Real-time PCR analysis of tailored and non-specific inocula for the removal of dimethyl sulfide in a biotrickling filter

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ABSTRACT. In this study, different inocula were compared for the removal of 20 ppmv of dimethyl sulfide (DMS) in a biotrickling filter. Three biotrickling filters were set up in parallel, inoculated respectively with *Thiobacillus thioparus* TK-m, sludge and sludge + *T. thioparus* TK-m + *Hyphomicrobium* VS. Taking into account 5 criteria (length of start-up period, maximal elimination capacity, influence of intermittent feeding and peak loadings, resistance to low pH), the performance of the biotrickling filters was compared. Overall, the non-specific inoculum (sludge) showed the best performance, and a small benefit was observed by adding the two DMS degrading strains to the sludge. To quantitatively monitor the bacterial populations, real-time PCR was used with universal bacterial primers and with newly developed primers for the specific detection of the introduced strains. In HANDS++, rather constant *Hyphomicrobium* VS and total DNA concentrations were observed on the rings after start-up. In THIO, the applied operational changes had a clear effect on the total DNA amounts. The protocol for detection of *T. thioparus* needs to be further optimized, however.

1 INTRODUCTION

Mixtures of reduced sulfur compounds are emitted to the atmosphere by a number of industries, like waste water treatment, Kraft pulping, animal rendering and composting (Ruokojärvi *et al*., 2001; Smet *et al*., 1996). Due to their obnoxious smell and low odor threshold, especially organic sulfur compounds like dimethyl sulfide (DMS) often cause odor nuisance in the surroundings. Depending on the waste gas characteristics, biofilters or biotrickling filters can be used to eliminate the odor. Optimizing the inoculation strategy can be important to maximize the DMS elimination with both technologies. In the case of biofiltration, inoculation with e.g. *Hyphomicrobium* can increase the DMS elimination capacity (Smet *et al*., 1996). For biotrickling filtration, inoculation is always needed, but the choice of inoculum can potentially influence the DMS removal and reactor stability.

To follow up the fate of introduced bacterial strains, a monitoring technique is needed that is quantitative, reproducible and specific. Plate counting techniques often fail to specifically detect low numbers of certain bacteria, especially in organic matrices like

sludge or compost. Real-time PCR could offer a solution for this problem. With this technique, the amount of DNA during amplification is continuously monitored by labelling of primers, probes or amplicon, mostly with fluorogenic molecules. The ideal amplification curve of a real-time PCR, when plotted as fluorescence intensity against cycle number, is a typical sigmoidal growth curve. The point at which the fluorescence passes from insignificant levels to clearly detectable is called the threshold cycle (C_T), and this value is used in the calculation of template quantity during quantitative real-time PCR (Mackay *et al*., 2002).

The goals of this study were (i) to evaluate the suitability of tailored and non-specific inocula for removing DMS in biotrickling filters and (ii) to develop and test primers for the specific detection of DMS-degrading bacteria in biofilm and organic matrices with real-time PCR.

2 MATERIALS AND METHODS

2.1 Experimental set-up

Three parallel biotrickling filters (THIO, HANDS, HANDS++) were set up, made of Plexiglas (internal diameter: 0.055 m) and each packed with 1 L of polyethylene KMB carrier rings (diameter: 10mm, height: 7mm, specific surface area: 333 $m^2\ m^{-3}$) (Kaldnes Miljøteknologi AS, Tønsberg, Norway). One liter of inoculum was added in a vessel below the reactor and circulated at a rate of 80 ml min^{-1}. In THIO, a suspension of *Thiobacillus thioparus* TK-m was used (0.07 g volatile suspended solids (VSS) l^{-1}), while in the other biotrickling filters sludge was used, originating from a membrane bioreactor treating landfill leachate. The HANDS biotrickling filter was inoculated with pure sludge (7.02 g VSS l^{-1}) while the HANDS++ reactor with sludge (900 ml), supplemented with 50 ml *Thiobacillus thioparus* TK-m (0.22 g VSS l^{-1}) and 50 ml *Hyphomicrobium* VS (0.49 g VSS l^{-1}). If the pH of the liquid medium was below 6.5, it was adjusted to 7 by adding 1 M NaOH. The air flow (dry air, Air Liquide) was provided in downflow mode at 1 l min^{-1}, providing an empty bed residence time (EBRT) of 60 s in each bioreactor. DMS was dosed in the air stream by a capillary diffusion system, as described previously (Smet *et al*., 1993). Sampling ports were provided in the tubing before and after each biotrickling filter.

