
# Microbial community analysis in biotrickling filters treating isopropanol air emissions

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# Microbial community analysis in biotrickling filters treating isopropanol air emissions

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# Microbial community analysis in biotrickling filters treating isopropanol air emissions

## Abstract

The evolution of the microbial community was analysed over one year in two biotrickling filters operating under intermittent feeding conditions and treating isopropanol emissions, a pollutant typically found in the flexography sector. Each reactor was packed with one media: plastic cross-flow-structured material or polypropylene rings. The communities were monitored by fluorescence in situ hybridisation (FISH) and denaturing gradient gel electrophoresis (DGGE) analysis of the 16S rRNA region. After inoculation with activated sludge, the biotrickling filters were operated using inlet loads (ILs) from 20 to 65 g C $m^{-3}$ $h^{-1}$ and empty bed residence times (EBRTs) from 14 to 160 s. Removal efficiencies higher than 80% were obtained with ILs up to 35 g C $m^{-3}$ $h^{-1}$ working at EBRTs as low as 24 s. There was an increase in the total percentage of the target domains of up to around 80% at the end of the experiment. Specifically, *Gammaproteobacteria* domain group, which includes the well-known volatile organic compund (VOC)-degrading species such as *Pseudomonas putida*, showed a noticeable rise in the two biotrickling filters of 26% and 27 %, respectively. DGGE pattern band analysis revealed a stable band of *Pseudomonas putida* in all the samples monitored, even in the lower diversity communities. In addition, at similar operational conditions, the biotrickling filter with a greater relative abundance of *Pseudomonas sp.* (19.2% *vs*. 8%) showed higher removal efficiency (90% *vs*. 79%). Results indicate the importance of undertaking a further in-depth study of the involved species in the biofiltration process and their specific function.

**Keywords**: biotrickling filters; denaturing gradient gel electrophoresis; fluorescence in situ hybridisation; microbial community; volatile organic compounds.

## 1. Introduction

Flexography is part of the printing sector and its industry grew by 4.5% from 2009 to 2010, comprising around 150,000 companies in Europe [1]. The main organic solvents used in flexography are ethanol and isopropyl alcohol, since they are used in the formulation of solvent inks, dilution and cleaning processes [2]. Stricter requirements for the control of volatile organic compounds (VOCs) from the use of solvents have been published by a new European Directive on industrial emissions (Council Directive 2010/75/EC), in which VOC emission limits have been established between values of 50 and 150 mg C $Nm^{-3}$ depending on the industrial activity. Thus, the flexographic industry requires treatment technologies to comply with the directive regulating VOC emission.

There are several techniques for treating VOC emissions, such as condensation, adsorption, absorption, thermal destruction and biotechnologies. Biotechnologies, including biofilters, biotrickling filters and bioscrubbers, are a potential alternative to conventional techniques for removing VOCs from emission streams with high flow rates and relatively low VOC concentrations. Moreover, these have proven to be both cost-effective and environmentally friendly [3]. Furthermore, biological treatment has been included as an end-of-pipe technique for waste gas abatement in installations for the surface treatment of substances using organic solvents [4].

Biotrickling filters (BTFs) use a specified inert packing material and involve a liquid phase, which trickles through the bed providing nutrients. The biofilm is developed on the packing surface, the microbial community being essential for the successful performance of the process. The effectiveness of the BTF process has been studied from the laboratory to the industrial scale treating aromatic and oxygenated compounds [5-13]. However, there is little

data in the literature on compounds related to the flexographic sector. For example, Chang and Lu studied the removal of isopropanol (IPA) using a trickled-bed air biofilter packed with coal and empty bed residence times (EBRTs) from 20 to 90 s [14]. Removal efficiencies (REs) between 90 and 99% were achieved for inlet loads (ILs) of between 45 and 88 g C $m^{-3}$ $h^{-1}$. Krailas and Pham reported an isopropanol elimination capacity (EC) of 276 g $m^{-3}$ $h^{-1}$ for an IL of 342 g $m^{-3}$ $h^{-1}$ using a downward flow biofilter [15]. Popov et al. evaluated a pilot-scale biotrickling filter at a flexographic printing facility employing an EBRT of 14 s [16]. Highly variable VOC emissions containing a mixture of alcohols and esters, with an average inlet concentration of 1.56 g $m^{-3}$, were treated with an average RE of 89%.

As reported above, main efforts have been made to improve the feasibility and robustness of the bioprocess; however, there are few studies focused on the characterisation of the bacterial population in BTFs. For this purpose, molecular biological techniques are useful for analysing microbial community structures during the removal of contaminants in the biological processes. Some of the applied techniques are polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP), denaturing gradient gel electrophoresis (DGGE) and fluorescence in situ hybridisation (FISH). PCR consists of amplifying nucleotide fragments and SSCP detects sequence variations (single-point mutations and other small-scale changes) through electrophoretic mobility differences. DGGE is based on the electrophoretic separation of PCR products with the same length, but with different sequences, on a linear denaturing gradient polyacrylamide gel [17]. FISH detects nucleic acid sequences by probes with fluorescent dyes that hybridises specifically to its complementary target sequence within the cell [18]. These techniques have been applied in the field of biofiltration [19,20]. For example, Khammar et al. studied the spatial structure of microbial communities in a biofilter treating a complex mixture of 11 VOCs using PCR-SSCP. The authors observed a

spatialisation of the biodegradation functions in the biofilter related to the stratification of microbial density and diversity along the filter bed [21]. In another study, Friedrich et al. applied FISH to determine the bacterial community in a full-scale industrial biofilter [22]. Results indicated that members of the *Betaproteobacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Cytophaga-Flavobacteria*, *Firmicutes* and *Gammaproteobacteria* were the most abundant groups, although a non-homogeneous distribution along the biofilter was observed, since the lower 50 cm of the biofilter bed was the most active in removing pollutants. The DGGE technique was applied to assess the impact of changing VOC emission composition on the bacterial community structure in a trickle-bed air biofilter during 192 days [23]. The results showed that the structure of the microbial community in the biofilter was different after each change in the inlet composition.

The purpose of this work was to characterise the microbial communities present in two biotrickling filters, each one with a different packing material (a plastic cross-flow-structured material or polypropylene rings) for treating isopropanol (IPA) emissions. The following specific objectives were developed: (1) application of FISH and DGGE to analyse any variation in the microbial communities following changes in the operating parameters of the BTFs; (2) comparison between the microbial communities in the two BTFs.

