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# Structural and functional responses of sewage microbial communities used for the treatment of a complex mixture of volatile organic compounds (VOCs)

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## Keywords

bioprocessing, degradation, diversity, ecology, microbial structure, pollutant, stress response.

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## Abstract

**Aims:** The aim of this work was to assess the impact of the applied mass loading on the selection of an efficient microbial community able to degrade a complex mixture of volatile organic compounds (VOCs).

**Methods and Results:** Two reactors were used and were supplied with a gaseous effluent containing 11 VOCs with different concentrations. The response of the microflora was monitored as a function of time: biodegradation activity, bacterial density and diversity. The results showed that the applied mass loading seems to have an impact on the functioning and the genetic structure of the bacterial community.

**Conclusions:** A high mass loading seems to induce a low efficient functioning in terms of elimination efficiency and a simplification of the genetic structure of the total bacterial community with the apparition of a dominant microflora. A low mass loading seems to favour a better functioning and allows to keep a healthier bacterial diversity.

**Significance and Impact of the Study:** In the treatment processes of gaseous effluents, it would be judicious to define the functioning parameters of the process to keep the diversity of important functional bacterial groups. These results provide also useful information about changes in microbial communities following natural or anthropogenic alterations in different ecosystems.

## Introduction

In recent years, biological processes have been found to be an attractive alternative to treat waste gases when compared to conventional thermal and physicochemical techniques. They are more environmentally and economically viable as it has already been proven in several applications (Devigny *et al.* 1999). The improvement in these biological processes (biofilter, biotrickling and bioscrubber) has led to a better control of key parameters (Dirk-Faitakis and Allen 2003; Lackey *et al.* 2003; Sanchez-Monedero *et al.* 2003; Canovai *et al.* 2004; Elmrini *et al.* 2004), enabling the application of these bioprocesses to be also extended to the removal of volatile organic compounds (VOCs), which are harmful for humans and the environment. The gaseous effluents emitted into the atmosphere

by industries are often a complex mixture of different VOCs. Therefore, to control and optimize this process on an industrial scale, it is interesting to study the removal efficiency of laboratory scale pilot units whose inlet gaseous effluent is a VOC mixture. Moreover, most of the studies have been carried out for one pollutant or a family of VOCs such as aromatic compounds or ketones or chlorinated compounds. (Roberge *et al.* 2001; Aizpuru *et al.* 2003; Delhomenie *et al.* 2003; Atoche and Moe 2004). It is therefore essential to take such gaseous effluents into consideration.

Although these studies have led to an improvement in the performance of biological processes, research into the microbial ecology of these bioreactors for gaseous compounds removal is necessary to optimize the management of this biological treatment system (Van Groenstijn and

Hesselink 1993). Recent breakthroughs in molecular techniques, which overtake the limitations of the cultural techniques, have been applied to characterize microbial community structure in biological reactors such as wastewater treatment plants (Collins *et al.* 2005), contaminated soils (Becker *et al.* 2006; Joynt *et al.* 2006) and biotechniques for air pollution control (Friedrich *et al.* 2002; Khammar *et al.* 2005). Friedrich *et al.* (2002) investigated the microbial diversity of a full-scale biofilter used for waste gas treatment in an animal-rendering plant and showed that microbial diversity was high and biofilters were commonly inoculated with uncharacterized micro-organisms. Moreover, in these ecosystems, microbial diversity has to be related to environmental parameters because physicochemical properties of pollutant mixtures such as water solubility, biodegradability, partition coefficients and inhibitory effects influence the structuring of degradation activities and microbial communities (Khammar *et al.* 2005).

The functioning of these biotechniques for air pollution control can be subdivided into two main periods: start-up and steady states. The start-up procedure may last from a few days to a few months and consists of the selection of the efficient microbial communities (Devinny *et al.* 1999). To improve this stage, that is to say to reduce the delay allowing the steady state to be reached, *microflora* implementation has been considered. Usually, this stage refers to the adaptation of a microbial *consortium* with a broad substrate capacity – such as activated sludge coming from wastewater treatment plants (wwtp) to the VOC mixture. It consists of forcing a gaseous effluent containing VOCs through the suspension of activated sludge. It is assumed that the selection of micro-organisms colonizing activated sludge and able to metabolize hardly biodegradable compounds would be favoured. An aggressive treatment is commonly used (Tellez *et al.* 2002): the *microflora* is supplied with air containing VOCs for which the concentration is high. The reactor is then seeded with this adapted microbial *inoculum*. What are the dynamics of the microbial community structure? Does this enrichment step involve a decrease of micro-organisms' diversity? Does this loss depend on the concentration of each compound introduced in the reactor?

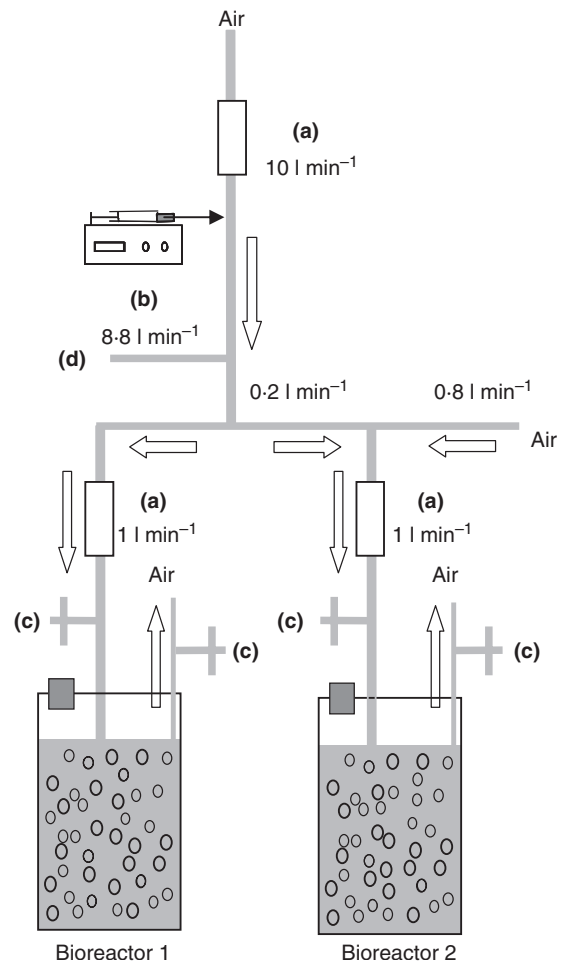
Then, the aim of this work was to evaluate the impact of the applied mass loading on this adaptation phase. Hence, the dynamics of the microbial community structure and the distribution of biodegradation efficiency in two parallel bioreactors filled with activated sludge and treating a controlled polluted airstream containing a complex mixture of VOCs, with different mass loading, were investigated. The complex mixture of VOCs with different polarity, solubility and biodegradability was representative

