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Inoculation and start-up of a biotricking filter removing dimethyl sulfide

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Abstract

Two *Hyphomicrobium* VS inoculation protocols were compared for start-up of a biotrickling filter removing dimethyl sulfide (DMS). One biotrickling filter (HBF 1) was filled with rings that were submerged in a nutrient medium containing *Hyphomicrobium* VS fed with DMS, another biotrickling filter (HBF 2) was similarly filled with rings that were submerged in nutrient medium, but continuously supplied with actively growing *Hyphomicrobium* VS and fed with methanol. Initially, about 40 times more *Hyphomicrobium* VS cells were attached to the rings in HBF 2. During the experiment, two to three times more *Hyphomicrobium* VS cells were still found to be present on the rings in HBF 2 compared to HBF 1. The maximal DMS elimination capacity at 90% removal efficiency of HBF 1 was 7.2 g m^{-3} h^{-1} after 30 days of operation. The elimination capacity decreased, however, when the inlet loading rate exceeded 15 g m^{-3} h^{-1} (200 ppmv inlet concentration). The performance of HBF 2 was much better, with an elimination capacity of 8.3 g m^{-3} h^{-1} (90% removal efficiency) after 2 days of operation, increasing to a maximum of 57 g m^{-3} h^{-1} at 92% removal efficiency. Microbial community analysis with denaturing gradient gel electrophoresis showed very different microbial communities in both biotrickling filters (Pearson correlation coefficient of 0%). Moreover, the decreased DMS elimination capacity of HBF 1 at higher influent loading rate corresponded with a drastic change of the microbial community on the rings. The latter observations suggest that the functional efficiency of the microbial community in a biotrickling filter can be related to its composition.

Keywords: Biotrickling filter; *Hyphomicrobium* VS; Inoculation; Dimethyl sulfide; Microbial ecology

1. Introduction

Biological waste gas treatment techniques can provide cost-effective solutions for treating odorous or solvent laden airstreams [1,2]. The most widely known and used technique is biofiltration, which is generally also the most economical one. A biofilter usually consists of an organic filter bed, containing microorganisms and nutrients, in which gaseous pollutants are degraded. Its major drawback is limited process control, potentially leading to dehydration and acidification, dependent on the waste gas characteristics. Biotrickling filters are more recent designs, in which microorganisms form a biofilm on an inert carrier material that is kept moist by circulation of a liquid medium. These bioreactors provide superior process control, which is an advantage especially for highly concentrated waste streams or waste streams containing acidifying pollutants, like sulfur, chlorine or nitrogen containing compounds. In biofilters, a sufficient number and a high diversity of microorganisms are generally present and therefore, inoculation is not frequently applied. Only in a few cases has it been shown that inoculation was beneficial, e.g. for removal of volatile organic sulfur compounds [3,4] or ethene [5]. In a

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number of other studies, inoculation was applied as a standard procedure, although its usefulness was not demonstrated or investigated [6–11]. In biotrickling filters, few microorganisms are initially present on the carrier material and inoculation is therefore always needed. Mostly (enriched) mixed microbial communities of unknown composition are used for this purpose [12–17]. Usually a standard inoculation protocol is applied, by recirculating the inoculum liquid over the packing for a few hours before or directly upon start-up of the biotrickling filters. It can take up to several weeks before the bioreactors are effective in removing the gaseous pollutants. To our knowledge, there are no studies attempting to shorten the start-up period or to increase the elimination capacity by modifying the inoculation protocol, although this could be of practical importance. One possible strategy is to maximize the number of useful, pollutant-degrading strains and to minimize the presence of microorganisms that are not directly metabolizing the pollutants, by inoculating with axenic cultures of microorganisms. However, only few studies have applied inoculation with axenic cultures (e.g. [18,19]) and little information is available on how microbial communities evolve during or after inoculation of biotrickling filters.

In recent years, molecular techniques have been increasingly applied to investigate microbial community composition in various ecosystems, including in biofilters and biotrickling filters. Cloning and sequencing of 16S rRNA is the most powerful technique for analysing microbial diversity in natural samples. However, for studying population changes, this approach is laborious, time-consuming and expensive. With genetic fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) or (terminal) restriction fragment analysis, multiple samples can be analyzed simultaneously, as is required for studying the complex dynamics of microbial communities [20].

In this study, the efficacy of the method of inoculation of a biotrickling filter with *Hyphomicrobium* VS was investigated to remove dimethyl sulfide from waste gases. Two different inoculation protocols are described, and the evolution of the *Hyphomicrobium* VS cell numbers and the biofilm microbial communities was investigated using plate-counting and denaturing gradient gel electrophoresis (DGGE), respectively.