## 2. Material and methods

### *2.1 BTF set-up and operating conditions*

The experimental system consisted of two identical laboratory-scale BTFs operating in parallel, named BTF1 and BTF2. The schematic of the experimental set-up is shown in Figure 1. Both BTFs were built by a methacrylate column with a total length of 126 cm and an

internal diameter of 14.4 cm. BTF1 was filled with a plastic cross-flow-structured packing material (Odourpack, Pure Air Solutions, the Netherlands) with a surface area of 410 $m^2$ $m^{-3}$, and BTF2 with a random packing material (Refill-Tech, Italy) consisting of polypropylene rings with a nominal diameter of 5/8" and a surface area of 348 $m^2$ $m^{-3}$. The set-up was completed with a 10-L recirculation tank and the recirculation solution was intermittently fed into the bioreactor (15 min every 1.5 h) in a counter-current mode with respect to the air flow. The liquid velocity was set to 10 m $h^{-1}$. A nutrient solution (21.65 g $KNO_3$ $L^{-1}$, 4.6 g $Na_3PO_4 \cdot 12H_2O$ $L^{-1}$, trace elements (μg $L^{-1}$): 12000 Ca, 1500 Fe, 2650 Mg, 605 Zn, 60 Co, 60 Mo, 55 Ni, 50 Cu, 45 B, 40 Mn, 8 I, 3 Se & 1 Cr; and vitamins (μg $L^{-1}$): 20 beta-Carotene, 70 B1, 70 B2, 95 B6, 10 B9, 0.1 B12, 3 C, 0.3 D, 500 E, 2 Biotin & 900 Niacin) was supplied into the recirculation tank using a peristaltic pump to maintain a supplied mass ratio of carbon to nitrogen of 35. The inoculum for both BTFs was an activated sludge obtained from a wastewater treatment plant located in Valencia (Spain). An inoculum volume of 0.5 L (TSS concentration of 3500 mg $L^{-1}$) was added to the recirculation tank and continuously flowed through the bed for 24 h.

The influence of IL and EBRT on the removal of isopropanol and microbial communities were analysed in three phases (A, B and C). Each phase was designed with a constant inlet concentration. In the phases A, B and C, it was adjusted to 1000, 500 and 250 mg C $Nm^{-3}$, respectively. For each phase, several IL step increases were carried out together with variations in the EBRTs of between 14 and 160 s (gas velocities between 258 and 23 m $h^{-1}$). Table 1 summarises the experimental plan and the biomass sampling events. The experiments were planned to simulate typical industrial emissions, hence an intermittent loading pattern was applied (16 h of feeding isopropanol per day, 5 days per week). During nights and weekends without isopropanol supply, the air flow rate was kept constant and the

water trickling was stopped. The first three biomass samples (day 60 at phase A-II, 100 at phase B-II and 130 at phase C-I) corresponded to experiments with an IL of 35 g C $m^{-3}$ $h^{-1}$ and a gradual reduction of the EBRT from 90 to 24 s.

In the last phase (D), the effects on the system re-start-up after a long period without isopropanol feeding was evaluated to check the robustness of the process and the impact on the microbial community. Macroscopic changes such as RE recovery and microscopic changes such as the microbial ecology structure were evaluated. During the period from day 164 to day 217 (referred to here as the starvation period), the supply of isopropanol was stopped, the air flow rate was adjusted to achieve an EBRT of 60 s and the water trickling pattern was reduced to 15 min per day. On day 217, VOC feeding was restored using the same intermittent pattern applied before (16 h of feeding isopropanol per day, 5 days per week) and the trickling of water for 15 min every 4 h was set. In phase D-I, an IL of 35 g C $m^{-3}$ $h^{-1}$ and an EBRT of 60 s were established. A few days after isopropanol feeding was restored (on day 217), a new biomass sample was obtained on day 227 (at phase D-I). Finally, the IL was twice increased at phase D-II and two biomass samples (days 245 and 276) were collected. Results of the removal of both BTFs during phases A, B and C have been previously published by the authors on San-Valero et al. [24]. A summary of these results are presented here in order to show the complete performance of both BTFs during whole experimental period (from phase A to D). The performance is used to correlate him with the microbial communities analysed from biomass samples taken throughout entire experimental period.

### *2.2 Fluorescence in situ hybridization (FISH)*

The FISH technique was carried out, adapting the procedure described by Amann et al. [25].

The procedure included the following steps: sampling, fixation, hybridisation with the respective probes for detecting the respective target sequences, image acquisition and analysis. The probes targeting general bacterial groups used in this work were: *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* related to the Proteobacteria domain, *Firmicutes* (low G+C Gram-positive bacteria) and *Actinobacteria* (high G+C Gram-positive bacteria). These groups have been previously identified as the main ones involved in VOC removal [26,27]. *Pseudomonas sp.*, *Pseudomonas putida*, *Bacillus* and *Mycobacterium* were the targeted species monitored since previous studies have indicated that these species play an important role in VOC degradation [28-32].

### *2.2.1 Sampling and fixation*

Biomass samples from the bioreactors were taken from three sampling ports located at different heights of the packed bed of the reactor: 20, 62 and 105 cm. They were mixed at a 1:1:1 ratio (wt) and disaggregated with an Ultra-Turrax (IKA® T18 basic, Germany), resulting in one sample for each BTF. The samples were fixed using the procedure described by Amann at al. [25]. According to the procedure, each sample was fixed with two methods: (1) to analyse Gram-negative cells where the sample was fixed with paraformaldehyde solution (4% in PBS). The sample was incubated at 4ºC for 2 h. Then, the biomass was pelleted by centrifugation for 3 min at 7000 rpm, washed with PBS, pelleted again and finally, re-suspended in PBS solution; (2) to analyse Gram-positive cells, the sample was centrifuged for 3 min at 7000 rpm and re-suspended with ethanol solution (96%).

### *2.2.2 Hybridisation*

In this study, a Cy5-labelled EUBmix as the general probe and Cy3-labelled specific probes (Thermo Fisher Scientific, Germany) were used. EUBmix consists of a 1:1:1 (vol) mixture of

the probes EUB338, EUB338II and EUB338III. Fluorochromes Cy5 and Cy3 with different wavelengths for excitation and emission allow simultaneous microscopic observations. The oligonucleotide probes used in this study and the percentage of formamide used for each probe are summarised in Table 2 [33]. Hybridisation was performed at 46 ºC for 2 h. After this step, the slides were rinsed for 18 min at 48 ºC, dried and mounted. For mounting, an anti-fading product (VECTASHIELD®, ATOM S.A, Spain) was used to avoid the loss of fluorescence.