of real industrial chemical emissions. One reactor was, with a mass loading that commonly supply biofilters ( $100\text{--}200\text{ g VOCs h}^{-1}\text{ m}^{-3}$  packing material), used for the treatment of such gaseous effluents; the second was supplied with a high mass loading. Specifically in this study, Single Strand Conformation Polymorphism (SSCP) of PCR amplified community structure 16S rDNA was used to compare readily the community structure of these two reactors.

## Materials and methods

### Reactor design

Two identical laboratory scale pilot units were used (Fig. 1). Each unit consisted of a 5-l glass reactor filled with 4 l of a suspended biomass sampled from the



**Figure 1** Pilot scale units. (a) Flow rate regulators; (b) syringe pump loaded with the mixture of 11 volatile organic compounds (VOCs); (c) gas sampling; (d) gas split.

activated sludge tank in a domestic sewage treatment plant of Saint Christol lez Alès (France). The liquid VOC mixture was continuously injected and vaporized into an air stream operating at  $10 \text{ l min}^{-1}$  via a syringe pump Precidor (Infors AG, Switzerland). Some of the polluted air ( $1 \text{ l min}^{-1}$ ) was forced through each reactor. The internal diameter of the gas diffuser was  $0.625 \times 10^{-2} \text{ m}$ . Both bioreactors were rightly homogenized by gas diffusion into activated sludge. The composition of the activated sludge was 4.5 g of chemical oxygen demand per litre, 1.1 g of biological oxygen demand per litre and 5.2 g of total suspended solids per litre. The volume of the liquid phase was maintained constant (4 l) by the regular addition of a mineral salt nutrient solution HCMM3 defined by Juteau *et al.* (1999). The experiment was performed at normal room temperature (20–25°C) and a neutral pH was maintained using a NaOH solution ( $1 \text{ mol l}^{-1}$ ).

### VOC mixture

Close to industrial realities, a wide range of VOCs were used. The mixture of VOCs contained 11 compounds divided in respectively oxygenated compounds with methanol, acetone, methyl ethyl ketone (MEK), methyl isobutyl ketone (MIBK), butyl and ethyl acetates (BA, EA), aromatic compounds with toluene, ethylbenzene, p-xylene and halogenated compounds with dichloromethane (DCM) and 1,2-dichloroethane (DCE) (Carlo Erba Reagenti, France, 99% puro). The mixture of pollutants was prepared by mixing equal masses of the VOCs mentioned above.

### Concentration of compounds in the inlet gas stream

The concentration of each compound in the inlet gas stream was 700 and 140  $\text{mg m}^{-3}$  for reactors 1 and 2 respectively. The applied mass loadings were then 115.5 and 23.1  $\text{g VOCs h}^{-1} \text{ m}^{-3}$  activated sludge for bioreactors 1 and 2 respectively. Both reactors were monitored for 38 days.

### Biodegradation monitoring

The biodegradation of VOCs was measured daily by monitoring the concentration of each compound at the sampling inlets and outlets. The reactor sampling points were directly connected, via Teflon tubes, to a gas chromatograph unit (HP 6890; Hewlett Packard) equipped with a flame ionization detector. A 30-m HP-5 capillary column was used with a carrier gas (Helium) flow rate of  $2 \text{ ml min}^{-1}$ . The gas sample was injected into the column via an automatic gas sampling valve (250  $\mu\text{l}$ ); hence, the gas samples were not stored.

The concentration of a compound was defined as follows: the area of the peak was directly related to the concentration via the calibration of the chromatograph.

The biodegradation monitoring was estimated by calculating the VOC removal efficiency as follows:

$$\text{RE} = \left(1 - \frac{C_o}{C_i}\right) \times 100 \quad (1)$$

$C_o$ : VOC outlet concentration ( $\text{g m}^{-3}$ ),

$C_i$ : VOC inlet concentration ( $\text{g m}^{-3}$ ),

RE: removal efficiency (%).

The error in the measurement of VOC concentration was assessed using an empty reactor. The pollutant concentration was determined nine times, enabling the relative standard deviation (RSD) to be estimated at 10%.

The concentration of VOCs in liquid phase concentration was estimated according to the standard addition method. An increasing volume of a liquid mixture of MEK, MIBK, toluene, ethylbenzene, p-xylene, DCM and DCE was supplied in four air-tight tablet bottles to obtain a concentration of 0, 25, 50 and 75 mg of each VOC per litre for each sample of activated sludge. Each tablet bottle containing a final liquid volume of 4 ml was stirred for 30 min at 20°C. The headspace was then analysed by gas chromatography as described above. The plot of the area of the peak as a function of the quantity of each added compound allowed the initial concentration to be estimated.

### Direct cell count

The bacteria from the bioreactor sludge were enumerated by fluorescence microscopy using staining with DAPI (4',6-diamidino-2-phenylindole, Sigma, USA) to determine the total bacteria number (Kepner and Pratt 1994).