2. Experimental

2.1. Microorganisms and media

Hyphomicrobium VS [21] was grown using mineral medium, containing 3 g L^{-1} K_2HPO_4, 3 g L^{-1} KH_2PO_4, 3 g L^{-1} NH_4Cl, 0.5 g L^{-1} $MgSO_4{\cdot}7H_2O$ and 0.01 g L^{-1} $FeSO_4{\cdot}7H_2O$ at pH 7, with the addition of 1% (v/v) methanol. *Hyphomicrobium* VS was initially cultured by adding 50 μL of the strain (kept at −80 °C in glycerol) to 5 mL of mineral medium containing 1% of methanol, and incubating the suspension for 5 days at 37 °C. For growing *Hyphomicrobium* VS on DMS, 20 mL of this pregrown culture was centrifuged and washed with saline (0.9% NaCl) twice and added to 1 L of mineral medium. This suspension was provided with 100 ppmv DMS in air (200 ml min^{-1}) until growth was visible ($4.0 \pm 1.0 \times 10^7$ cells mL^{-1}). For starting the growth of *Hyphomicrobium* VS in a chemostat, 20 mL of the pregrown culture was added to 2 L of mineral medium. The chemostat was subsequently aerated and supplied with fresh, sterile mineral medium at 75 mL h^{-1} and methanol at a 1% influent concentration. The hydraulic residence time of this reactor (26.7 h) was slightly greater than the *Hyphomicrobium* VS doubling time (about 24 h). The effluent of this chemostat was used in the second inoculation protocol (see below).

2.2. Biotrickling filter construction

The biotrickling filter is made of plexiglass (internal diameter: 0.045 m) and packed with 1 L of polyethylene carrier rings (diameter: 10 mm, height: 7 mm, free volume: 75.6%, specific surface area: 333 m^2 m^{-3}) (Kaldnes Miljøteknologi AS, Tønsberg, Norway). The carrier rings for the DMS degrading biofilters were coated with polydimethylsiloxane (PDMS) prior to packing of the reactor to improve the adhesive properties of the rings. Therefore, a 10% PDMS solution was prepared by mixing 10% (v/v) of 2-compontent RTV (0.9 vol of RTV 615 A and 0.1 vol of RTV 615 B) (GE Bayer Silicones, Bergen Op Zoom, The Netherlands) in hexane for 30 min at 70 °C. The rings were subsequently immersed in the solution and dried overnight for evaporation of the solvent. Finally, the coated rings were baked in an oven at 105 °C for 1 h, to ensure good adhesion of the PDMS on the rings. After coating, the PDMS density on the rings was 2.13 mg PDMS m^{-2}, or 20 μm theoretical film thickness.

The liquid medium was recirculated over the biotrickling filter at 150 mL min^{-1}. The recycled liquid medium was refreshed in a semi-continuous mode, by replacing 10% of the medium every day (hydraulic residence time 10 days), in order to keep the nutrient concentrations sufficiently high and to prevent sulfate accumulation (<15 g L^{-1}). In the medium for *Hyphomicrobium* VS, the 3 g L^{-1} NH_4Cl was replaced by 6.7 g L^{-1} KNO_3 to prevent growth of autotrophic ammonium oxidizing bacteria. If the pH was lower than 6.5, it was adjusted to 7 by adding 1 M NaOH manually. The air flow (dry air, Air Liquide) was provided in upflow mode (countercurrent with the liquid medium), at 0.5 L min^{-1}, providing an empty bed residence time (EBRT) of 120 s in each bioreactor. DMS was dosed in the air stream by a capillary diffusion system, as described by Smet et al. [22]. The system consists of one or more 4 mL vessels containing the liquid DMS, placed in a thermostatic water bath and each connected with the main air stream with a diffusion capillary. A concentration gradient between a vessel and the upper outlet of the diffusion capillary forces the compound to diffuse through the capillary. The DMS mass flux to the air stream is dependent on the capillary dimensions, water bath temperature and total pressure in the main air stream. Additional overpressure is provided by forcing the main air stream through capillary

tubing before entering the reactor, to minimize the effect of varying atmospheric pressures. In this case the concentration of DMS in the air stream was regulated by the number of vessels connected to the air stream. Gas sampling ports were provided in the tubing before and after the biotrickling filter.

2.3. Inoculation procedure and biotrickling filter experiments

A scheme of both inoculation procedures used in this study is shown in Fig. 1. In the first DMS degradation experiment (HBF 1), 1 L of rings were inoculated by submerging them for 24 h in 1 L of *Hyphomicrobium* VS culture, already adapted to degrade DMS ($4.0 \pm 1.0 \times 10^7$ cells mL^{-1}). An air stream containing 100 ppmv of DMS was bubbled through this reactor at a flow rate of 200 mL min^{-1}. After 24 h, the biotrickling filter column was packed with the inoculated rings and the operation of the reactor started. In a second inoculation protocol (HBF 2), the coated rings were inoculated by incubating 1 L of the rings in a 2 L Erlenmeyer. An actively growing *Hyphomicrobium* VS culture was continuously added to this Erlenmeyer, at a rate of 75 ml h^{-1}, from a chemostat of the bacteria. Oxygen was supplied into the flask by air sparging. After 5 days the rings were added to the biotrickling filter and the operation of the reactor started.