### *2.2.3 Image acquisition and analysis*

Slides were examined using a Leica TCS SP confocal laser scanning microscope (CLSM, Leica Microsystems, Germany). The microscope is equipped with two He-Ne lasers with light emissions at 543 and 633 nm. Each image captured was formed with two pictures, one corresponding to EUBmix and the other to the specific probe. Five images were acquired for the negative control (without probes) and 30 for the stained samples (with specific and general probes). Specific probes were quantified as the proportion of EUBmix-labelled bacteria using image analysis based on the methodology developed by Jubany et al. [34]. This method was implemented in the Matlab® software. First, the thresholds for general and specific probes were calculated as the minimum intensity value satisfying the condition: the proportion of pixels with intensity lower or equal to the threshold value in the negative control images is greater or equal to a value of 99.9%. These thresholds were used to exclude the autofluorescense of the sample in the quantification procedure. The quantification of images for the stained samples was carried out as the sum of the pixels with intensity higher than the threshold intensity for the general and specific probes. Finally, the ratio of the sum of the pixels for the specific probe to that for the general probe was estimated.

### *2.3 DNA extraction and PCR amplification*

DNA was extracted using a JETQUICK tissue DNA Spin kit (Genycell biotech, Spain) from the biomass samples taken on days 60, 100, 227 and 276 (phases A-II, B-II, D-I and D-II, respectively). DNA was stored at -20 ºC until analysis. The extracted DNA was used as the target DNA in PCR to amplify the 16S rRNA coding regions, using two universal primers for the bacterial domain, primer F357GC: 5´CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG3´ and primer R518: 5´ATTACCGCGGCTGCTGG3´. A GC clamp was added to the forward primers to facilitate the DGGE. PCR was performed in 50-µl reaction volume using a mixture containing 0.1 units/µl of Taq DNA polymerase, 3.2 mM dNTPs, 3 mM $MgCl_2$ 32 mM $(NH_4)_2SO_4$, 134 mM Tris HCl and 0.2 µM of each primer (EuroClone, Italy).

PCR was executed in a thermal cycler (LongGene Scientific Instruments, Hangzhou) using the temperature cycling conditions described by Muyzer and Ramsing [17]. It consisted of 20 cycles of: 94 ºC for 1 min, 65 ºC for 1 min, a gradual decrease in the temperature using 0.5 ºC increments until 55 ºC, followed by 72 ºC for 3 min. The PCR reactions followed with 10 cycles of: 94 ºC for 1 min, 55 ºC for 1 min and 72 ºC for 3 min. A final extension at 72 ºC for 7 min was undertaken as the final step.

### *2.4 DGGE analysis*

Five µl of the PCR product was separated on a 2% agarose gel at 100 V for 30 min to verify the correct amplification before DGGE. For DGGE analysis, the PCR product generated from each sample was separated on an 8% acrylamide gel using a linear denaturant gradient increasing from 35% to 60% using the KuroGel Verti 2020 DGGE System (VWR

international Eurolab S.L.). DGGE was performed using 20 µl of PCR product in 1xTAE buffer at 60 ºC with a sequence of 50 V for 5 min, 150 V for 120 min and 200 V for 60 min.

## 3. Results and discussion

### *3.1 BTF performance*

The performances of the two biotrickling filters treating IPA emissions, BTF1 and BTF2, are presented in Figure 2a and 2b, respectively. The performance was evaluated in terms of inlet load (IL), elimination capacity (EC) and removal efficiency (RE). Table 3 summarises the performance of both BTFs on the days the biomass samples were taken. As can be observed in Figure 2, approximately two weeks after inoculation, the systems were able to achieve stable REs of around 80% (phase A-I, 160 s of EBRT and IL of 20 g C $m^{-3}$ $h^{-1}$). A decrease in the EBRT to 90 and 50 s at phases A-II and A-III, respectively, caused a decrease in RE for both BTFs with values varying from 60 to 85%. In phase B, the inlet concentration was set at 500 mg C $Nm^{-3}$ and the EBRT was adjusted to 90, 50 and 25 s for phases B-I, B-II and B-III, respectively. REs greater than 90% were obtained in phases B-I and B-II, whereas in phase B-III, the RE dropped to values of 60-70%. In phase C, the inlet concentration was set at 250 mg C $Nm^{-3}$ and the EBRT was adjusted to 24 and 14 s for phases C-I and C-II, respectively. In these phases with the most demanding operational conditions, the performances of the two systems were slightly different. For example, in phase C-I using an IL of 35 g C $m^{-3}$ $h^{-1}$ and an EBRT of 24 s, RE values of around 80% and 88% were observed for BTF1 and BTF2, respectively. Prior to the starvation period, the minimum EBRT (14s) was applied at phase C-II and the RE decreased to values of 49% and 60% for BTF1 and BTF2, respectively.

The starvation period took place from day 164 to day 217 to evaluate the re-start-up of the process after restoring VOC feeding. Macroscopic changes such as RE recovery and microscopic changes such as the microbial ecology structure were evaluated. On day 217, VOC feeding was restored using the same intermittent pattern applied before (16 h of feeding isopropanol per day, 5 days per week) and the trickling of water for 15 min every 4 h was set. In phase D-I, an IL of 35 g C $m^{-3}$ $h^{-1}$ and an EBRT of 60 s were established. Ten days after restoring VOC feeding, both BTFs achieved REs as high as 90%, similar to those observed during phase B-II, demonstrating that the BTFs could handle long periods without VOC feeding. In the last phase (D-II) where the IL was twice increased to 65 g C $m^{-3}$ $h^{-1}$, average RE values of 72% and 80% were reached in BTF1 and BTF2, respectively.

### *3.2 Monitoring of bacterial community by FISH*

The monitoring of the bacterial community in the biofilm samples of both BTFs was carried out by FISH over the entire experimental period in which different operational conditions were tested (Table 1).

Figure 3 shows the evolution of the relative abundance of the general bacterial groups for the two BTFs, BTF1 (Figure 3a) and BTF2 (Figure 3b), on days 60, 100, 130, 227, 245 and 276. The results were expressed as the percentage of EUBmix-stained cells (hereafter defined as total bacteria). The total percentages of the target domains calculated as the sum of each general bacterial group are summarised in Table 4 for BTF1 and BTF2

Total percentage values ranged from 29.7 to 79.2% and from 69.4 to 88.1% for BTF1 and BTF2, respectively. It is worth noting that the higher value of the total identified bacterial groups was observed at the last phase in both BTFs (79.2 % and 88.1% for BTF1 and BTF2, respectively). This could be explained by the specialisation of the microbial community to

groups and/or species more suitable for degrading the pollutant (IPA) during the year of experimentation. Other authors have indicated that the development of highly diverse communities could be enhanced in environments with high resource availability [35]. For example, Álvarez-Hornos et al. studied microbial populations in pilot-scale biotrickling installed in a plastic coating factory to control complex emissions with highly variable VOC composition [36]. The authors reported high bacterial diversity and heterogeneity, with total percentages of the same bacterial groups as those in this study varying between 23% and 56% relative to the EUBmix counts, with no predominant groups.