A volume sample (2 ml) was diluted in 9 volumes of 1% sodium hexametaphosphate, and homogenized using a blender (Ultra Turax, T25 basic, IKA, Staufen, Germany) for 2 min at  $19\,000 \text{ rev min}^{-1}$ . Then, 1 ml of this suspension was incubated with 3.7% formaldehyde for 30 min, before counter-staining by DAPI at a final concentration of  $20 \mu\text{g ml}^{-1}$  for 1 h, in the dark, at an agitation of  $200 \text{ rev min}^{-1}$ .

Stained bacteria were recovered on  $0.2 \mu\text{m}$ -polycarbonate membrane filter (Millipore GTBP, USA) by microfiltration. The filters were then mounted on microscope slides in Mounting Medium (Sigma Aldrich, USA) and observed using an epifluorescence microscope (DMLB, Leica, Germany) equipped with a UV excitation filter (BP 340–380 nm) and an LP 425 barrier filter. For each slide, 30 fields were counted. The ability of the data was expressed in terms of relative standard deviation (RSD).

### Analysis of total DNA by PCR-SSCP (Single Strand Conformation Polymorphism)

The DNA extraction method has already been described (Godon *et al.* 1997). The DNA amplified target was the V3 region of the 16S rDNA gene for the bacteria domain (primers W104 and W049) (Table 1). Primer W104 was labelled by a 6-FAM fluorochrome. Each reaction tube contained 100 ng of each primer, 10 mM dNTPs, 1X PFU Turbo DNA polymerase reaction buffer with 25 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 1.25 U Pfu Turbo DNA polymerase (Stratagene) and 1 µl of purified template DNA adjusted to a total volume of 50 µl.

The reaction mixtures were placed in a thermocycler (Perkin Elmer) at 94°C for 30 s. Three-stage cycles were then performed 30 times: at 94°C for 30 s, at 61°C for W104/W49 for 30 s, 72°C for 30 s and a final stage at 72°C for 15 min.

The PCR products were diluted depending on the band intensity on the 2% agarose gel. A 1 µl of diluted PCR products was mixed with 18.8 µl of loading buffer TSR (Applied Biosystem) and 0.2 µl of internal weight standard Genescan-400 HD Rox (Applied Biosystem). The double-strand helix was then separated for 2 min at 94°C, and immediately cooled in an ice-water bath for 10 min, ensuring that single-stranded products were obtained. Capillary electrophoresis SSCP analysis was performed on an ABI PRISM 310 automated DNA sequencer (Applied Biosystem) equipped with a capillary tube filled with a polymer, for 30 min at 12 kV and a temperature of 32°C. A sample volume of 5 µl was loaded onto the polymer [Genescan polymer (7% w/w) at 8 mg ml<sup>-1</sup>, Glycerol 10%, TBE 1X].

In SSCP fingerprint, each 16S rDNA molecule has a reproducible migration which corresponds to one peak in SSCP pattern. Data processing was performed with the GENESCAN software (Applied Biosystems). The samples were aligned using the internal lane standard peaks as follows: seven peaks of the standard, which framed the sample peaks, were assigned a value using the scan numbers and the same values were used for the different

**Table 1** Primers used to amplify the 16S rDNA and their characteristics

Name	Sequence	Position*	Reference
W104	5'-6-Fam -TTACCGCGCTGC TGGCAC-3'	R533	Zumstein <i>et al.</i> 2000
W049	5'-ACGGTCCAGACTCCTAC GGG-3'	F331	Zumstein <i>et al.</i> 2000
W018	5'-GAGTTTGATCMTGGCTC AG-3'	F9	Godon <i>et al.</i> 1997
W031	5'-TTACCGCGGCTGCTGGC AC-3'	R500	Zumstein <i>et al.</i> 2000

\*Positions were defined by the *Escherichia coli* sequence.

samples (Zumstein *et al.* 2000). This software also allows co-migrated peaks to be detected.

### Amplification, sequencing and phylogenetic analysis

The bacterial community of bioreactor 2 was inventoried by using partial 16S rDNA sequence. These sequences had been amplified with the universal W031 primer and W018 bacterial domain primer (Table 1).

Each reaction tube contained 1U of red Taq DNA polymerase (Sigma Aldrich), 1 µl DNA, 5 of Taq Red 10× buffer, 40 mmol of dNTP (Promega), 0.2 µg of each primer (Sigma Aldrich) adjusted to a final volume of 50 µl. Three-stage cycles were then performed 30 times: at 94°C for 1 min, 1 min at 55°C, 1 min at 72°C and a final stage at 72°C for 10 min. The amplification products were verified on 2% agarose gel.

Firstly, the amplification products were purified (QIA-quick PCR purification kit, Qiagen), then inserted into the pGEMt-vector as recommended by the TOPO cloning kit manufacturer (Invitrogen). The clones (186) were seeded on two microplaques in the presence of 2YT containing ampicillin and glycerol. The sequencing was achieved by Millegen society (Toulouse, France). The sequences marked 'bad' according to the software PHRED analysis were removed (Ewing and Green 1998; Ewing *et al.* 1998). From this analysis, four sequences were discarded. The other sequences counted 500 pb. The sequences were compared with EMBL data (December 2006). The processing of the sequences was performed by using ARB software (Lehrstuhl für Mikrobiologie, Technische Universität München, Freising, Germany) (Ludwig *et al.* 2004). The previous sequence database of ARB, named SSU January2003, was completed with some 16S rDNA sequences that had previously been isolated from activated sludge samples (Bond *et al.* 1995; Christensson *et al.* 1998; Hesselmann *et al.* 1999; Lapara *et al.* 2000; Dabert *et al.* 2001; Crocetti *et al.* 2002; Juretschko *et al.* 2002; Wagner and Cloete 2002; Chouari *et al.* 2003; Eschenhagen *et al.* 2003) and from biofilter samples (Khammar *et al.* 2005).

Every sequence was treated as follows: alignment of sequences by using tools of ARB software, manual corrections of alignments were performed before introducing the sequences in a reference tree by using the parsimony method and the determination of operational taxonomic unit (OTU) by performing the similarity matrix. The cloned sequence types with more than 97% identity were considered to belong to the same OTU.