Thiobacillus thioparus TK-m (Kanagawa and Mikami, 1989) was grown in medium, containing 5 g l^{-1} $Na_2S_2O_3.5H_2O$, 4 g l^{-1} K_2HPO_4, 0.5 g l^{-1} $MgSO_4.7H_2O$, 0.4 g l^{-1} $(NH4)_2SO_4$, 0.25 g l^{-1} $CaCl_2$, 0.02 g l^{-1} $FeSO_4.7H_2O$ and 2 ml bromocresol purple solution at pH 7. *Hyphomicrobium* VS (Pol *et al*., 1994) was grown in 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} $MgSO_4.7H_2O$, 0.01 g l^{-1} $FeSO_4.7H_2O$ and 1 % methanol at pH 7.

2.2 DMS analysis

DMS gas concentrations were analysed with a GC 8000 Top gas chromatograph (CE Instruments) equipped with a flame ionisation detector (detection limit 0.13 ppmv). A 30 m DB-5 column (J&W Scientific; internal diameter 0.53 mm; film thickness 1.5 µm) was used with helium as a carrier gas. Gas samples of 1 ml were injected with a gastight pressure-lock precision analytical syringe (Alltech Ass.). In the experiments with 0.5 ppmv DMS influent concentration, GC-MS analysis was used for both the influent and effluent of the biotrickling filters. Using a modified GC injector system, 10 ml gas sample was injected quantitatively on a Tenax tube. DMS was desorbed in a thermal desorption unit (Markes International), with a 20 ml min^{-1} helium flow during 1 min at

50 °C and 7 min at 250 °C. The desorbed DMS was concentrated in a cold trap (-10 °C) and was injected in the GC (TRACE 2000 GC, Thermo, Belgium) by desorption for 3 min at 270 °C. The following temperature program was used: from 35 °C to 40 °C at 1 °C min^{-1} and from 40 °C to 220 °C at 20 °C min^{-1}. The GC column used was a VF-1ms (30 m x 0.25 µm x 1 µm). With a split flow of 5 ml min^{-1}, the sample was sent to a quadrupole mass spectrometer (TRACE WE-250 DSQ, Thermo, Belgium), using electron impact ionization. The source temperature was 220 °C and the scan speed 1578 mass units s^{-1}. The detection limit for DMS in a 10 ml gas sample was 4 ppbv. Response factors for GC and GC-MS measurements were determined by analyzing external standards prepared as headspace concentrations.