Regarding the evolution of the general bacterial groups, their composition changed over time and was influenced by the operational conditions in both BTFs, although there were some patterns that could be considered. As seen in Figure 3, the Gram-negative bacteria identified (*Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*) were more abundant than the Gram-positive bacteria identified (*Firmicutes* and *Actinobacteria*). A deep examination of the results indicated different trends for each group. For example, members of *Alphaproteobacteria* and *Betaproteobacteria* were the most fluctuating groups, with values ranging between 4% and 27% and 7% and 22%, respectively. *Firmicutes* and *Actinobacteria* groups presented a less oscillating behaviour, especially in BTF2 with an average value of 12% for both groups. The percentage of the *Gammaproteobacteria* group increased throughout the experimental period in both BTFs, being the most abundant group at the end of experiment with values of 26% and 27% for BTF1 and BTF2, respectively. This rise could be explained by the ability of *Gammaproteobacteria* species to degrade VOCs [30-32,37,38], enabling them to develop better in environments rich in VOC. It should be noted that in BTF2, where greater RE and greater quantity of biomass attached to the packing (visual inspections during the tested period) were obtained in comparison with BTF1, the

*Gammaproteobacteria* group was the most abundant in almost all samples, with values higher than 20%. As an example, Figure 4 shows a relatively high quantity of *Gammaproteobacteria* in BTF2 on day 276.

Regarding changes in certain bacterial species, Figure 5 represents their time-dependent changes in BTF1 (Figure 5a) and BTF2 (Figure 5b) on days 60, 100, 130, 227, 245 and 276. The total percentage of identified species calculated as the sum of *Pseudomonas sp., Bacillus* and *Mycobacterium* presented a pattern similar to that observed with the general bacterial groups. For example, from day 100 onwards (phase B-II), the total percentage of identified species in BTF2 was higher than that in BTF1, achieving an average value of 34.3% in comparison with an 18.4%, respectively.

A detailed analysis of the species in both BTFs showed a slight variation for *Bacillus* with values ranging between 1.5% and 10.5%, whereas *Mycobacterium* presented a greater fluctuation with values between 0.7 and 16%, especially during the days after the starvation period when it displayed the highest values (16.0% and 14% for BTF1 and BTF2, respectively). This could be explained by the fact that *Mycobacterium* is better protected against stressful periods without substrates and nutrients, due to the characteristics of its cell membrane. Regarding *Pseudomonas sp.*, a common species in environments rich in VOCs [29-31], several issues can be highlighted. As seen in Figure 5, the observed percentage of *Pseudomonas sp.* was always higher than that of *Pseudomonas putida*. The samples from BTF2 presented *Pseudomonas sp.* as the predominant species, with values ranging between 8% and 29%. In addition, these percentages were higher than those observed in BTF1, where *Pseudomonas sp.* showed a stable behaviour with an average value of 4.3%. These results agree with the bacterial evolution previously presented in Figure 3, where the abundance of *Gammaproteobacteria* in BTF2 was higher than that in BTF1. In fact, the most abundant group in BTF2 was *Gammaproteobacteria* and the most abundant species *Pseudomonas sp.* The differences between the ecological communities observed in the two BTFs could be attributed to different internal environment characteristics of the biofilm of each system. The use of two packing materials with different structure and surface area could elicit changes

such as transfer to and diffusion into the biofilm of VOC, oxygen and nutrients or different degrees of biomass detachment, wetted area and distribution of irrigation. The oxygen mass transfer coefficients ($k_La$) of the packings have been estimated by San-Valero et al. [24], with a similar value for both materials of approximately 50 $h^{-1}$ at a liquid velocity of 10 m $h^{-1}$ and values of 125 and 175 $h^{-1}$ for random and structured packing, respectively, when a liquid velocity of 30 m $h^{-1}$ was used. All these changes at the microscopic scale affect the microbial community of the biofilm, which finally result in different behaviours at the macroscopic scale. In fact, BTF2, with a higher abundance of species belonging to *Gammaproteobacteria* such as *Pseudomonas sp.*, produced greater REs than BTF1 (Table 3). For example, at phases C-I and D-II, RE values of 81.4 and 79% were observed for BTF1, and 88.7 and 90% for BTF2. It is worth noting that at the liquid velocity applied in the study, 10 m $h^{-1}$, the packing materials of both BTFs present a similar value of the $k_La$ (50 $h^{-1}$). Therefore, the greater RE reached in BTF2 could be due to the higher population of these species, since the random packing (BTF2) does not present better mass-transfer properties. Changes in the performance of the system with changes in the microbial community show the importance of further studying the involved species during the removal process.

### *3.3 Monitoring of bacterial community by DGGE*

In parallel to FISH analyses, DGGE was performed to compare the 16SrDNA fragments of bacteria in the samples collected from both BTFs on different operational days. DGGE band patterns for samples taken on days 60, 100, 227 and 276 are presented in Figure 6. The samples were also compared to a control sample of an enriched batch culture of *Pseudomonas putida*. Analysis of DGGE patterns revealed changes in the structure of the bacterial community during the experimental period. After 276 days of operation, there was a decrease in the number of bands in both BTFs, indicating lower diversity in the microbial community at the end of the experiment. The fact that this was also observed in the FISH analyses could be explained by the specialisation of specific groups and/or species that are more suitable for degrading the pollutant. It should be noted that despite the difference in the bacterial community structure as revealed by the DGGE patterns, some bands appeared in all the

samples analysed, indicating that several species could survive these conditions because of their ability to survive in environments rich in VOCs and/or participate in their degradation. As shown in Figure 6, one of these stable bands matched the culture enriched with *Pseudomonas putida*, corroborating the presence of this species in environments with VOCs.