### Results

Two parallel bioreactors, which were inoculated with activated sludge from an urban wastewater treatment plant,

were designed to run under identical operating conditions (Fig. 1). Each reactor was supplied with a complex synthetic gaseous effluent containing oxygenated, aromatic and chlorinated compounds, but the concentration of each compound differed. The concentration of each pollutant was  $700 \text{ mg m}^{-3}$  and  $140 \text{ mg m}^{-3}$  for bioreactors 1 and 2 respectively. The pollutant removal, the dynamics of microbial densities and the microbial diversity were monitored as a function of time.

### Pollutant removal for bioreactors 1 and 2

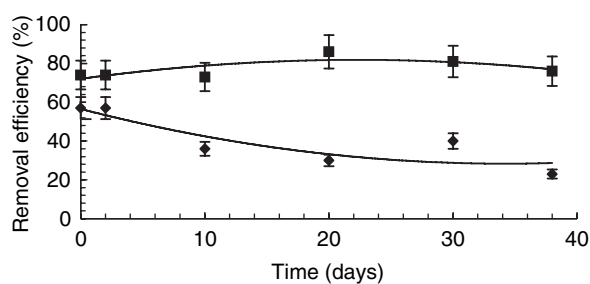
Figure 2 presents the elimination efficiency of VOCs, expressed in percentages, for both bioreactors.

It can be noted that elimination efficiency measured at the beginning of the experiment was 55% for bioreactor 1 and 75% for bioreactor 2 (Fig. 2). This result can be explained by the aqueous solubility of the compounds. The aqueous solubility given by the Henry coefficient (at  $20^\circ\text{C}$ , partition of compound in aqueous phase upon gas phase) (Sander 1999) is as follows: weak for aromatic ( $0.13 \text{ mol l}^{-1}$  per atm) and chlorinated compounds ( $0.36 \text{ mol l}^{-1}$  per atm for DCM and  $0.73 \text{ mol l}^{-1}$  per atm for DCE) and high for esters ( $3.1 \text{ mol l}^{-1}$  per atm for butyl acetate and  $6.2 \text{ mol l}^{-1}$  per atm for ethyl acetate), ketones ( $2.2 \text{ mol l}^{-1}$  per atm for MIBK,  $20 \text{ mol l}^{-1}$  per atm for MEK and  $30 \text{ mol l}^{-1}$  per atm for acetone) and methanol ( $220 \text{ mol l}^{-1}$  per atm).

For bioreactor 1, the elimination efficiency measured decreased from 60% to 30% at  $t = 26$  days and remained stable until  $t = 38$  days. For bioreactor 2, the elimination efficiency remained close to 80% from the beginning to  $t = 38$  days.

Table 2 presents the elimination efficiencies obtained for oxygenated, aromatic and chlorinated compounds for both bioreactors.

For bioreactor 1, the oxygenated compounds were removed and the elimination efficiency reached 45% whereas for bioreactor 2, oxygenated compounds were



**Figure 2** Removal of volatile organic compounds (VOCs) for both bioreactors as a function of time.  $\blacklozenge$  Bioreactor 1,  $\blacksquare$  Bioreactor 2, — polynomial curve (order 2).

**Table 2** Elimination capacity (EC:  $\text{g removed VOCs h}^{-1} \text{ m}^{-3}$  activated sludge) and elimination efficiency (EE: %) reached for volatile organic compounds (VOC) chemical groups. The applied mass loading (ML:  $\text{g VOCs h}^{-1} \text{ m}^{-3}$  activated sludge) is indicated

Compounds	Bioreactor 1			Bioreactor 2		
	ML	EC	EE	ML	EC	EE
Oxygenated	63	28.4	45	12.6	12.6	100
Aromatic	31.5	0	0	6.3	3.5	55
Chlorinated	21	0	0	4.2	2.1	50

NR, not removed.

completely eliminated and the elimination efficiency of aromatics and halogenated compounds reached 55% and 50% respectively.

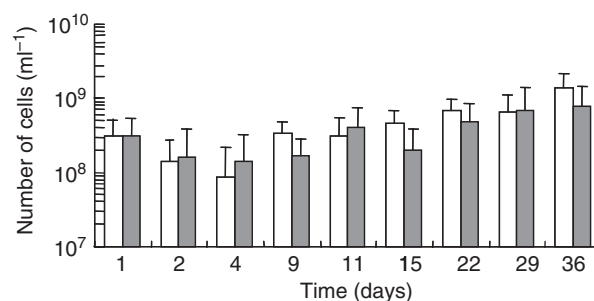
All compounds of each chemical family behaved similarly for bioreactor 2. For bioreactor 1, the esters were completely removed while the elimination efficiency of MEK, methanol and acetone and MIBK decreased from values close to 100% at the beginning of the experiment to 30% and 25% and 0% respectively at  $t = 38$  days.

The liquid phase concentrations have been estimated to 720 and 40  $\text{mg VOCs per litre}$  for bioreactors 1 and 2 respectively.

These results seem to show a negative correlation according to the microflora functioning: a better elimination efficiency was reached when the bioreactor was supplied with the complex VOCs mixture at a low concentration. Moreover, different chemical families of compounds were removed.

### Quantitative Microbial Dynamics

The cell densities were monitored as a function of time for 38 days (Fig. 3). The results obtained by enumerating total bacteria stained with DAPI show that the bacterial densities followed the same evolution. The number of total bacteria reached at  $t = 36$  days remains similar:



**Figure 3** Colonization dynamics for both bioreactors.  $\square$  Bioreactor 1,  $\blacksquare$  Bio-reactor 2.

$1.4 \times 10^9$  and  $7.7 \times 10^8$  cells  $\text{ml}^{-1}$  for bioreactors 1 and 2 respectively. The flocs were destroyed as described in Material and Methods section.

