable strains isolated from samples collected in one laboratory peat biofilter used for the treatment of this complex mixture. An appropriate and original experimental system was used that enabled key parameters such as the concentration of gaseous compounds and the biomass growth to be taken into consideration. Toluene was used as reference molecule and the strains (13) isolated at the bottom of the reactor were considered. The results obtained show that only a *Rhodococcus* strain exhibited interesting toluene degradation capabilities.

1. Introduction

The removal of Volatile Organic Compounds (VOCs) from contaminated airstreams has become a major air pollution concern (Leson and Winer, 1991). These pollutants are subject to severe environmental constraints because of their negative impact on health (Moretti and Mukhopadhyay, 1983) and on environment (Ruddy and Carroll, 1993). Improvement of the biofiltration process, commonly used for the removal of odorous compounds, has led to a better control of key parameters such as the moisture content of the medium, temperature or pH (Devinny et al. 1999, Van Groenestijn and Kraakman, 2005; Prado et al., 2006; Vergara-Fernandez et al., 2007), enabling the application of biofiltration also to be extended to the removal of volatile organic compounds (VOCs). In a biofilter, the waste gas is forced to rise through a layer of packed porous material and pollutants contained in the gaseous effluent are oxidized or converted into biomass by the action of microorganisms previously fixed on the packing material. The quantity of water in the packing remains constant (Fanlo et al., 1998).

The biofiltration process is based on two principal phenomena: i) the transfer of contaminant from the air to the water phase or to the support medium, ii) the bioconversion of pollutants to biomass, metabolic end-products or carbon dioxide and water. The pollutant is biodegraded in the biofilm, which contains various organisms growing on the surface of the solid medium.

The diversity of biofiltration mechanisms and their interaction with the microflora mean that the biofilter is defined as a complex and structured ecosystem (Malhautier et al., 2005). As a result, in addition to operating conditions, research into the microbial ecology of biofilters is required in order better to optimize the management of this biological treatment system. More and more studies are carried out to elucidate community structures in gas biofilters (Friedrich et al., 2003; Khammar et al., 2005; Sercu et al., 2005; Cai et al., 2006; Shim et al., 2006; Chung, 2007).

Industrial gaseous emissions into the atmosphere are mostly complex mixtures of VOCs (Fanlo et al. 1998). It is therefore essential to take such gaseous effluents into consideration and to study the biodegradation mechanisms of such complex mixtures. When VOCs are introduced simultaneously into the reactor, the performance of the process is dependent on the interaction between the contaminants. For mixed pollutants, the order of biodegradation follows the potential biodegradability of the compounds involved. Micro-organisms first use the pollutants that provide the most abundant energy import. It has been observed that, for a laboratory biofilter supplied with a complex mixture of VOCs (11 pollutants) at a concentration of 50 mg. m^3 for each compound, oxygenated compounds are removed first and more efficiently compared with aromatic and halogenated compounds (Khammar et al., 2005). A stratification of degradation activities in function of depth has also been highlighted. Oxygenated compounds are removed at the top of the column and aromatics at the bottom. Moreover, Khammar et al. (2005) characterized the culturable microflora having colonized this biofilter and members of gram positive bacteria with high GC content and *Proteobacteria* have been isolated. Hence, it is probable that the genera of bacteria isolated from samples collected at the bottom of the column possess aromatic degradation capabilities, given that these compounds are removed at this depth.

The aim of this work therefore focuses on the growth detection of reference culturable strains during VOCs degradation. The VOCs degradation capabilities of the strains isolated from samples collected at the bottom of the biofilter were measured. An appropriate and original experimental system was then used, allowing key parameter such as the concentration of gaseous compounds and the biomass growth to be considered. Toluene was used as reference molecule.

2. Material and Methods

2.1 Chemicals

Toluene was obtained from Carbo Erba Reagenti, 99% (Rodano, Italy). A stock solution was made up as follows. Pure liquid toluene was injected by means of a 10-µL syringe into sterile 500-ml flasks containing 250 mL of mineral salt solution and sealed with a Teflon-coated cap (Interchrom; Montluçon, France), to attain a concentration in the gas phase of around 8 $g.L^{-1}$, after water-air compound equilibration according to Henry's law.

2.2 Bacterial Strains

Different strains were isolated as previously described (Khammar *et al.*, 2005). During the present investigation, the microorganisms were routinely maintained at 4°C on a Trypticase Soy Agar medium diluted ten-fold (TSA1/10). Before inoculation, a bacterial suspension was obtained by preparing a TSA1/20-grown liquid culture of each strain.