## 4 Conclusions

Two biotrickling filters treating intermittent emissions contaminated with isopropanol were monitored over almost one year and the microbial communities were analysed by FISH and DGGE. Both BTFs were inoculated with the same inoculum, the same pollutant was removed by the two systems, and the same operational and maintenance conditions were applied, the only difference between the two being the type of packing material used. Under these conditions, the random packing material showed a slightly higher capacity for removal. At the end of the experiment, the relative abundance of the *Gammaproteobacteria* group was observed to increase in both BTFs, with values of around 26%. This rise could be associated with the increase in the species belonging to this group such as *Pseudomonas sp.*, predominant species in BTF2 with values between 8% and 29%, while in BTF1, it exhibited a stable trend with an average value of 4.3%. Moreover, DGGE analysis confirmed the presence of *Pseudomonas putida* in all the samples monitored, even in the less diverse communities observed at the end of the experiment. The differences in the microbial community of both BTFs could be linked to the difference in the performances of the systems. For example, BTF2 displayed a removal efficiency of 90% compared to the 79% shown by BTF1 at phase D-II. Furthermore, in BTF2, the percentage of the common species present in VOC-rich environments such as *Pseudomonas sp.* was twice as much as that observed in BTF1 (19.2% and 8%, respectively). Results show the importance of further studying the involved species during the removal process, e.g. future studies include the application of new

molecular biological tools as next-generation sequencing methods.

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Table 1. Experimental plan for the removal of isopropanol under intermittent loading conditions. EBRT: empty bed residence times; IL: inlet load.

| | PHASE A | | | PHASE B | | | PHASE C | | PHASE D | |
|---|---|---|---|---|---|---|---|---|---|---|
| | I | II | III | I | II | III | I | II | I | II |
| Days | 0-48 | 49-69 | 70-90 | 91-97 | 98-104 | 105-125 | 126-132 | 133-163 | 217-228 | 229-300 |
| Sampling event on day | | 60 | | | 100 | | 130 | | 227 | 245, 276 |
| Inlet concentration, mg C $Nm^{-3}$ | | 1000 | | | 500 | | | 250 | 500 | 1000 |
| IL, g C $m^{-3}$ $h^{-1}$ | 20 | 35 | 65 | 20 | 35 | 65 | 35 | 65 | 35 | 65 |
| EBRT, s | 160 | 90 | 50 | 90 | 50 | 25 | 24 | 14 | 60 | 50 |

Table 2. List of oligonucleotide probes used in this study

| Probe | Specificity | Sequence, 5'–3' | Formamide, % |
|---|---|---|---|
| EUB338[a] | *Most Bacteria* | GCTGCCTCCCGTAGGAGT | 0 – 50 |
| EUB338-II[a] | *Bacteria not detected by EUB338* | GCAGCCACCCGTAGGTGT | 0 – 50 |
| EUB338-III[a] | *Bacteria not detected by EUB338* | GCTGCCACCCGTAGGTGT | 0 – 50 |
| ALF968 | *α–Proteobacteria* | GGTAAGGTTCTGCGCGTT | 20 |
| BET42A | *β–Proteobacteria* | GCCTTCCCACTTCGTTT | 35 |
| - | *Competitor for BET42A* | GCCTTCCCACATCGTTT | 35 |
| GAM42A | *γ –Proteobacteria* | GCCTTCCCACATCGTTT | 35 |
| - | *Competitor for GAM42A* | GCCTTCCCACTTCGTTT | 35 |
| HGC69A | *Actinobacteria (high G+C Gram-positive bacteria)* | TATAGTTACCACCGCCGT | 25 |
| - | *Competitor for HGC69A* | TATAGTTACGGCCGCCGT | 25 |
| LGC354A[b] | *Firmicutes (low G+C Gram-positive bacteria)* | TGGAAGATTCCCTACTGC | 35 |
| LGC354B[b] | *Firmicutes (low G+C Gram-positive bacteria)* | CGGAAGATTCCCTACTGC | 35 |
| LGC354C[b] | *Firmicutes (low G+C Gram-positive bacteria)* | CCGAAGATTCCCTACTGC | 35 |
| PS56a | *Pseudomonas sp.* | GCTGGCCTAGCCTTC | 0 |
| Ppu56A | *P. putida* | GCTGGCCTAACCTTC | 0 |
| REX72 | *Bacillus* | TGGGAGCAAGCTCCCAAAG | 20 |
| Myc657 | *Mycobacterium* | AGTCTCCCCTGYAGTA | 30 |

[a] EUB338, EUB338-II and EUB338-III were used in the mixture $EUB_{mix}$

[b] LGC354A, LGC354B and LGC354C were used in the mixture $LGC_{mix}$

Table 3. Operational and performance parameters of both BTFs on the sampling days. EBRT: empty bed residence time; IL: inlet load; RE: removal efficiency.

| Sampling day (Phase) | EBRT, s | | Inlet concentration, mg C $Nm^{-3}$ | | IL, g C $m^{-3}h^{-1}$ | | RE, % | |
|---|---|---|---|---|---|---|---|---|
| | BTF1 | BTF2 | BTF1 | BTF2 | BTF1 | BTF2 | BTF1 | BTF2 |
| 60 (A-II) | 90 | 90 | 1021 | 1035 | 41.0 | 38.0 | 85.6 | 86.6 |
| 100 (B-II) | 52 | 51 | 530 | 517 | 34.0 | 33.4 | 89.6 | 90.9 |
| 130 (C-I) | 27 | 27 | 298 | 295 | 37.0 | 38.8 | 81.4 | 88.7 |
| *Starvation period from day 164 to 217* | | | | | | | | |
| 227 (D-I) | 59 | 60 | 632 | 630 | 35.3 | 34.5 | 89.7 | 94.6 |
| 245 (D-II) | 48 | 49 | 898 | 1051 | 61.0 | 71.0 | 77.0 | 77.0 |
| 276 (D-II) | 48 | 48 | 1091 | 1095 | 75.0 | 75.0 | 79.0 | 90.0 |

Table 4. Total percentage values of the bacterial groups in both BTFs

| Sampling day (Phase) | ***Total percentage value*** | |
|---|---|---|
| | *BTF1* | *BTF2* |
| 60 (A-II) | 29.7 | 69.4 |
| 100 (B-II) | 62.2 | 76.5 |
| 130 (C-I) | 64.9 | 60.1 |
| *Starvation period from day 164 to 217* | | |
| 227 (D-I) | 72.9 | 76.1 |
| 245 (D-II) | 63.9 | 61.9 |
| 276 (D-II) | 79.2 | 88.1 |

**Figure 1.** Schematic of the experimental set-up.

**Figure2.** Performance of the BTFs in removing isopropanol, (a) BTF1 and (b) BTF2. Arrows represent the days when biomass samples were taken. (◆) Inlet Load, (✕) Elimination Capacity and (□) Removal Efficiency.

**Figure 3.** Time-dependent changes in bacterial community composition using general FISH probes. Results corresponding to (a) BTF1 and (b) BTF2.