The applied mass loading seems to have an impact on the removal of compounds in terms of elimination efficiency and chemical nature of the biodegraded chemicals without having an influence on the densities of the total bacterial community.

### Structure of Total Bacterial Communities

The SSCP method was used to define the structure of total bacterial diversity at the dominant level in activated sludge samples collected as a function of time for both bioreactors (Fig. 4).

On a given SSCP pattern, each peak can be correlated with the V3 16S rDNA sequence of one micro-organism. The SSCP profile analysis at the beginning of the experiment ( $t = 0$ ) reveals 25 peaks. Between  $t = 0$  and  $t = 38$  days, the evolution of the genetic structure differs for both bioreactors. For bioreactor 1, the SSCP analysis of total bacterial DNA extracted from samples reveals 15

dominant peaks among which 10 peaks are dominant on the left of the pattern, whereas for bioreactor 2, the number of peaks remains stable without any dominant peak. Hence, at  $t = 38$  days, the SSCP patterns obtained for both reactors are different.

A simplification of the genetic structure of the total bacterial community is then observed for bioreactor 1 supplied with the highest mass loading. Moreover, a dominant bacterial community appeared. However, when the bioreactor was supplied with the VOC complex mixture at low concentration, a large bacterial diversity was maintained.

As bacterial consortium in the VOC-degrading aerobic reactor 2 achieved the highest elimination efficiency with the biodegradation of aromatics and chlorinated compounds, it has been chosen to perform a partial characterization of this consortium. A total of 182 sequences retrieved from 16S rDNA were clustered to 90 OTU (Table 3 and Fig. 5). The majority of the sequences (85%) was split into five divisions: 31 OTU (37% of the sequences) are related to the group CFB, 16 OTU (17% of the sequences) and 14 OTU (20%) are related to the  $\alpha$ -Proteobacteria and  $\beta$ -Proteobacteria subdivision respectively, 4 OTU (10%) are related to Cyanobacteria, 5 OTU (7%) are closed to the sequences of group TM7. The remaining sequences are distributed throughout the following divisions: *Planctomycetes* (6 OTU/7 sequences), firmicutes (5 OTU/7 sequences),  $\epsilon$ -Proteobacteria (2 OTU/3 sequences), *Verrucomicrobium* (1 sequence), *Actinobacteria* (3 OTU/4 sequences), OP11 (1 OTU), *Chlorobi* (1 OTU), *Chloroflexi* (1 OTU/2 sequences). Among the OTU, 29% are represented by a single sequence. The coverage (C) reached 71%; it was calculated according to the formula defined by Good (1953) (Wagner *et al.* 2002):

$$C = \left(1 - \frac{n}{N}\right) \times 100 \quad (2)$$

$N$ : Number of all sequences in the 16S rRNA gene clone library,  $n$ : Number of OTUs consisting of only one sequence.

The percentage of similarity with the sequences previously defined in Genbank database ranges from 88 % to 100 %. A similarity higher than or equal to 97% with a sequence contained in the public information database is observed for 39 OTU, representing 99 sequences. Six dominant OTUs (23 sequences) are close to sequences coming from cultivable organisms: 10 sequences are close to *Novosphingobium hassiacum* (AJ416411), 2 sequences are close to *Sphingomonas sp.* JS1 (AJ427917), 1 sequence is related to *Sphingopyxis sp.* C-1 (AB161684), 3 sequences are related to *Hyphomicrobium vulgare* IFAM

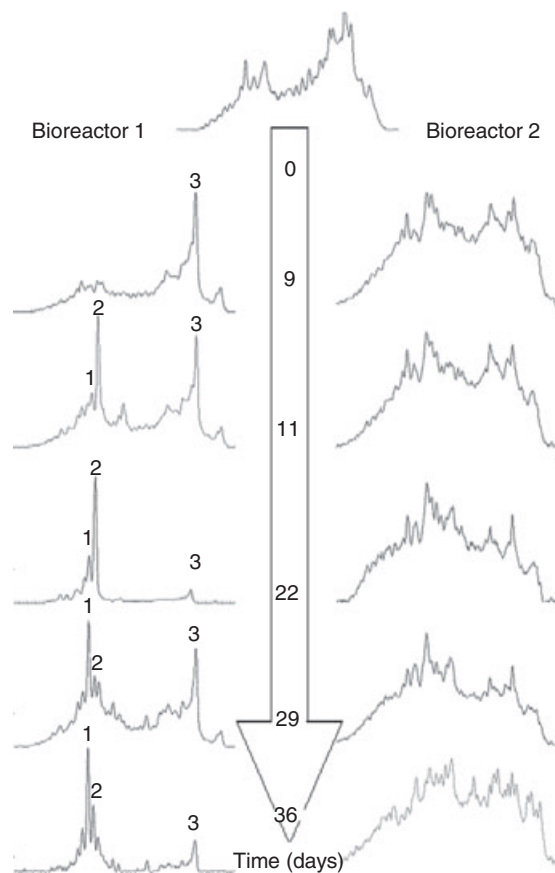


Figure 4 Bacterial community dynamics for bioreactors 1 and 2.