2.4. Analytical methods

Dimethyl sulfide concentrations were measured by a Varian 3700 chromatograph equipped with a flame ionization detector and a 30 m CP-SIL 5CB column (Chrompack, internal diameter 0.53 mm, film thickness 5 μm). A Pressure-Lok Precision Analytical Syringe (Alltech Ass.) was used for injecting 1 mL gas samples. Sulfate and nitrate concentrations were measured by analyzing the recycling medium with ion chromatography, using an IC 761 Compact Ion Chromatograph (Metrohm) with a metrosep A supp 5 column and a metrosep A 4/5 guard column. The mobile phase consisted of a 3.2 mM Na_2CO_3 and 1 mM $NaHCO_3$ buffer, supplemented with 5% acetone, supplied at a flow rate of 0.5 mL min^{-1}. The

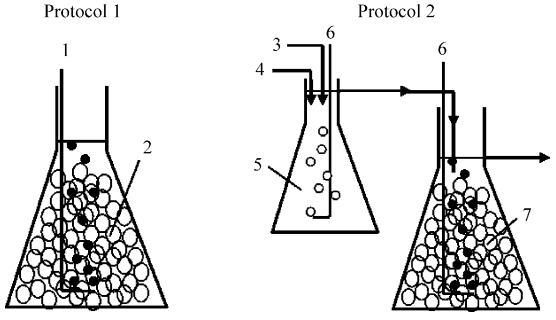

Fig. 1. Schematic representation of the inoculation protocols used. A: (1) DMS inlet, (2) rings + mineral medium + *Hyphomicrobium* VS; B: (3) methanol, (4) mineral medium, (5) *Hyphomicrobium* VS + mineral medium, (6) air and (7) rings + mineral medium + *Hyphomicrobium* VS.

pH of the liquid recycle was measured with a pH electrode (Jenway 3310).

2.5. Microbial community analysis

The diversity of the microbial community in both the liquid recycle medium and the biofilm on the carrier rings was analyzed by plate counts and by denaturing gradient gel electrophoresis (DGGE). For the liquid, 2 mL samples were taken from the storage tank. For the biofilm sampling, all rings were removed from the biotrickling filter, mixed and five rings were randomly taken. Those five rings were sonicated for 10 min in 5 mL of sterile deionized water with a Branson 2200 sonicator, operating at 47 kHz. Counting of *Hyphomicrobium* VS colony forming units (CFUs) was performed by plating 100 μL from a dilution series of the sample material on agar plates, using mineral medium containing 15 g L^{-1} agar. Each dilution was supplemented with 1% filter-sterilized methanol (modified from Pol et al. [21]). Isolation on nutrient agar was performed for samples from HBF 1.

The analysis of the microbial community diversity was performed in three subsequent steps, as described by Boon et al. [23]: first the DNA was extracted from the samples and purified; subsequently the purified DNA was amplified by PCR and finally the DNA strands were separated using denaturing gradient gel electrophoresis (DGGE). DNA extraction was performed by mixing 2 mL of liquid sample with 4 mL of sterile 10 mM Tris–HCl [pH 9] and 3 g of sterile glass beads. The suspension was homogenized three times for 90 s in a bead beater (B. Braun Biotech International, Melsungen, Germany), with 10 s of cooling between each run. After adding 160 μL of lysozyme (50 mg mL^{-1}) and gentle mixing for 10 min, 300 μL of 20% SDS was added and the sample was further mixed manually for 10 min. Subsequently, 1 mL of 8 M ammoniumacetate was added and the sample was centrifuged for 15 min at 3000 g (4 °C). An amount of 4 mL of chloroform/isoamylalcohol (24:1) (Fluka) was added to the supernatant and mixed manually until a homogenous suspension was obtained. The suspension was centrifuged again for 15 min at 3000 g (4 °C) and the water phase was recovered. After adding 0.8 vol of 100% isopropanol, the DNA was precipitated for 1 h at −20 °C. The DNA was finally centrifuged at 18,000 g for 25 min (4 °C), the pellet was dried at room temperature and the extracted DNA was dissolved in 100 μl of sterile deionised water. The crude DNA extract was further purified with Wizard DNA Clean-Up System (Promega, Madison, Wisconsin, USA), according to manufacturer instructions and the purified DNA was finally recovered in 50 μl DNase- and RNase-free filter-sterilized water and frozen at −20 °C until further analysis. DNA amplification was performed with the bacterial primers P338FGC and 518R, using the PCR Core System I (Promega). An amount of 1 μL of the DNA sample was added to 24 μL of the master mix in a Biozym Minicycler thermocycler. The final concentrations of the different compounds in the mastermix were: 0.2 μM of each