**Figure 4.** *Gammaproteobacteria* in BTF2 on day 276. (a) General probe and (b) specific probe.

**Figure 5.** Time-dependent changes in bacterial community composition using specific FISH probes. Results corresponding to (a) BTF1 and (b) BTF2.

**Figure 6.** DGGE banding patterns of bacterial 16S rRNA from samples on days 60, 100, 227 and 276 in (a) BTF1 and (b) BTF2. Sample of a culture enriched with *Pseudomonas putida* was also included.

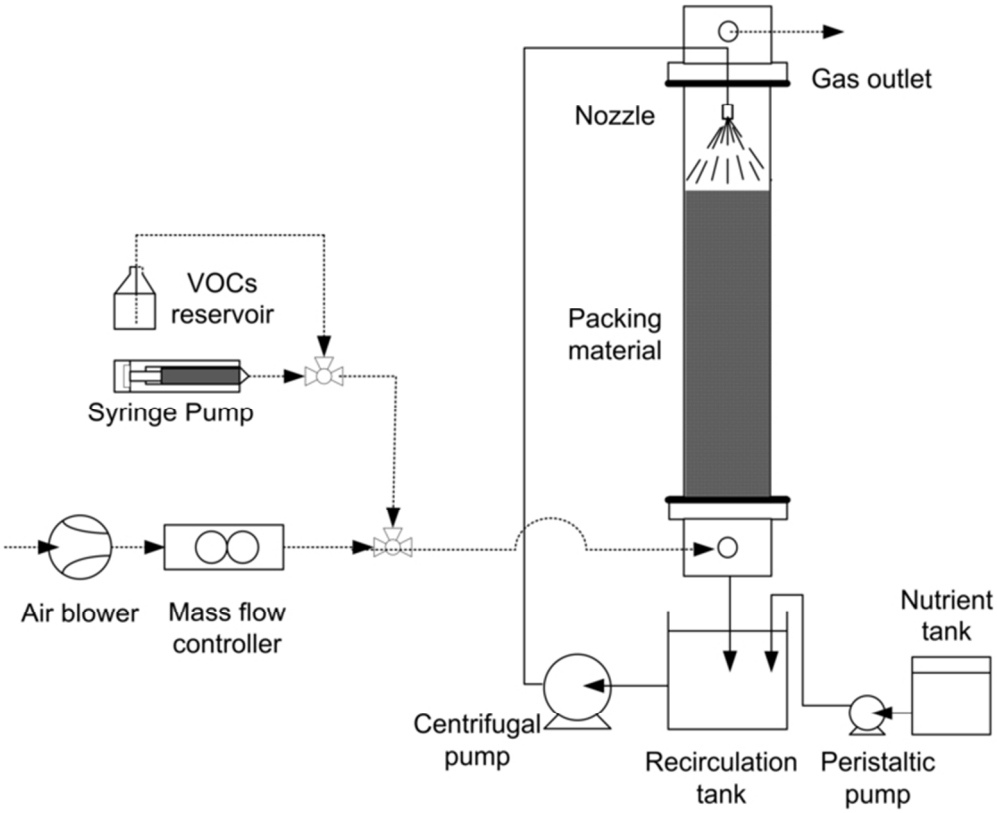


Figure 1. Schematic of the experimental set-up.
64x52mm (300 x 300 DPI)

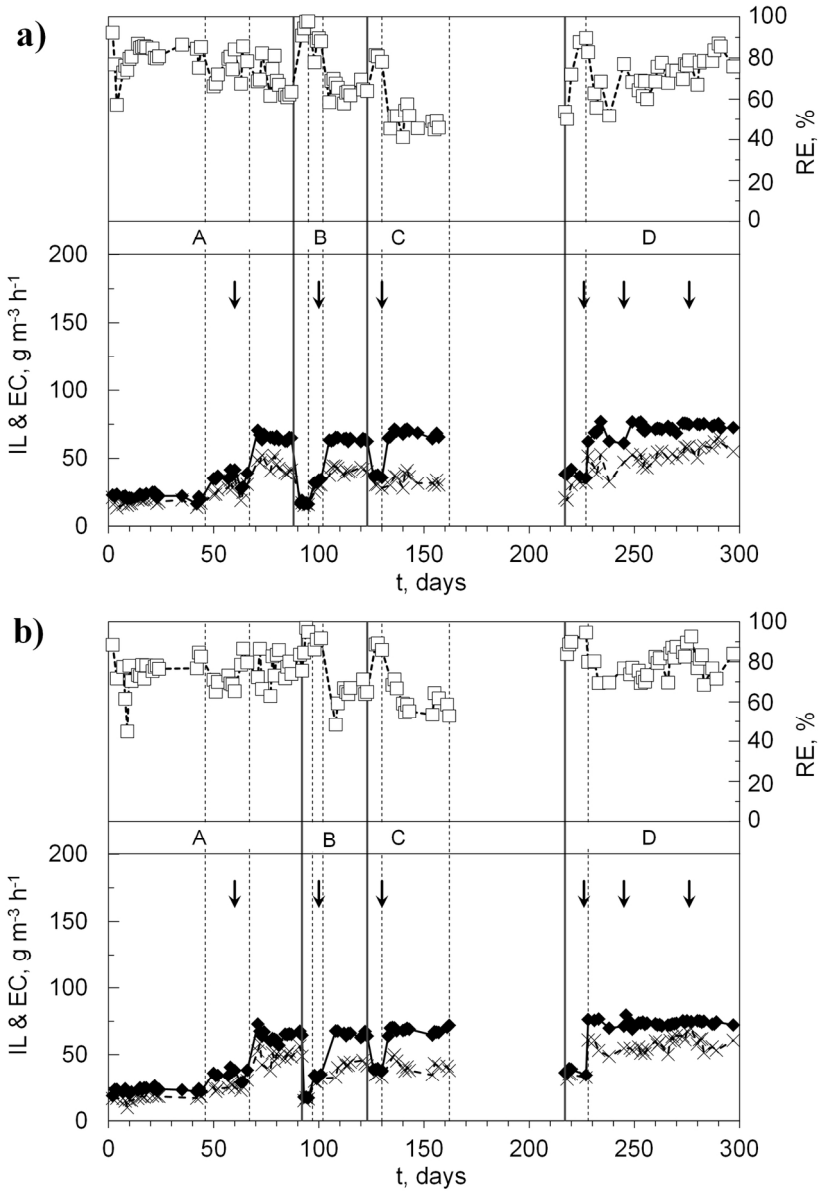


Figure 2. Performance of the BTFs in removing isopropanol, (a) BTF1 and (b) BTF2. Arrows represent the days when biomass samples were taken. (■) Inlet Load, (X) Elimination Capacity and (o) Removal Efficiency
131x191mm (300 x 300 DPI)