**Table 3** Phylogenetic affiliation of partial 16S rDNA sequence from COVELI operational taxonomic unit (bioreactor 2)

Phylum and sub-phylum	OTU	Number of clones Libraries		Closest micro-organism or uncultured clone in GenBank database name (accession number)	Similarity %	
		D1	D2			
<i>CFB</i>	COVELI D1A01	2		Clone PHOS-HE21 (AF314419)	98	
	COVELI D1A03	1		Clone RSC-II-66 (AJ252690)	93	
	COVELI D1A05	3	2	Clone pLW-48 (DQ066991)	94	
	COVELI D1A06	5	5	Clone PHOS-HE21 (AF314419)	98	
	COVELI D1A08	2	3	Clone RSC-II-66 (AJ252690)	95	
	COVELI D1A09	2		Clone Toolik_Jun2005_shruborg_191 (DQ510084)	98	
	COVELI D1A10	1		Clone Kas178B (EF203208)	100	
	COVELI D1B02	2		Clone 965016F12.x1 (DQ064918)	93	
	COVELI D1B05	2		Clone TW11-23 (DQ501362)	96	
	COVELI D1B08	1		Clone RSC-II-66 (AJ252690)	93	
	COVELI D1B11	1	2	Clone 26 (DQ413085)	98	
	COVELI D1C03	1		Clone Blhii45 (AJ318145)	96	
	COVELI D1D02	2	1	Clone 72-ORF19 (DQ376575)	95	
	COVELI D1D10	1	1	Clone D53-49 (DQ463228)	93	
	COVELI D1D11	1	2	Clone 965018F10.x1 (DQ065018)	96	
	COVELI D1E12	2	1	Clone TH-22 (AB185009)	95	
	COVELI D1F03	1		Clone M07 (AF495415)	89	
	COVELI D1F04	1	1	Clone Cont08 (AY651310)	99	
	COVELI D1G04	2		Clone isolate E53-26 (DQ444150)	97	
	COVELI D1G07	2		Clone RSC-II-66 (AJ252690)	91	
	COVELI D1H02	2		Clone Blhii45 (AJ318145)	96	
	COVELI D1H04	1		Clone ORSFC1_h06 (EF393375)	92	
	COVELI D2A05		1	Clone TW11-23 (DQ501362)	96	
	COVELI D2C03		1	Clone LiUU-9-122 (AY509312)	88	
	COVELI D2D03		1	Clone RSC-II-66 (AJ252690)	93	
	COVELI D2D10		1	<i>Chryseobacterium caeni</i> strain N4 (DQ336714)	97	
	COVELI D2E07		1	Clone nmt ct5 (DQ070860)	96	
	COVELI D2E11		2	Clone Kas178B (EF203208)	96	
	COVELI D2F11		1	Clone RSC-II-66 (AJ252690)	92	
	COVELI D2G08		1	Clone AKYH1028 (AY921686)	96	
	COVELI D2H08		1	Clone 965016F12.x1 (DQ064918)	94	
		Total	38	28		
	<i>Planctomyces</i>	COVELI D1A02	1		Clone C2 (AY268227)	94
COVELI D1A07		1		Clone c5LKS47 (AM086132)	94	
COVELI D1C09		1		Clone E101-12 (DQ001639)	90	
COVELI D2A12			2	Clone A12 (AF234727)	96	
COVELI D2F06			1	Clone CY0ARA026D10 (BX294726)	95	
COVELI D2G05			1	Clone K20-53 (AF145848)	98	
	Total	3	4			
<i>α-Proteobacteria</i>	COVELI D1A12	1		Clone DR550SWSAEE43 (DQ354731)	99	
	COVELI D1B07	8	2	<i>Novosphingobium hassiacum</i> strain (AJ416411)	98	
	COVELI D1B10	2		Clone TC2 (DQ297946)	99	
	COVELI D1C10	1		Clone TH-31 (AB185018)	96	
	COVELI D1D06	1		Clone mle1-13 (AF280850)	99	
	COVELI D1D09	1		Clone N-C-98 (AB201614)	98	
	COVELI D1E04	1		Clone Bk_6A (EF209343)	99	
	COVELI D1E10	1	1	<i>Sphingomonas</i> sp. strain JS1 (AJ427917)	97	
	COVELI D1G11	1		<i>Sphingomonas</i> sp. strain Y49 (DQ821731)	99	
	COVELI D1H05	1		Clone DSSD33 (AY328732)	95	
	COVELI D2A02		2	Clone G12 (AF485399)	97	