A suspension of isolated *Rhodococcus erythropolis* strain in 100 µL of sterile water was produced in a 1.5-mL tube. DNA was extracted from cells by using the kit NucléoSpin Tissue (Macherey-Nagel, Hoerdt, France) according to the manufacturers' instructions. The SSU rDNA was then amplified by PCR using the rDNA universal primer W002 (5'GNTACCTTGTTACGACTT3', position R1492) with the rDNA bacteria primer W018 (5'GAGTTTGATCMTGGCTCAG3', position F9) for 16S rDNA amplification. DNA products were sequenced (Millegen, Toulouse, France). A sequence of 1500pb was carried out and used for comparison with sequences available in a database (Genbank). Moreover, a SSU rDNA sequence of 450 bp was amplified by PCR using two *Rhodococcus erythropolis* specific primers (5'CGTCTAATACCGGATATGACCTCCTATC3', position F165) and (5'GCAAGCTAGCAGTTGAGCTGCTGGT3', position R633) (Stackebrandt et al., 1988).

2.3 Degradation Kinetics

Tests were performed in 500-ml sterile flasks containing 250 mL of mineral salt solution and sealed with a Teflon-coated cap (Interchrom; Montluçon, France). The mineral salt nutrient solution HCMM2 (Ridgway et al. 1990) was modified by Juteau et al. (1999) and called HCMM3. The flasks are directly connected to a HP5890 gas chromatograph *via* sterile 1/16" Teflon-tubes. Toluene was added as the sole carbon and energy source. The stock solution described above allowed liquid toluene volume to be injected in order to attain an initial concentration in the gas phase in a range of around 30 to 2000 $mg.m^{-3}$. Pure liquid toluene was injected by means of a 10-µL syringe in

order to attain an initial concentration in the gas phase in a range of around 3000 to 5000 mg.m^{-3}. After water-air substrate equilibration according to physical laws and air-tightness control (24 hours), flasks were inoculated with a bacterial suspension (3 mL) containing 10^9 cells per mL. Incubation was performed at 28°C at an agitation speed of 200 rpm. The biodegradation was monitored by on-line gas phase measurement of substrate consumption until it was exhausted.

Toluene contents were determined by injecting gas samples into an HP5890 Series gas chromatograph (Hewlett Packard, Palo Alto, USA). The gas sample was injected into the column every twenty minutes *via* an automatic gas sampling valve operated by the laboratory technical staff. A 30-metre HP-1 capillary column (Hewlett Packard, Palo Alto, USA) was used with a carrier gas (Helium) flow rate of 15 mL. min^{-1}. The temperatures of the column and the flame ionization detector were 40°C and 220°C respectively. The detection limit obtained for toluene was 1 mg. m^{-3}.

The maximum toluene consumption rate was obtained from experimental data by using linear regression between three consecutive experimental values.

2.4 Microbiological Procedures

In order to measure the bacterial growth, for each experiment and concomitantly with the experimental flask, a control with no carbon source was inoculated with the bacterial suspension (3 mL) containing 10^9 cells per mL. This flask allows the initial bacterial count to be achieved. Bacteria were enumerated by fluorescence microscopy using staining with 4',6-diaminido-2-phenylindole (DAPI) (Sigma, USA) to define the total number of bacteria (Rodriguez et al. 1992).

Firstly, 20 mL-bacterial suspensions coming from the experimental and control flasks were sampled and treated as follows. Cells were then fixed with 3.7 % formaldehyde for 30 min before staining with DAPI at a final concentration of 1.25 µg. mL $^{-1}$ for 15 minutes in the dark. Stained bacteria were recovered by microfiltration through 0.2 µm polycarbonate membrane microfilters (Millipore GTBP, Ireland). The microfilters were then mounted on microscope slides in Mounting Medium (Sigma, USA) and examined using an epifluorescence microscope (Leica DMLB) equipped with a blue excitation filter (BP 340-380 nm) and a barrier filter LP 425.

3. Results

Different strains were isolated from a peat biofilter on ten-fold diluted Trypticase Soy Agar medium (Table 1) (Khammar *et al.*, 2005).