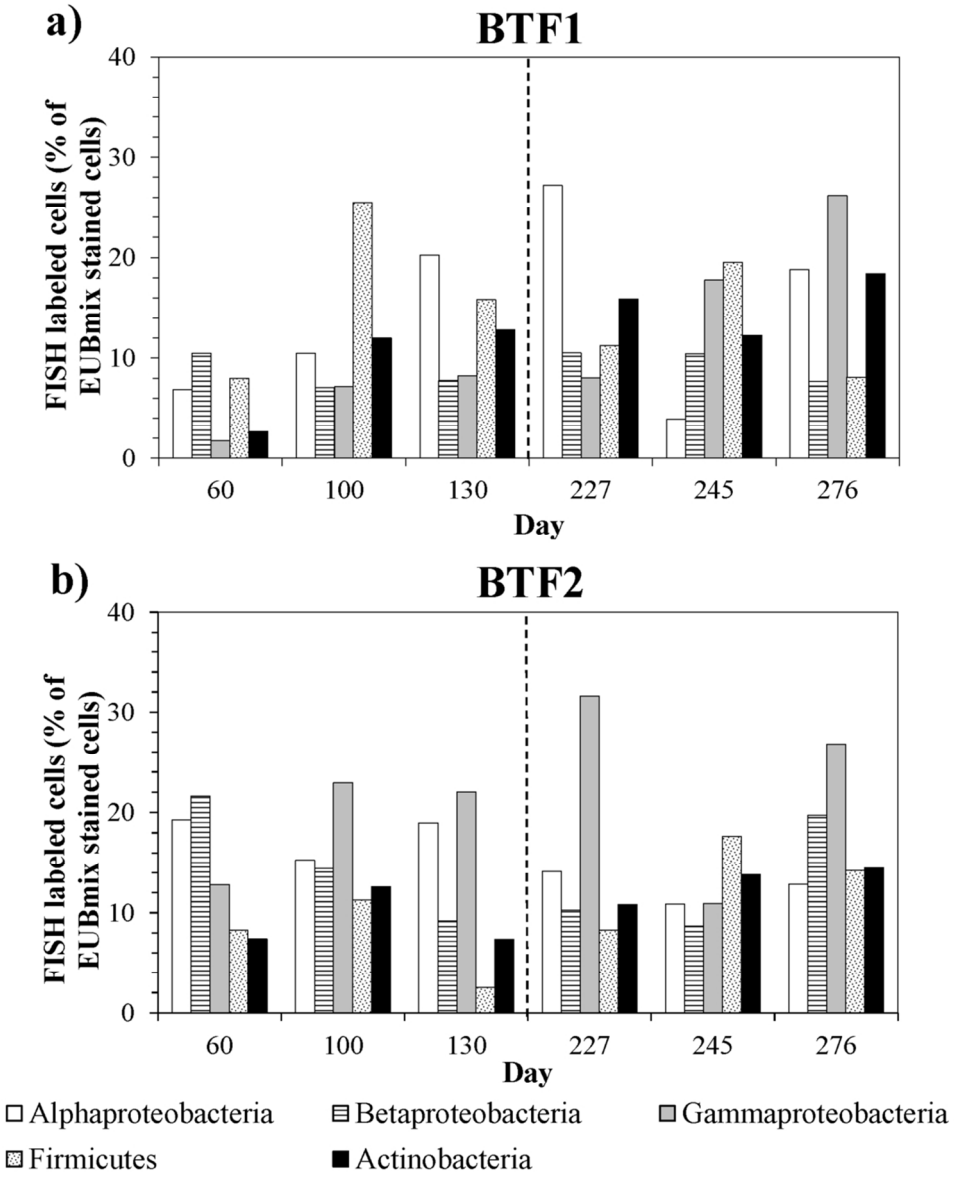


Figure 3. Time-dependent changes in bacterial community composition using general FISH probes. Results corresponding to (a) BFT1 and (b) BTF2.
99x123mm (300 x 300 DPI)

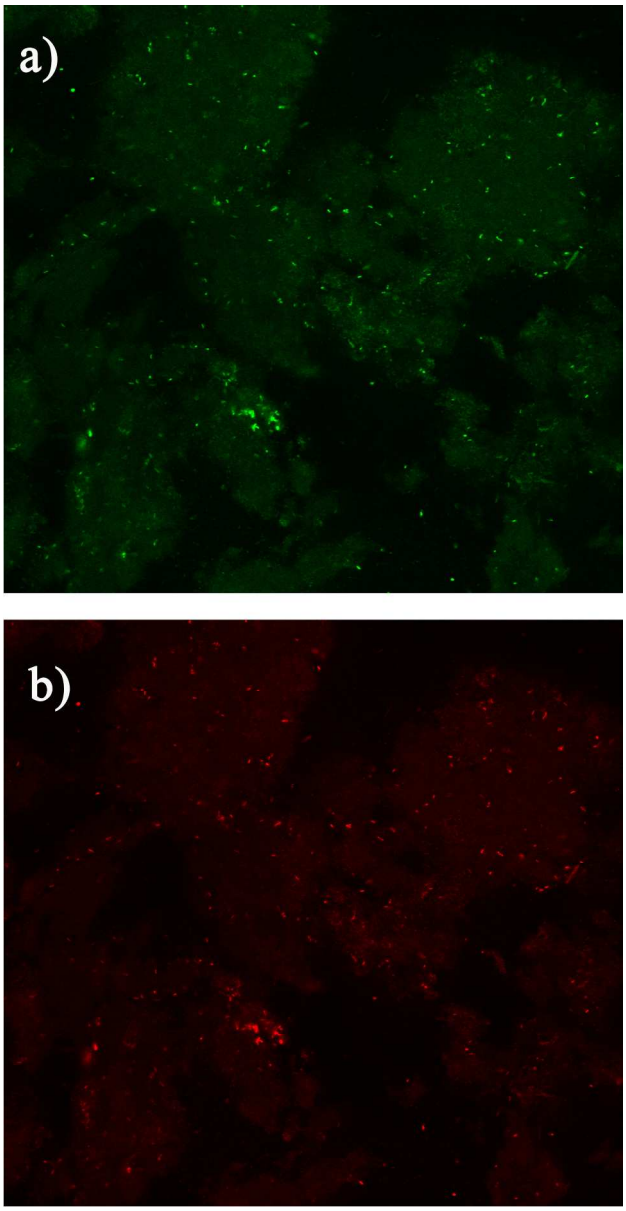


Figure 4. Gammaproteobacteria in BTF2 on day 276. (a) General probe and (b) specific probe.
154x300mm (300 x 300 DPI)