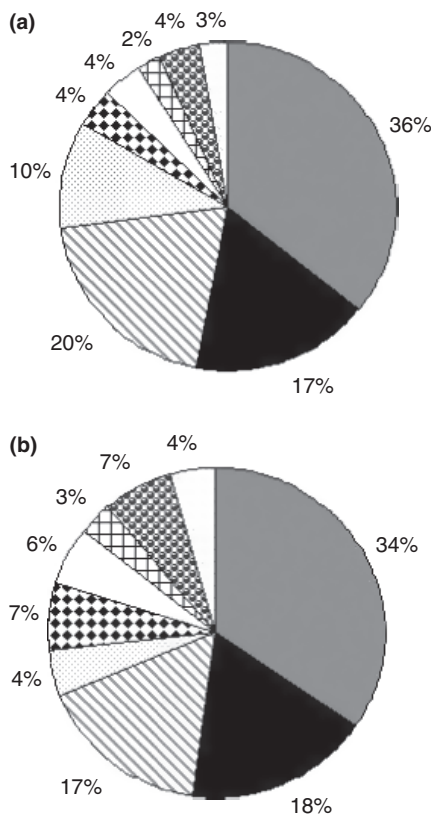


**Table 3** (Continued)

Phylum and sub-phylum	OTU	Number of clones Libraries		Closest micro-organism or uncultured clone in GenBank database name (accession number)	Similarity %
		D1	D2		
	COVELI D2B06		1	Clone EV818CFSSAHH203 (DQ337011)	89
	COVELI D2B10		3	Clone G74 (DQ480486)	99
	COVELI D2B11		1	Clone MP104-SW-b11 (DQ088792)	99
	COVELI D2D04		1	Clone TH-127 (AB185006)	95
	COVELI D2E03		2	Clone BCM3S-13B (AY102903)	94
	Total	18	13		
<i>β-Proteobacteria</i>	COVELI D1A04	1		Clone EV818SWSAP42 (DQ337076)	99
	COVELI D1B01	2		Clone 178up (AY212629)	100
	COVELI D1C01	1		Clone M26_Pitesti (DQ378246)	94
	COVELI D1D04	1	3	Clone Spb153 (AJ422165)	100
	COVELI D1E03	1	3	Clone SX2-47 (DQ469218)	99
	COVELI D1E11	2	4	Clone R1-B35 (AB195767)	99
	COVELI D1F02	1	1	Clone DOK_CONFYM_344 (DQ828607)	96
	COVELI D1F08	2		Clone B80 (EF029321)	92
	COVELI D1F12	1	7	Clone M26_Pitesti (DQ378246)	97
	COVELI D1H06	1		Clone LO13-11 (AF358003)	97
	COVELI D2B08		1	Clone Run-SP 97 (AB247485)	93
	COVELI D2D08		1	Clone 237ds5 (AY212684)	97
	COVELI D2D11		1	Clone HKT437 (DQ439603)	93
	COVELI D2F05		1	Clone 178up (AY212629)	96
	COVELI D2F07		1	Clone RAN-135 (AY499453)	97
	Total	13	23		
<i>ε-Proteobacteria</i>	COVELI D1C05	1		Clone Rs127 (AM110055)	95
	COVELI D2D01		1	Clone AT-s2-18 1 (AY225653)	97
	COVELI D2E10		1	Clone IC-61 (AB255073)	93
	COVELI D2E12		1	Clone TH-127 (AB185006)	97
	COVELI D2F09		2	Clone Blgi18 (AJ318136)	97
	COVELI D2G01		1	Clone 342B (AY571850)	93
	Total	1	6		
LGCGPB	COVELI D1B03	2		Clone CC 103 (DQ228214)	99
	COVELI D1D07	2		Clone GWS-BW-H199 (AY731370)	96
	COVELI D1H08	1		Clone B1NR70D7 (AY957892)	94
	COVELI D2B07		1	Clone BSV78 (AJ229222)	91
	COVELI D2C12		1	<i>Clostridium</i> sp. EBR-02E-0046 (AB186360)	90
	Total	5	2		
HGCGPB	COVELI D1E08	1		Clone DOK_BIODYN_clone368 (DQ828052)	99
	COVELI D1F09	2		Clone SMS9-86WL (AY043909)	96
	COVELI D2F03		1	<i>Gordonia defluvii</i> strain J5 (AY650266)	97
	Total	3	1		
<i>Cyanobacteria</i>	COVELI D1A11	8	5	Clone TH-31 (AB185018)	97
	COVELI D1F11	2	2	Clone B1NR70D7 (AY957892)	94
	COVELI D2A11		1	Clone B1NR70D7 (AY957892)	93
	COVELI D2G02		1	Clone D5 (AY375144)	95
	Total	10	9		
<i>Verrucomicrobium</i>	COVELI D1G08	1		Clone S040 (AY037575)	98
OP11-derived 1	COVELI D2C02		1	Clone DA18 (AY193187)	92
<i>Chlorobi</i>	COVELI D2H04		1	Clone F13-30 (AF495427)	96
<i>Chloroflexi</i>	COVELI D2B05		2	Clone A4b (AF234686)	90

OP11-derived: phylum defined by Harris *et al.* (2004).

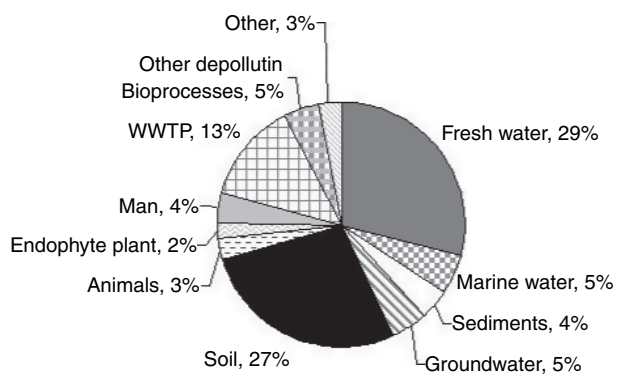
CFB, *Cytophaga*, *Flexibacter*, *Bacteroides* phylum.



**Figure 5** Distribution of the 16S rDNA sequences and operational taxonomic unit (OTU) from bioreactor 2 in Phylum. (a) Distribution of sequences. (b) Distribution of OTU. (□, CFB; ■,  $\alpha$ -Proteobacteria; ▨,  $\beta$ -Proteobacteria; ▩, Cyanobacteria; ▤, Planctomyces; □, LGCGPB; ▥, HGCGPB; ▦,  $\epsilon$ -Proteobacteria; ▧, Autres).

MC-750 (Y14302), 6 sequences are related to the strain HTCC333 (AY429712) and the last sequence is close to *Gordonia bronchialis* C2 (AY262331).

The environmental distribution of sequences by habitat type, for which the similarity is higher than 95%, was



**Figure 6** Distribution of the 16S rDNA sequences which have more than 95% similarity with operational taxonomic unit (OTU) from bioreactor 2 in function of their environmental origin.

performed (Fig. 6). For each OTU, the origin of the sequences with more than 95% similarity, has been determined (Fig. 6). These sequences have been obtained from total DNA extracted from environmental samples. The sequences defined during this inventory are not specific sequences found in activated sludge. 29% come from fresh water, 27% come from soil and 13% from activated sludge. Among the sequences closest to our sequences, 18% come from the treatment of polluted effluent such as in wwtp.

## Discussion

### Elimination capacity of the microbial communities

The elimination capacity has been calculated for both bioreactors. First, the results show that for two different types of feeding, the global elimination capacity obtained for bioreactor 1 was slightly superior to the global elimination capacity measured for bioreactor 2. Hence, it is also necessary to consider the elimination efficiency for both bioreactors. Oxygenated compounds were completely eliminated in bioreactor 2. The removal of aromatic and chlorinated compounds was equivalent and reached 50–55%. Easily biodegradable compounds are known to prevent the elimination of more recalcitrant VOCs (Veir *et al.* 1996; Deshusses *et al.* 1999). More accurately, in biofilters used for the treatment of VOCs for which the concentration was  $100 \text{ mg m}^{-3}$ , the removal of aromatics (80%) was higher than the removal of halogenated compounds (60%) (Aizpuru *et al.* 2001). As expected in bioreactor 2, the empty bed residence time (4 min) of the compounds would favour the availability of halogenated compounds for the microflora; the empty bed residence time of the same compounds in biofilters is of 36 s. Nevertheless, these results have to be compared carefully as biofilters treating a complex mixture of VOCs have to be considered as heterogeneous systems. It has been effectively shown that the spatialization of biodegradation functions directly depends on the chemical nature of pollutants (Khammar *et al.* 2005).