primer, 200 μM of each dNTP, 1× *Taq* DNA Polymerase, 10× reaction buffer, 1.5 mM $MgCl_2$, 400 ng μL^{-1} of bovine serum albumin (Hoffmann-La Roche, Basel, Switzerland) and 1.25 U/50 μL of *Taq* DNA polymerase in DNase- and RNase-free filter-sterilized water (Sigma–Aldrich Chemie, Steinheim, Germany). After each PCR amplification round, the size of the PCR product was verified on a 1% agarose gel. After PCR samples were stored at −20 °C. DGGE was performed using the Bio-Rad D Gene System (Bio-Rad, Hercules, CA, USA) based on the protocol by Muyzer et al [24]. PCR products were loaded onto a 8% (w/v) polyacrylamide gel in 1X TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4). The polyacrylamide gels were made with denaturing gradients ranging from 45 to 70% (100% denaturant contains 7 M urea and 40% formamide). After the electrophoresis (16 h, 60 °C, 38 V), gels were stained with SYBR Green I nucleic acid gel stain (1:10,000 dilution, FMC BioProducts, Rockland, ME, USA) during 20 min with agitation. The stained gel was immediately photographed on a UV transillumination table with a Video Camera Module (Vilbert Lournat, Manre-la Vallé, France). The processing of the DGGE gels was done with the Bionumerics software 2.0 (Applied Maths, Kortrijk, Belgium). To compare banding patterns in DGGE gels, the Shannon Diversity Index (H) [25] was calculated as follows, for a sample on day '*i*':

$$H_i = -\sum_{j=1}^{S} P_j \ln P_j$$

$$P_j = \frac{n_j}{N}$$

with n_j is the intensity of peak '*j*' in the DGGE banding pattern, *N* the sum of all peak intensities in the DGGE banding pattern and *S* the number of peaks in the DGGE banding pattern.

3. Results

3.1. DMS degradation and inoculation protocol

In Fig. 2, the DMS influent and effluent concentration, inlet loading rate (B_v) and elimination capacity (EC) of the first biotrickling filter inoculated with *Hyphomicrobium* VS (HBF 1) are shown. The DMS influent concentration of this 47-day-long experiment was gradually increased from 45 to about 200 ppmv. The first day of operation, a removal efficiency of 58% was observed. After an adaptation period of 16 days, a stable removal efficiency of 90 ± 1% was obtained for the next 11 days, at influent concentrations varying between 44 and 91 ppmv. During a further increase of the DMS influent concentration, some temporary decreases of the removal efficiency were observed, mostly due to sharp increases of the influent concentration. However, on day 37, at a DMS influent concentration of 117 ppmv, the removal efficiency decreased sharply to 68%. The highest observed EC during this experiment at $\eta > 90\%$ was 7.2 g m^{-3} h^{-1}, while the maximum EC (EC_{max}) was 9.3 g m^{-3} h^{-1} on day 42 ($\eta = 68\%$). After the DMS influent concentration was further increased up to 200 ppmv, the removal efficiency decreased below 50%. In addition, the EC also decreased from 8.3 ± 0.8 g m^{-3} h^{-1} (days 41–44) to 5.2 ± 0.2 g m^{-3} h^{-1} (days 45–47). The pH value of the circulating liquid was controlled between 5.9 and 7 during the entire experiment. Because the maximum DMS elimination capacities were low compared with literature data [14], even after 47 days of reactor operation, it was decided to start up a new biotrickling filter to try to increase the maximum EC.

The second DMS degrading biotrickling filter (HBF 2) was started, after using an adapted *Hyphomicrobium* VS inoculation protocol. The main differences between both protocols were (i) the carbon source, being DMS in the first protocol and methanol in the second and (ii) the inoculation reactor, operated in batch mode or continuously supplied with actively growing bacteria in the first and second protocol, respectively. In Fig. 3, the DMS influent and effluent concentration, inlet loading rate and elimination capacity of the HBF 2 are shown. After 2 days of operation, a removal efficiency of 90% was already obtained, at DMS influent concentrations of about 120 ppmv (EC = 8.3 g m^{-3} h^{-1}). At this point, the elimination capacity was larger than obtained in HBF 1, at similar removal efficiency. The DMS influent concentration was gradually increased to a final concentration of 600–800 ppmv between days 63 and 72. During this concentration increase, the DMS removal efficiency of HBF 2 remained between 88 and 99% (days 3–72), except for some