2.3 Nucleic acids extraction and real-time PCR

To test the linear range of detection of *Hyphomicrobium* VS in sludge, 0.5 ml sludge samples (2x concentrated) were spiked with 50 µl of a tenfold dilution series (10^0 – 10^{-7}) of *Hyphomicrobium* VS culture (1.28 x 10^{10} CFU ml^{-1}). For sampling the biofilm in the biotrickling filters, all rings were removed, mixed and five rings were randomly picked. Those 5 rings were sonicated for 10 min in 5 mL of sterile deionized water with a Branson 2200 sonicator, operating at 47 kHz. The extract was concentrated to 0.5 ml by centrifugation for 5 min at 3000 x g and removing excess supernatant. Nucleic acid extraction was performed according to the protocol of Griffiths *et al*. (2000). Firstly, 0.5 ml of sample was mixed with 0.5 g of RNase free glass beads (0.1 – 0.11 mm), 0.5 ml hexadecyltrimethylammonium bromide (CTAB) extraction buffer and 0.5 ml phenol-chloroform-isoamyl alcohol (25:24:1) (pH 8). Samples were lysed for 3 x 30 s in a bead beater (B. Braun Biotech International, Melsungen, Germany) with 10 s between each run, and the aqueous phase containing the nucleic acids was extracted after separation by centrifugation (3000 x g) for 5 min. After removing phenol by mixing with an equal volume of chloroform-isoamyl alcohol (24:1), the nucleic acids were precipitated from the aqueous phase with 2 volumes of 30 % (w/v) polyethylene glycol 6000 – 1.6 M NaCl for 2 h at room temperature. Nucleic acids were pelleted by centrifugation (18000 x g) for 10 min at 4 °C, washed with 1 ml ice cold 70 % (vol/vol) ethanol, centrifuged again (18000 x g) for 10 min at 4 °C and air dried prior to resuspension in 100 µl DNase- and RNase-free filter sterilised water (Sigma-Aldrich Chemie, Steinheim, Germany). 75 µl of the crude extract was further purified with Wizard DNA Clean-Up System (Promega, Madison, Wisconsin, USA), according to the manufacturer's instructions.

Real-time PCR analysis was performed by adding 1 µl of purified DNA extract with 24 µl mastermix, using an ABI PRISM 7000 Sequence Detection System (Applied Biosciences, Foster City, USA) and the ABI PRISM 7000 SDS software. The mastermix was prepared by mixing 10 µl SYBR® GREEN PCR Master Mix (Applied Biosciences, Warrington, UK), 0,75 µl of each primer (10 pM) and 12.5 µl of filter sterile DNase and RNase free water for each 24 µl. The temperature program used consisted of the following steps: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. All samples were analyzed in triplicate, including a negative control. In the case of specific primers, melting curves were analyzed to verify amplification of the correct DNA template. For amplifying the total 16S rDNA, the primers P338F and P518r were used (Boon *et al.*, 2002). Standard curves were analyzed using known amounts of the plasmid containing *Hyphomicrobium* VS DNA, used for sequencing purposes.

2.4 Primer construction

To obtain amplified *Hyphomicrobium* VS DNA, 1 ml of liquid culture was boiled for 5 min and PCR amplification was performed on the crude extract using the PCR Core System I (Promega) and primers P63F and R1378r, according to the protocol of Boon *et al.* (2002). Amplification of the correct fragment was verified on a 1% agarose gel and to ensure the purity of the DNA, the PCR product was cloned by using a TOPO TA Cloning kit with vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA). Three clones with insert were sent for sequencing to ITT Biotech (Bielefeld, Germany). Based on the obtained DNA sequence, primers were constructed using the PrimRose software (Ribosomal Database Project, www.rdp.cme.msu.edu). For *Thiobacillus thioparus* TK-m the primers were constructed similarly using the sequence available for *Thiobacillus thioparus* in the NCBI (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) database.

3 RESULTS AND DISCUSSION

3.1 Biotrickling filtration of dimethyl sulfide

The biotrickling filters were operated for a period of 119 days. According to the parameters that were investigated, different experimental periods were distinguished (Table 1).

Table 1. Description of experimental periods during DMS biotrickling filtration.

Period	Days	Description
I	1-28	Start-up period
II	29-51	Increasing DMS loading rate
III	52-71	DMS peak concentrations
IV	72-75	Intermittent DMS loading
V	88-95	Decreasing pH
VI	98-119	0.5 ppmv DMS influent concentration

During period I, the length of the start-up period was compared for the three reactors, at a DMS influent concentration of 19.0 ± 3.7 ppmv (3.0 ± 0.6 g m^{-3} h^{-1}). As is shown in Figure 1, HANDS and HANDS++ reached a DMS removal exceeding 99.5 % 10 days after start-up. For THIO, however, it was necessary to add *Thiobacillus thioparus* culture suspension twice on top of the column before the same DMS removal efficiency was obtained after 22 days.