Biofilter depth	Species	Group	Similarity	Accession number
0.7 m	Uncultured eubacterium WR 857 *(PAH polluted soil)*	ND	94%	AY216882
0.7 m	*Micrococcus luteus*	HGC%	98%	AY216883
0.7 m	Actinomycete *(chlorophenol degrading reactor)*	HGC%	94%	AY216884

Biofilter depth	Species	Group	Similarity	Accession number
0.7 m	*Gordona polyisoprenivorans*	HGC%	99%	AY216885
0.7 m	*Burkholderia sp.*	β-*Proteobacterium*	97%	AY216886
0.7 m	Unidentified *(hydrocarbon seep sediment)*	β-*Proteobacterium*	99%	AY216887
0.7 m	*Pietermaritzburg bacteria*	γ-*Proteobacterium*	87%	AY216888
0.7 m	*Burkholderia sp.*	β-*Proteobacterium*	97%	AY216889
0.7 m	*Frateuria aurantia*	γ-*Proteobacterium*	92%	AY216878
0.9 m	Clone HPS-54 *(tropical soil sample)*	ND	97%	AY216890
0.9 m	Actinomycete *(faeces from African millipede)*	HGC%	94%	AY216891
0.9 m	*Burkholderia sp.*	β-*Proteobacterium*	97%	AY216683
0.9 m	*Rhodococcus erythropolis*	HGC%	99%	DQ518913
0.9 m	*Massilia timonae*	β-*Proteobacterium*	97%	AY216892

Table 1: Phylogenetic affiliation of best matching sequences of microbial strains isolated from samples collected at 0.7 and 0.9 m in depth in a peat biofilter.

These strains were isolated at the bottom of the column where aromatics were removed. Among the isolated strains, only *Rhodococcus erythropolis* (Accession number DQ518913) presented toluene degradation capabilities when the applied concentration varied between 30 to 6000 $mg.m^{-3}$. The maximum toluene consumption rate varied between 6.5 and 920 µg toluene. h^{-1}. The identification of this strain has been confirmed by using 16S rDNA specific primers of *Rhodococcus erythropolis*.

For each experiment, the thermodynamic equilibrium was reached and maintained for 24 hours before inoculating cells. For all experiments carried out, the relative standard deviation of toluene quantity measurement, obtained from the 24 hours-thermodynamic equilibrium test, varies between 0.6 and 2.4 %.

Figure 1 shows the obtained results for three initial toluene quantities 200, 400 and 6380µg that correspond to gas concentration varying between 165, 330 and 5270$mg.m^{-3}$ respectively. The results showed that toluene quantity introduced in the flask was completely biodegraded by microorganisms. These experiments highlighted that the lag phase was longer as the initial toluene quantity increased.