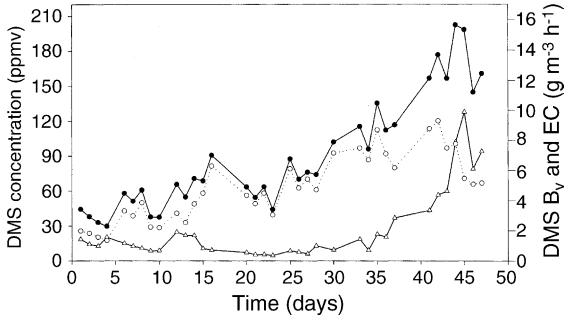

Fig. 2. Left axis: DMS inlet (●) and outlet (△) concentration; right axis: DMS loading rate (●) and elimination capacity (◯) for HBF 1.

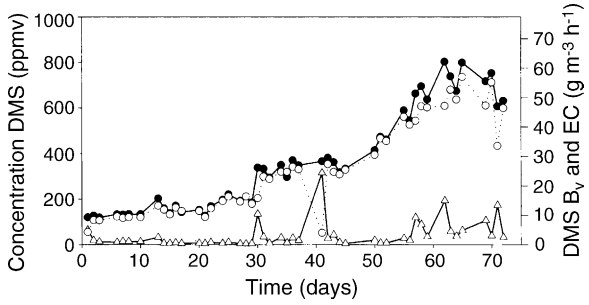

Fig. 3. Left axis: DMS inlet (●) and outlet (△) concentration; right axis: DMS loading rate (●) and elimination capacity (◯) for HBF 2.

temporary decreases. The decreases in removal efficiency on days 30, 57 and 62 can be explained by increases of the DMS influent concentration with steps of 120 to 200 ppmv. Adaptation periods of 1 to 2 days were required for the microorganisms to adapt to higher DMS influent concentrations. The lower removal efficiency ($\eta = 14\%$) on day 41, on the other hand, can be explained by a temporary decrease of the pH of the liquid medium to 5. This low pH was caused by a delayed change-out of the medium for 3 days, causing accumulation of H_2SO_4, but again, recovery of the removal efficiency was obtained after 1 day. All other measured pH values remained between 6.1 and 7. The highest EC obtained with HBF 2 was 57 g DMS m^{-3} h^{-1} ($\eta = 92\%$) on day 65.

3.2. Microbial community dynamics

In Table 1, the amount of *Hyphomicrobium* VS cells on the carrier rings and in the recycling medium are shown for HBF 1 and HBF 2. Shortly after inoculation, the cell number on the rings for HBF 2 was about 40 times higher than for HBF 1 ($(1.4 \pm 0.5) \times 10^9$ CFU $ring^{-1}$ and $(3.4 \pm 1.3) \times 10^7$ CFU $ring^{-1}$, respectively). However, after inoculation the number of *Hyphomicrobium* VS increased in HBF 1 and decreased in HBF 2. After 20 days the mean number of *Hyphomicrobium* VS on the carrier rings was about two to three times greater in HBF 2. Moreover, significant amounts of *Hyphomicrobium* VS were present in the liquid phase of both reactors, although these numbers were more variable than those for the bacteria attached to the rings.

Plating on nutrient agar showed that six different colony types of heterotrophs were present in HBF 1 (results not shown). Amplified DNA extracts of the two most important (orange and white) colonies were loaded on DGGE gels, to compare their occurrence in DNA extracts from the biofilm samples. For HBF 2, heterotrophic plate counts were not performed, and only DGGE was used to assess microbial diversity.

Fig. 4 shows the DGGE profiles for the biofilm microbial communities in HBF 1 and HBF 2. The bands corresponding to *Hyphomicrobium* VS were determined by comparing the positions of the bands with that of a pure *Hyphomicrobium* VS culture and are marked in Fig. 4 with a square. Other marked bands were compared on a second gel to confirm their equal position in the gel, which is less clear in Fig. 4 due to a slightly uneven gradient distribution in the gel (data not shown). The white and orange colonies that were found on the nutrient agar plates appeared to be invisible or very faint in the mixed communities of HBF 1 and 2.