During period II, the DMS inlet loading rate (B_V) was systematically increased to determine the maximal elimination capacities (EC_{max}) of each reactor. For all biotrickling filters, a removal efficiency exceeding 99.5 % was observed up to a B_V of 10 g m^{-3} h^{-1}. The EC_{max} values, calculated as the plateau value after plotting the elimination capacity versus the B_V, were 25-35 $g.m^{-3}.h^{-1}$ for THIO, 25-28 $g.m^{-3}.h^{-1}$ for HANDS and 30-34 $g.m^{-3}.h^{-1}$ for HANDS++. No major differences existed regarding the EC_{max} values for the different biotrickling filters; only for HANDS++ a slightly higher value was observed. During period VI, 0.5 ppmv DMS was sent to the reactors, but > 99.2 % removal efficiencies were observed for all reactors.

During period III a peak concentration of 555 ppmv DMS was applied for 1 hour, after which the removal efficiency of 20 ppmv of DMS was determined. The time needed before the removal efficiency exceeded 95 % again, was 110 min for THIO, 75 min for HANDS and 60 min for HANDS++. These responses were very similar, which means

that no toxic or other stresses were expected in either reactor. The small differences in recovery observed were probably due to the different partitioning behaviour of DMS in mineral medium (THIO) versus sludge (HANDS and HANDS++).

In period IV, the operation under intermittent DMS loading was investigated. The following influent regime was applied 4 times: 8-10 hours with 20 ppmv DMS (1 l min^{-1} gas flow rate) followed by 14-16 hours without DMS loading or gas flow (idle period). Two hours after the first idle period, the DMS removal efficiencies after stabilization were > 90 % in all biotrickling filters. After the next three idle periods, no significant decrease of the DMS removal efficiency after stabilization was observed for HANDS++ (> 99.5 %) and only a small decrease for HANDS (> 98 %). After 4 idle periods, HANDS++ removed > 99 % of the DMS one hour after re-dosing DMS, while HANDS > 90 %. For THIO, however, the time for > 90 % removal of DMS increased gradually (Figure 2). After the 4th idle period, a DMS removal efficiency of only 69 % was attained after 8 hours.

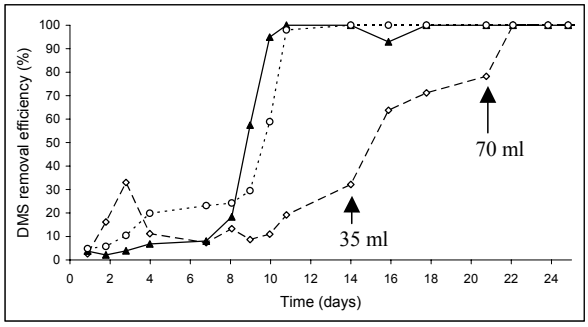

Figure 1. DMS removal efficiency during the start-up period for THIO (◊), HANDS (▲) and HANDS++ (○). Arrows show the addition of the indicated volume (ml) of *Thiobacillus thioparus* culture to THIO.

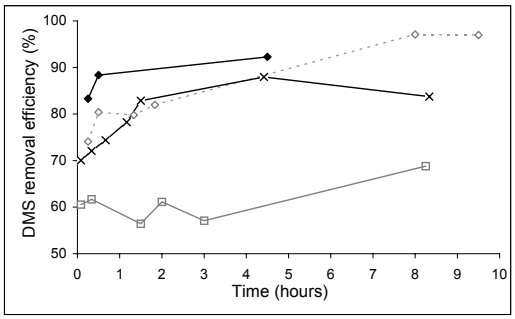

Figure 2. DMS removal efficiency in THIO after first (♦), second (◊), third (×) and fourth (□) idle period.

During period V, the influence of low pH was investigated. In Figure 3, the effect of pH on the DMS removal efficiency is shown, at 20 ppmv DMS influent concentration. In THIO, the removal efficiency decreased to about 45 % at pH 5, while in HANDS and HANDS++ a value of about 90% was maintained.