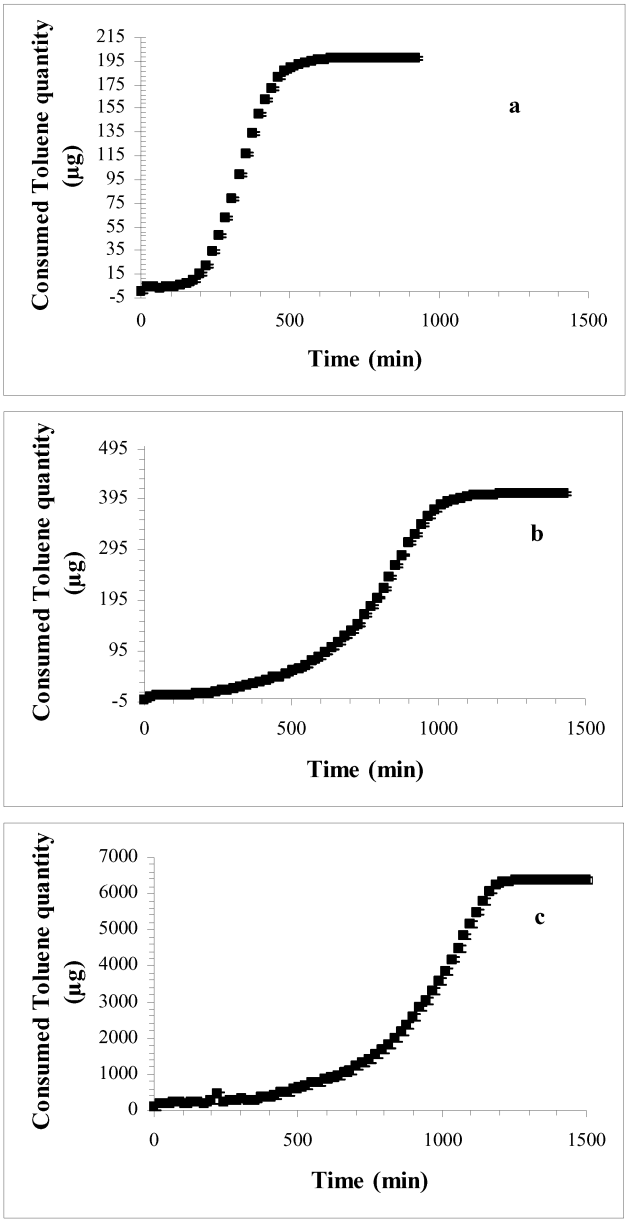

Figure 1: Toluene degradation kinetics. a: initial toluene quantity : 195 µg; b: initial toluene quantity: 395 µg; c: initial toluene quantity: 6300 µg. ■ *Toluene consumed for biodegradation assay. RSD (%), obtained from the 24 hours-thermodynamic equilibrium test, is indicated.*

Bacteria have been counted for each experiment. At the beginning of the assay, the bacterial number remained stable around 10^6-10^7 bacteria.mL^{-1}, whatever the

experiment. No bacterial growth was detected for toluene concentration inferior to 2000 mg. m^{-3}, since the counts obtained at the end of the experiment were equivalent to the initial ones. For toluene concentrations ranged between 2000 and 6000 $mg.m^{-3}$, a bacterial growth was detected, the number of cells increased with a factor 10.

4. Discussion

The system used was original and presents certain advantages.
i) Before investigating degradation kinetics and therefore inoculating microorganisms, it is of prime importance to check that thermodynamic equilibrium is reached. The transfer of pollutants from the gas phase to the liquid phase must therefore be controlled. This transfer of pollutants occurs according to physical laws. Concentrations in the water will be proportional to those in the air and the constant of proportionality is Henry's constant. Models of biofiltration generally support this since they treat the biofilm like water by using Henry's law to predict mass transfer rates inside the biofilm where degradation occurs (Miller and Allen, 2004). ii) The stability of the toluene concentration in the headspace has to be checked. This first step allows then the air-tightness of the flask to be controlled. iii) The gas sample is injected into the column *via* automatic gas sampling valve that allow the time lapse between two samples to be reduced. An analysis can then be performed every 20 minutes. iv) The volume sampled (250 µL) is low and does not allow the headspace to be depleted with toluene.
The results obtained show that only a *Rhodococcus erythropolis* strain exhibited toluene degradation capabilities. It has been shown that no less than 99% of the microorganisms observable in nature cannot be cultured using conventional techniques (Amann et al. 1995). Then, it can be suggested that uncultured strains present aromatic biodegradation activities. Furthermore, it will be interesting to consider the biodegradation activities of the complex microflora.
Some authors (April et al., 1998; Bell et al., 1999; Gulensoy and Alvarez, 1999; Woertz and Kinney, 2000) showed the ability of micro-organisms to use several VOCs as a single carbon source. Chemical pollutants which can be degraded by *Rhodococci* range from simple hydrocarbons through chlorinated hydrocarbons, aromatic hydrocarbons and nitroaromatics to chlorinated polycyclic aromatics such as polychlorinated biphenyls (PCBs) (Bell et al., 1998, Goodfellow et al., 2004). Hence, it is not surprising that the *Rhodococcus erythropolis* strain isolated from a biofilter removing a complex VOC mixture containing aromatics exhibits toluene biodegradation capabilities.
It has been observed that the lag phase was longer as the initial toluene quantity increased. It can be assumed that the cells have to adapt their metabolism (enzymes synthesis) to more and more toxic functioning conditions. Nevertheless, the obtained results highlight that this *Rhodococcus* strain achieves to adapt to toxic functioning conditions.
No microbial growth was detected by counting the total number of cells when the concentration is inferior to 2000 $mg.m^{-3}$. It seems probable that, for these toluene concentrations, the energy obtained from the toluene biodegradation are used for maintenance energy and not available for biosynthesis and cell growth.
These results showed that this system seems to be appropriate for estimating the biodegradation ability of pollutants by a bacterial strain or a microbial consortium. This

information is important because the detection of the biodegradation activity of microorganisms could be used as indicator of the functionality of a microbial consortium, in term of pollutant biodegradation. This system could also be used to study the interactions between substrates by considering the degradation capabilities of a mixture of compounds.

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