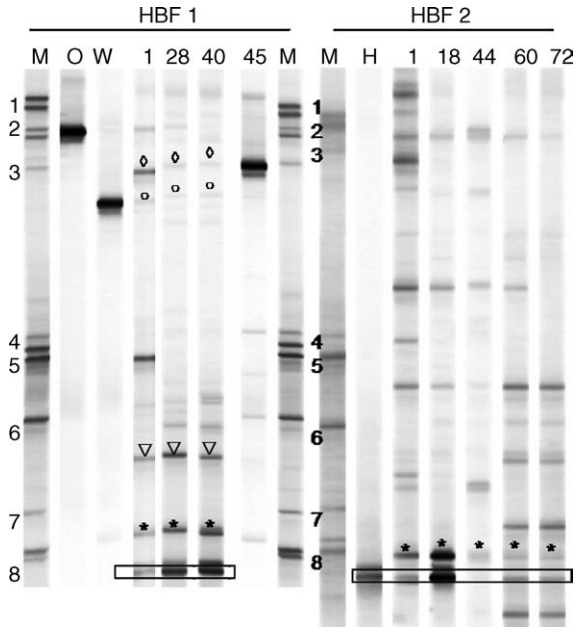

Fig. 4. DGGE paterns of the biofilms in HBF 1 and HBF 2. The bands corresponding with *Hyphomicrobium* VS are shown in squares. The banding patterns of the isolated orange and white colonies and the pure *Hyphomicrobium* VS culture are marked as O, W and H, respectively. Equal reference markers (M) are incidated with numbers from 1 to 8. Series of equal bands are marked with (*), (▽), (◯) and (◊). The dates of the samples are indicated above the gel picture.

Visual inspection of the gel indicates that the microbial community in HBF 1 biofilm changed slightly between the inoculation and day 28 and then remained unchanged until day 40. Five major bands present on day 1 (*Hyphomicrobium* VS and the bands marked with symbols) remained present during the first 40 days of reactor operation. Only one major band had disappeared by day 40. Between day 40 and 45, however, a sudden change in microbial community composition was observed, with one band becoming very dominant. At the same time, the band corresponding to *Hyphomicrobium* VS disappeared.

The microbial community in the biofilm of HBF 2 evolved after inoculation during some 44 days. Visual inspection of

Table 1
Hyphomicrobium VS plate counts for the biotrickling filters after the first (HBF 1) and second (HBF 2) inoculation protocol (results are shown for the biofilm on the rings and for the recycle liquid)

Day	Biofilm (CFU $ring^{-1}$)		Liquid (CFU ml^{-1})	
	HBF 1	HBF 2	HBF 1	HBF 2
0	$(3.4 \pm 1.3) \times 10^7$	$(1.4 \pm 0.5) \times 10^9$	$(7.6 \pm 2.1) \times 10^7$	–
17		$(3.8 \pm 0.8) \times 10^8$		$(1.0 \pm 5.0) \times 10^5$
20	$(1.9 \pm 0.4) \times 10^8$		$(3.0 \pm 1.0) \times 10^8$	
44		$(4.2 \pm 5.0) \times 10^8$		$(3.0 \pm 7.0) \times 10^6$
47	$(1.3 \pm 0.3) \times 10^8$		$(6.0 \pm 4.0) \times 10^6$	
71		$(5.3 \pm 0.5) \times 10^8$		$(2.0 \pm 1.0) \times 10^7$

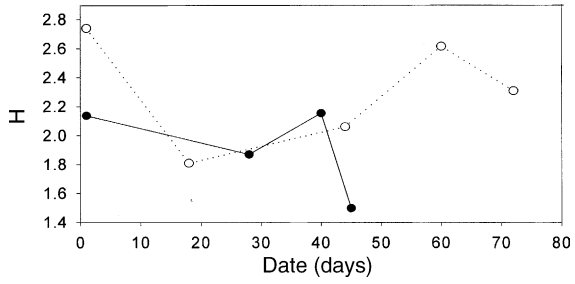

Fig. 5. Shannon diversity index (H) for the biofilm samples of HBF 1 (●) and HBF 2 (○).

the gel patterns shows that 10 out of the 16 ribotypes, representing the microorganisms initially present on the inoculated rings, had disappeared on day 60, while at that time about five new ribotypes were present. It took between 44 and 60 days before a stable microbial community was obtained. In the banding patterns, the band representing *Hyphomicrobium* VS can clearly be distinguished (except for day 44), next to a diversity of ribotypes that were not present in the initial inoculum.

In Fig. 5, the evolution of the Shannon Diversity Index (H) for the biofilm samples of HBF 1 and HBF 2 is shown. The diversity was rather constant in HBF 1 initially ($H = 2.1 \pm 0.2$), but then decreased to a minimum of 1.5 at the end of the experiment. In HBF 2, however, a value greater than 2.0 was maintained during the experiment, except for a decrease to 1.8 after reactor start-up.