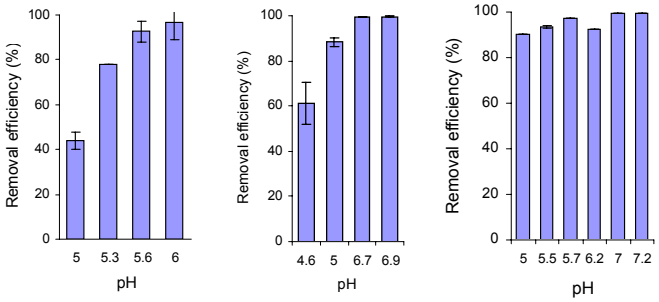

Figure 3. DMS removal efficiency at different pH values for THIO (left), HANDS (middle) and HANDS++ (right).

3.2 Development and application of real-time PCR

By using the BLAST algorithm and the NCBI database with the obtained DNA sequence for *Hyphomicrobium* VS, the strain was identified as *Hyphomicrobium methylovorum* (100% similarity). For specific detection of the strain, the forward primer HM-F (5'-TACTAGGTGGCTGCGCATTGT-3') and the reverse primer HM-R (5'-GGGAATTCCAGCAATGGACAG-3') were selected. The combination of both primers showed no mismatches for the target strain, all other strains in the database had at least one mismatch. Agarose gel electrophoresis showed successful PCR amplification of a ~200 bp fragment of pure *Hyphomicrobium* VS DNA, while no amplification was observed using the purified DNA extract of a compost sample. After comparing the real-time PCR amplification of pure *Hyphomicrobium* VS DNA at different primer concentrations (100, 300, 900 nM), a primer concentration of 300 µM was selected. The linear range of detection of DNA from *Hyphomicrobium* VS was first determined for the purified plasmid extract, used for sequence determination. In this case a linear range of 6 orders of magnitude was obtained (10^{-1} to 10^{-7} dilution). After spiking a dilution series of *Hyphomicrobium* VS in sludge, however, a linear range of only 2 orders of magnitude was obtained, starting from 1.28 x 10^{9} CFU (g sludge)$^{-1}$ (Figure 4). The protocol was very sensitive, however, enabling detection of 1.28 x 10^{2} CFU (g sludge)$^{-1}$. Real-time PCR analysis on tenfold dilutions of the original samples confirmed that no inhibition of the amplification occurred, since ten times lower DNA amounts were consistently measured. The amount of *Hyphomicrobium* VS culture that was added to the sludge during inoculation of the biotrickling filters (10^{8}-10^{9} CFU [g sludge]$^{-1}$) should be quantifiable, based on the linear detection range that was found.

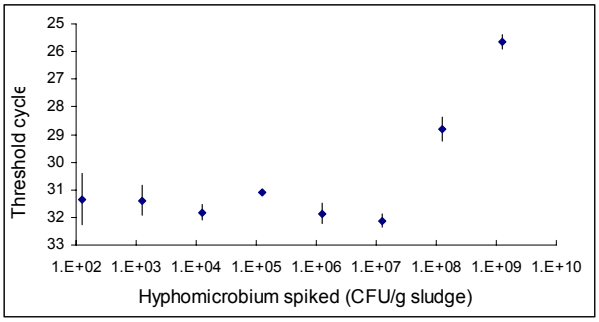

Figure 4. Threshold cycle (C_T) versus the amount of *Hyphomicrobium* VS spiked in the sludge samples (n=3).

In Figure 5, the total DNA amount per ring is shown for THIO, HANDS and HANDS++ for different sampling dates. For THIO, a 400-fold increase could be observed between day 11 and 53, after which the amount of DNA decreased again. The increase as well as decrease of the total DNA amount in THIO could be related with the operating conditions. Firstly, on days 14 and 21 biomass was added and until day 51 the loading rate was increased, leading to higher biomass concentrations. Afterwards, intermittent DMS loading was applied, the pH was decreased and very low DMS concentrations were applied, leading to decreasing DNA amounts. More representative results would be obtained, however, by using the *Thiobacillus thioparus* specific primers. For HANDS and HANDS++, a faster increase of the total DNA concentrations occurred after start-up, compared with THIO. In addition, the total DNA amount remained more stable afterwards.