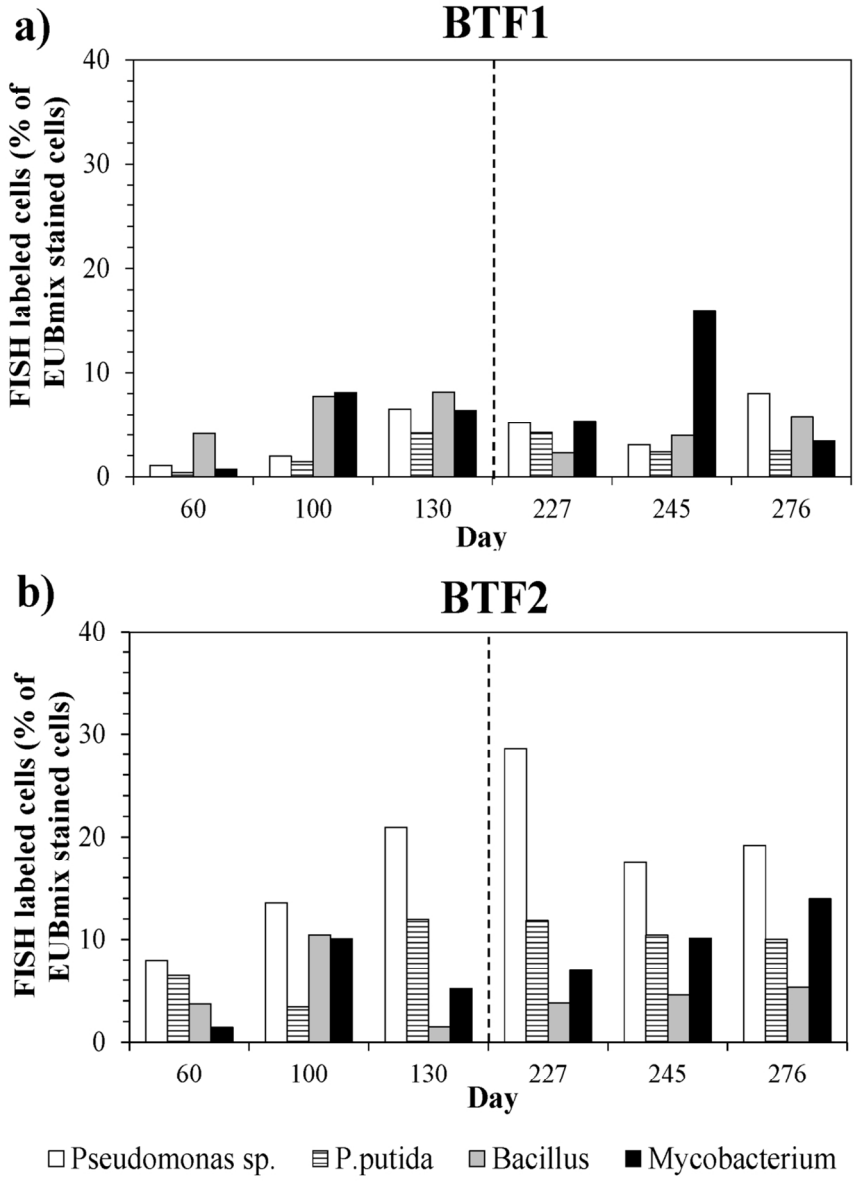


Figure 5. Time-dependent changes in bacterial community composition using specific FISH probes. Results corresponding to (a) BFT1 and (b) BTF2.
107x144mm (300 x 300 DPI)

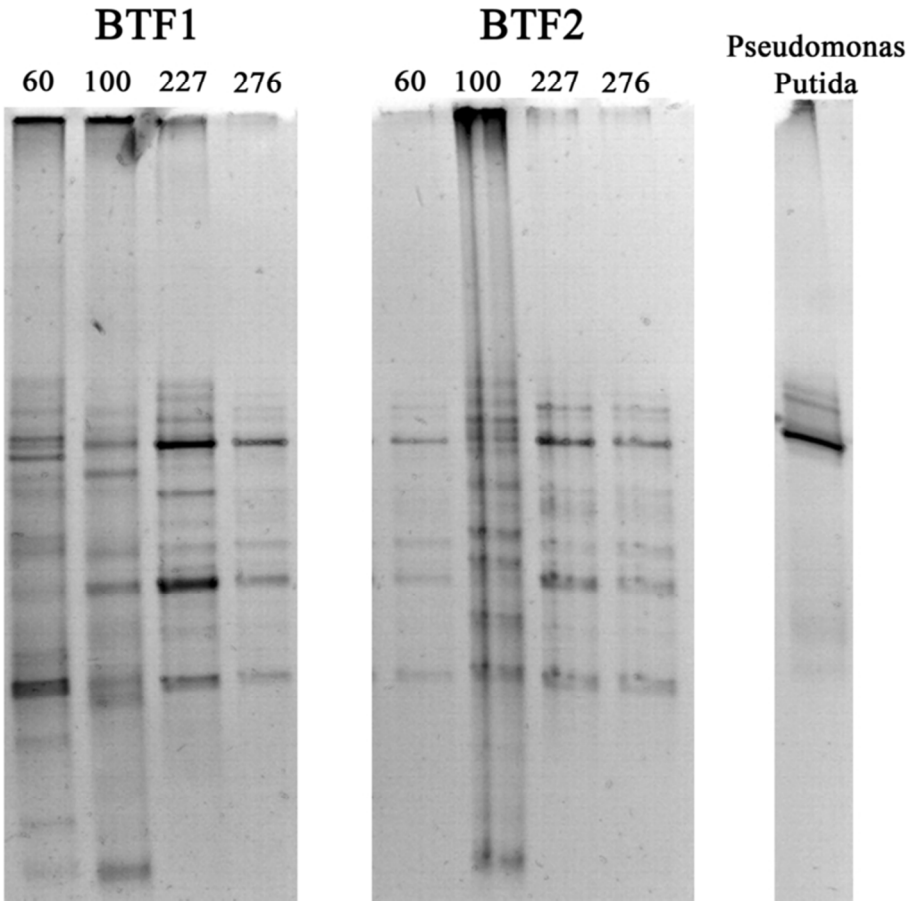


Figure 6. DGGE banding patterns of bacterial 16S rRNA from samples on days 60, 100, 227 and 276 in (a) BTF1 and (b) BTF2. Sample of a culture enriched with Pseudomonas putida was also included.
73x67mm (300 x 300 DPI)