4. Discussion

The DMS removal efficiency and the EC of HBF 1 were not as good as was initially expected, based on comparison with literature data. The EC_{max} was 9.3 g m^{-3} h^{-1} ($\eta = 68\%$), which compares unfavorably with values in the literature from about 16 g m^{-3} h^{-1} ($\eta = 71\%$) [21] to as large as 71 g m^{-3} h^{-1} ($\eta > 99\%$) [14]. Besides the low EC_{max} in the reactor, a decreasing EC was observed after the application of loading rates exceeding 15 g m^{-3} h^{-1}. It is likely that inhibition of the biological activity occurred, possibly due to toxicity of the high DMS influent concentrations (about 200 ppmv). Smet et al. [3] have previously shown that high DMS concentrations can exert a toxic effect on a Hyphomicrobium enrichment culture, especially at concentrations exceeding 300 ppmv. In the experiments with HBF 1, the operational parameters were controlled as follows: pH 6 to 7; sulfate concentration <15 g L^{-1}; nitrate concentration 2.5–5.3 g L^{-1} and hydraulic residence time of the circulated medium 10 days. Because these conditions should provide good growing conditions for *Hyphomicrobium* VS [14,21], it was expected that the cause of the overall low removal efficiencies in HBF 1 was related to the composition or activity of the microbial community that colonized the rings rather than due to suboptimal operational conditions. After starting HBF 2, the DMS removal efficiencies obtained and the elimination capacities confirmed that the 2nd inoculation protocol provided superior start-up and long-term stability of the biotrickling filter, even at DMS influent concentrations up to 800 ppmv (inlet loading = 62 g m^{-3} h^{-1}). Reactor operation revealed that the DMS removal efficiency was sensitive to DMS step increases of 50 ppmv or more and to a pH decrease below 5.

After the first inoculation protocol, the microbial diversity of the biofilm was investigated by plating on nutrient agar and by DGGE analysis. Rather low colony diversity (six different colony types) was obtained with the plate counts, compared with the 13–15 ribotypes observed on the DGGE patterns of HBF 1. Comparing the corresponding DGGE bands of the two major colony types with the actual banding pattern of the total biofilm microbial community showed that the isolated microorganisms constituted only minor part of the biofilm microbial community. Therefore, plate-counting was discontinued for microbial community analyses in HBF 2, because of apparent selective enrichment for only a portion of the bacteria present. Wagner et al. [26] have also observed that culture dependent community structure analysis of activated sludge produced partial and heavily biased results when compared with oligonucleotide probing.

When *Hyphomicrobium* VS cell counts and DGGE patterns for the HBF 1 and HBF 2 biofilms were compared, a major influence of the inoculation protocol on the microbial community and its evolution could be observed. In HBF 1, about 40 times lower *Hyphomicrobium* VS cell numbers were initially present on the rings compared to those in HBF 2. One of the reasons for this is probably the faster growth rate of *Hyphomicrobium* VS on methanol than on DMS. According to de Zwart et al. [27], growth rates of *Methylophaga sulfidovorans*, a representative for methylotrophs in microbial mats, are about four times lower on DMS ($\mu = 0.08\ h^{-1}$) than on methanol ($\mu = 0.3\ h^{-1}$). Also Pol et al. [21] estimated the maximum growth rates of *Hyphomicrobium* VS on methanol and dimethyl disulfide to be about 0.14 and 0.065 h^{-1}, respectively. After 20 days of reactor operation, the difference in the numbers of *Hyphomicrobium* VS cells was much less, but they remained present on the rings of HBF 2 at an average of a factor of two to three times more than in HBF 1. The high initial *Hyphomicrobium* VS cell number of $(1.4 \pm 0.5) \times 10^9$ CFU $ring^{-1}$ could not be sustained during reactor operation, indicating that a maximal carrying capacity of about 4 to 5×10^8 CFU $ring^{-1}$ was reached. At the end of the experiment with HBF 1, a slight decrease of the DMS EC was observed. One can infer from the data in Table 1 that a significant decrease of the *Hyphomicrobium* VS cell numbers in the biofilm is not an explanation for the decrease in HBF 1 performance. The decreased EC could possibly be due to a decreased DMS degrading activity of *Hyphomicrobium* VS, due to toxicity or other effects.

Besides the different *Hyphomicrobium* VS cell numbers, clear differences could be observed in the DGGE banding patterns of both reactors. Immediately after inoculation, a very