The *Hyphomicrobium* VS counts in HANDS++ were rather stable (results not shown), being 2.02 ± 1.15 pg DNA ring^{-1}, with no significant differences between all measurements (Kruskal Wallis test, $p = 0.05$). The corresponding mean C_T value was 27.6 ± 1.2, confirming that the measurement fell in the linear range of detection, based on the standard curve in Figure 4. The relative amount of *Hyphomicrobium* VS DNA compared with total bacterial DNA is shown in Table 2. This percentage decreases after day 11, to a relatively stable value between 0.27 an 0.47 %. Only on day 98 a small peak was observed, due to the low total DNA amount. Based on the amounts of VSS added initially, the relative abundance of *Hyphomicrobium* VS in the sludge medium was 0.39%. It appears that the relative abundance of the strain on the rings remains very close to that initial value, showing that *Hyphomicrobium* VS was able to establish a stable population in the sludge.

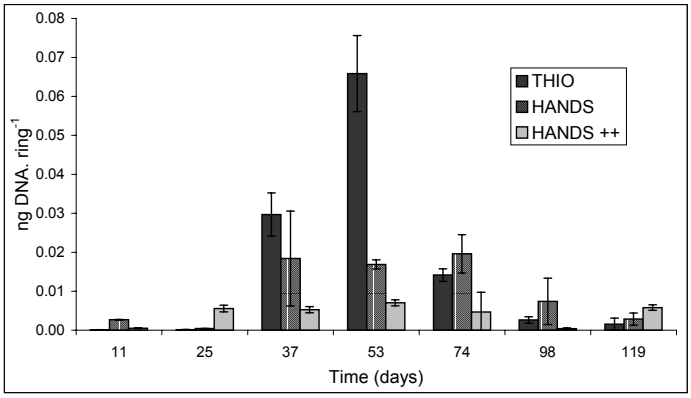

Figure 5. Amount of 16S rDNA on the rings of THIO, HANDS and HANDS++ (n=3).

Table 2. Percentage of *Hyphomicrobium* VS DNA on the rings of HANDS++.

Time (days)	Hyphomicrobium VS (%)
11	4.88
25	0.28
37	0.42
53	0.49
74	0.36
98	2.52
119	0.27

For *Thiobacillus thioparus* TK-m, only preliminary results are available. The following primers were selected: TTF 374-393 (5'-CCAGCAGCCGCGAATACGTA-3', TTF 502-521 (5'-AGTCTAGAGTGCGTCAGGAG-3') and TTR 565-584 (5'-TCGCCATTGGTGTTCCCTCC-3'). The combination of both primers showed no mismatches for the target strain, all other strains in the database had at least one mismatch. Agarose gel electrophoresis showed successful PCR amplification of a 211 bp fragment of pure *Thiobacillus thioparus* TK-m DNA, while no amplification was observed using the purified DNA extract of a pure sludge or *Hyphomicrobium* VS DNA sample. The protocol for real-time PCR is still being established, because the standard protocol showed considerable amplification of negative control and unspiked sludge samples.

4 CONCLUSIONS

After investigating the DMS removal efficiencies during different operational conditions, it can be concluded that HANDS++ exhibited the best DMS removal. The performance of HANDS was somewhat less under certain conditions, but especially the difference with THIO was significant. A real-time PCR protocol for detecting *Hyphomicrobium* VS in sludge matrices was developed, showing that the strain could survive after inoculation in a biotrickling filter. The evolution of the total bacterial DNA in THIO could be related with the operational parameters. After further development of the *Thiobacillus thioparus* real-time PCR protocol, the behaviour of the two inoculated strains in HANDS++ will be compared and the amount of *Thiobacillus thioparus* in THIO will be investigated.

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