For bioreactor 1, among oxygenated compounds, the biodegradability for esters was greater than methanol and ketones. The same result was obtained by Aizpuru *et al.* (2001). It has been assumed that various interactions occurred between components of the mixture and could modify the efficiency obtained for one single component.

Moreover, the liquid phase concentration estimates (720 and 40 mg VOCs per litre for bioreactors 1 and 2 respectively) highlighted that the difference between the removal efficiency in the two bioreactors seems to depend more on the biological kinetics than on the mass transfer rate of VOCs from the bubbles to the liquid phase.

## Two bacterial communities dynamics

While both reactors have been seeded with the same inoculum, the bacterial community dynamics in both reactors is different as they have been supplied with a gaseous effluent which differed in terms of mass loading. When the bioreactor was supplied with the VOC complex mixture at a low concentration, a large bacterial diversity is kept. However, a simplification of the genetic structure of the total bacterial community was then observed for bioreactor 1 supplied with the higher mass loading.

For bioreactor 1, significant modifications of the bacterial genetic structure occurred and a decrease in bacterial diversity was then observed. This phenomenon has already been observed in soil and sediment studies after the contamination of the microflora with pollutants such as mercury, copper, cadmium (Rasmussen and Sorensen 2001; Ranjard *et al.* 2006), fuel (Stephen *et al.* 1999) or polycyclic aromatic hydrocarbons (Langworthy *et al.* 1998), and during the start-up phase of bioreactors supplied with cattle manure (Chachkhiani *et al.* 2004) or aromatic compounds (Stoffels *et al.* 1998).

It has already been shown that the presence of more readily degradable compounds prevent the use of more recalcitrant VOCs (Deshusses *et al.* 1999). This explanation could result in selection for a less diverse community in reactor 1. According to the work of Ranjard *et al.* (2006), it could also be suggested that, in our study, below a critical concentration level, the VOC concentration could induce the enrichment and predominance of some types of microorganisms. Beyond this critical concentration, a decrease in diversity was observed in bioreactor 1.

Furthermore, the applied mass loading seems to have no influence on the densities of all bacteria. At the end of the experiment, the number of bacteria is similar for both bioreactors. Table 2 indicates that the total rate of VOC consumption is similar in both bioreactors. Assuming that the oxygenated compounds in bioreactor 1 will have a little lower biomass yield than the aromatic compounds in bioreactor 2, the overall growth of biomass might be expected to be similar. Moreover, it is likely that the mass of VOCs fed to the biomass is used for energy and maintenance, and not for growth, which results in a relatively small increase during the experiments. This result also seems to illustrate the growth of a bacterial community, which succeeds in adapting to toxic functioning conditions in bioreactor 1 for which the concentration of compounds in the bacterial suspension was 720 mg VOCs per litre.

## High bacterial diversity

The molecular analysis of the bacterial community in bioreactor 2 reveals a wide species diversity that has been

evidenced by the number of peaks (SSCP) and OTU number (inventory). A similar level of diversity has been noted in biofilters supplied with VOC mixtures (Friedrich *et al.* 2002; Khammar *et al.* 2005), in pollution removal processes with a free biomass (Lapara *et al.* 2000; Wagner *et al.* 2002) and in anaerobic digestion tanks (Zumstein *et al.* 2000). The species richness of the microbial communities of five laboratory-scale reactors and three wwtp has been analysed by 16S rRNA gene phylogenetic inventories established by using bacterial primers (Wagner *et al.* 2002). It is obvious that the number of plants analysed by the 16S rRNA approach is too low to infer ultimate conclusions. However, already in the available studies, the members of 13 bacterial divisions, out of 36 divisions currently identified for the bacterial domain (Hugenholtz *et al.* 1998; Rappe and Giovannoni 2003), were detected, indicating considerable diversity in wwtp. *Proteobacteria* are abundant in each library and represent more than 50% of the clones in five out of the eight surveys. *Betaproteobacteria* are the most frequently retrieved, members of this division. Molecular isolates affiliated to the *Bacteroidetes*, the *Chloroflexi* and the *Planctomycetes* were retrieved in significant numbers in several of the libraries. Our results demonstrate that *Proteobacteria* and *Bacteroidetes* were also retrieved in significant numbers in the library. The *Planctomycetes* and the *Chloroflexi*, yet, were not frequently retrieved whereas the *Cyanobacteria* and the TM7 group represent 10% and 7% of the obtained clones respectively.

Furthermore, the inventories of bioreactors used for the treatment of polluted air containing VOCs mixture by using molecular tools have to be considered to explore the richness species and more accurately the functional bacterial communities.

## Conclusion

This work highlights that the applied mass loading seems to influence the functioning and the genetic structure of the bacterial community during the enrichment phase. (i) A high mass loading seems to induce a low effective functioning in terms of elimination efficiency and chemical nature of removed compounds and a simplification of the genetic structure of the total bacterial community with the apparition of a dominant microflora. (ii) A low mass loading seems to favour a more efficient functioning and allows to maintain a greater bacterial diversity. Wagner *et al.* (2002) reported that wastewater treatment plants with low functional redundancy due to the presence of a low diversity of bacteria of a certain functional group are more sensitive to failure of the respective process than plants harbouring a high diversity of the same bacterial group. Then, in the gaseous effluent treatment processes,

it would be of interest to define functioning parameters of the process to keep the diversity of important functional bacterial groups.

Even if bioreactors 1 and 2 should have been operated in triplicate and the substrate mixture under investigation remains complex, the results obtained in this study provide useful information about the changes in microbial communities following natural or anthropogenic alterations in different ecosystems.

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