different microbial community was present in both biofilters (Pearson correlation coefficient 0%). This difference is probably due to the different carbon source that was used in both inoculation protocols, being DMS for HBF 1 and methanol for HBF 2. As previously mentioned [28], more varieties of microorganisms, e.g., members of the genera *Pseudomonas*, *Sphingomonas*, *Alcaligenes*, *Methylosinus* and *Methylobacterium,* are able to grow on methanol than DMS. Microorganisms able to metabolize DMS are rare and therefore the diversity in the HBF 1 biofilm is initially lower. The change in carbon source from methanol to DMS after starting HBF 2 can also explain the slowly evolving microbial community in this reactor. It took between 44 and 60 days before a stable community was observed, somewhat longer than observed by Tresse et al. [15], who found that it took about 35 days before the biofilm composition in a biotrickling filter treating styrene was adapted to the reactor operating conditions. In the Tresse et al. case, however, an inoculum already exposed to styrene was used, which may explain the shorter adaptation period. In HBF 1 the initial change, as observed in the DGGE pattern, was much less pronounced, because DMS was used as carbon source during the inoculation protocol and during the biofiltration experiments. The DGGE pattern of both HBF 1 and HBF 2 showed that a diverse microbial community had developed already on day 1. Because it was not possible to perform all manipulations during inoculation in a completely sterile manner, colonization could have occurred. Next to *Hyphomicrobium* VS a number of other bacteria are known to partially or fully metabolize DMS, like other *Hyphomicrobium* spp., *Thiobacillus* spp. and even *Pseudomonas* spp. or *Acinetobacter* spp. [28], enabling other bacteria to grow on excreted metabolites. When methanol was used as a carbon source during the 2nd inoculation experiment, even larger range of bacteria could grow in the medium or in the biofilm, which could explain the higher diversity of HBF 2 ($H = 2.7$) compared with that of HBF 1 ($H = 2.1$) on day 1.

When the reactor performance and microbial community analysis data were combined, two major observations were made. Firstly, in HBF 2, which performed much better than HBF 1 in terms of EC and removal efficiency, about twice as many *Hyphomicrobium* VS cells were present on the carrier material than in HBF 1. The benefit of the second inoculation protocol is apparent, namely that a greater number of *Hyphomicrobium* VS cells were initially present and remained on the packing throughout the entire period of reactor operation. Apart from the initial change after inoculation, this number remained quite constant during the whole experiment for both biotrickling filters, as shown in Table 1. The EC in both reactors increased, however, e.g., from 4 to about 50 g m^{-3} h^{-1} in HBF 2. Therefore no correlation existed between *Hyphomicrobium* VS cell numbers in the biofilm and the EC of HBF 2. Other authors have also not observed correlation between cell numbers and EC in a biofilter treating toluene, and therefore have suggested limitation by important environmental factors [29]. In the present study it can be expected that the activity of the *Hyphomicrobium* VS cells in HBF 2 was greater than in HBF 1, because an increase in cell numbers by a factor of two to three was related to a six-fold increase of the EC_{max}. When the Shannon Diversity Index was examined, no clear difference could be observed that related to the different ECs of the two biotrickling filters. There is a possibility, however, that by using the second inoculation protocol, other DMS-degrading bacteria besides *Hyphomicrobium* VS developed in HBF 2, which were not present in HBF 1. If so, that could then explain an increase in the DMS EC in HBF 2. A second major observation in this study was the sudden shift in the microbial community composition of HBF 1, when the EC of DMS decreased after 45 days of operation. This shift was illustrated by a reduction of the diversity index H to a value of 1.5, and reflected that one invading ribotype became dominant, as is clear in the DGGE gel pattern at day 45 (Fig. 4). Possibly, this exerted a negative effect on the DMS-degrading activity of *Hyphomicrobium* VS. According to Jiang and Morin [30] many factors can potentially influence the susceptibility of a community to biological invasions, including habitat disturbance and community structure. In this case, this could mean that the high DMS inlet concentrations or the community structure after the first inoculation protocol, respectively, were potential important factors. None of the physico-chemical parameters analyzed could, however, be related with the sudden change of the microbial community. Some authors have also attempted to relate microbial diversity to the functioning of reactors. In some cases positive [31,32] but in other cases no correlations [33] were reported. From the plate counts it was clear that although one new ribotype became dominant in the DGGE gels, *Hyphomicrobium* VS numbers on the rings remained high (1.3×10^8 CFU $ring^{-1}$). The observation that *Hyphomicrobium* VS was no longer visible in the gel patterns can be attributed to the fact that DGGE banding patterns only show bacteria present at levels greater than about 1 or 2% of the total bacterial community [24].

5. Conclusions and recommendations for further study

In this study, it was shown that the inoculation protocol can strongly influence the biofilm microbial community of a biotrickling filter treating DMS, and therefore reactor performance. A second inoculation protocol was shown to result in a superior outcome. Most likely because a greater number of *Hyphomicrobium* VS cells were introduced and ultimately maintained on the rings during reactor operation, allowing for a very rapid start-up and prolonged DMS-degrading activity, even at DMS concentrations exceeding 800 ppmv. More research about the microbial ecology in biotrickling filters is needed, however, to explain the causes and effects of changes in the microbial community composition, and especially to understand the link between community composition and reactor efficiency. Future work planned includes (i) excising bands from DGGE gels for species identification, (ii)

real-time PCR with newly designed primers to quantify inoculated bacteria and (iii) comparison of the quantities of DNA and RNA to distinguish genetic presence versus activity.

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