Evaluating the application of *Microbacterium* sp. strain BR1 for the removal of sulfamethoxazole in full-scale membrane bioreactors

A. Fenu, B. M. R. Donckels, T. Beffa, C. Bemfohr and M. Weemaes

ABSTRACT

Microbacterium sp. strain BR1 is a bacterial strain that recently received attention for its capability to mineralize sulfamethoxazole (SMX) and other sulfonamides. In this study, the survival of *Microbacterium* sp. in municipal sludge waters was tested in batch experiments to explore optimal process conditions. Inoculation of *Microbacterium* sp. was subsequently performed in a pilot membrane bioreactor (MBR) operated in two configurations: treating full-scale MBR permeate (post-treatment) and treating raw municipal wastewater. SMX removal by *Microbacterium* sp. could not be proved in any of the configurations, except for SMX concentrations far higher than the ones normally found in municipal wastewater. By use of molecular tools (fluorescence *in situ* hybridization analysis) a low capability to survive in activated sludge systems was assessed. After inoculation, *Microbacterium* sp. was reduced to a small fraction of the viable biomass. The observed growth rate appeared to be many times lower than the one of typical activated sludge micro-organisms. Possibilities of application in full-scale municipal wastewater treatment are scarce.

Key words | membrane bioreactor (MBR), *Microbacterium*, sulfamethoxazole (SMX), wastewater treatment

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INTRODUCTION

Sulfamethoxazole (SMX) is widely used as an antibiotic (Kümmerer 2009). Although conventional wastewater treatment technology has shown to significantly reduce the impact on the environment in terms of eco-toxicity (Muñoz *et al.* 2008), many pharmaceutical compounds, such as SMX, are poorly degradable and are therefore not fully removed in wastewater treatment plants (WWTPs) (Clara *et al.* 2005; Joss *et al.* 2005). Moreover, state-of-the-art post-treatment technologies such as sand filtration, coagulation, flocculation and flotation, fail to completely remove SMX because of its hydrophilic nature (Nakada *et al.* 2007).

As a consequence, SMX is still present in WWTP effluent, and concentrations up to 1.7 μg/L and 1.9 μg/L have already been reported by Loos *et al.* (2013) and Miao *et al.* (2004), respectively. In surface waters, SMX has been detected at concentrations of μg/L, which is in line with other antibiotics (Sim *et al.* 2011). In addition, the presence of SMX in water bodies can promote the development of antibacterial resistance (Cooper *et al.* 2008).

Novel technologies are developed that allow a better removal of micro-pollutants, including SMX, from wastewater. One such technology is bio-augmentation, i.e. the addition of specialized microbial strains to enhance or enable the degradation of certain compounds. Bouju *et al.* (2012) demonstrated that *Microbacterium* sp. strain BR1, a Gram-positive bacterium isolated from a membrane bioreactor (MBR) treating effluent contaminated with several pharmaceuticals, was able to grow on SMX as main source of carbon and energy. This was later confirmed by Ricken *et al.* (2013), who also discovered the metabolic pathway that enabled the degradation of sulfonamide antibiotics.

In this study, the applicability of *Microbacterium* sp. strain BR1 to degrade SMX in full-scale operation was tested and evaluated. More precisely, the objectives were: (1) to investigate whether *Microbacterium* sp. was able to survive when inoculated in an activated sludge culture and under realistic, non-optimal conditions; and (2) to evaluate to what extent SMX could be removed in activated sludge systems.

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MATERIALS AND METHODS

Bacterial strain cultivation and detection

Microbacterium sp. cells were acclimatized to SMX by growing them in 25% (vol/vol) Standard I medium, consisting of 3.75 g/L peptones, 0.75 g/L yeast extract, 1.5 g/L NaCl and 0.25 g/L D-(+)-glucose at pH 7.2–7.4, enriched with 0.5 mM SMX. The cultures were cultivated on a rotary shaker (130 rpm) at a controlled temperature of 28 °C until an optical density (at 600 nm) of 1.2 was reached. This typically occurred after approximately 40 hours.

The growth of *Microbacterium* sp. was monitored using a commercial fluorescence *in situ* hybridization (FISH) kit (VIT® *Microbacterium* sp. *BR1* Kit). Probe design and *in silico* specificity testing was carried out using the ARB software package. For FISH analysis, fresh activated sludge was fixated by 1:1 (vol/vol) dilution in pure ethanol in 15 mL sterile vials stored at −20 °C prior to the analysis. The probe mix EUB labelled with 6-FAM was used for viable bacterial cell counts. Total cell count was performed by DAPI (4′,6-diamidino-2-phenylindole) DNA staining.

Preliminary batch experiments

Prior to the experiments with the pilot MBR (see below), two series of dedicated batch tests were performed. In a first series of batch experiments (noted as A), the ability of the microbial community already present in the full-scale MBR ('activated sludge') to degrade SMX was tested and compared with the removal efficiencies observed on the full-scale MBR. During this experiment, dissolved oxygen (DO), temperature and pH were respectively controlled at 5 ± 0.2 mg/L, 20 °C and pH 6.5 ± 0.1. The experiment was performed in a 3 L vessel for a duration of 4 days and mixing was carried out at 300 rpm. The activated sludge concentration was 4 g MLSS/L (MLSS: mixed liquor suspended solids), a typical concentration in WWTPs.

A second round of experiments (B) was conducted to study the survival of *Microbacterium* sp. in non-sterile conditions, i.e. raw influent wastewater. The experiment was conducted under controlled conditions (pH 6.5 ± 0.1 and 5 ± 0.1 mgO_2/L) and at different temperatures (10, 15, 20, 25, 30 °C). The survival of *Microbacterium* sp. was evaluated after 4 and 8 days by measuring total bacterial cell count and *Microbacterium* sp. through FISH analysis.

Full-scale and pilot membrane bioreactor

The experiments were performed at the full-scale WWTP of Schilde (Schilde, Belgium), operated by Aquafin NV, treating 5,500 m³/day of municipal wastewater.

It consists of an anoxic tank, an aerobic tank and an MBR unit equipped with hollow fibre membranes. Fine bubble aeration in the aerobic tank is provided through diffusers and controlled on a fixed DO set-point.

Two inoculation experiments were performed using a pilot MBR (Figure 1) composed of two separated biological compartments (anoxic and aerobic) with total volume of 1,000 L. A submerged membrane was placed into the aerobic compartment and air scouring was provided (1.8 Nm^3/h) to prevent clogging of the membranes. Fine bubble aeration was provided through plate diffusers and pH was controlled by automatic dosing of concentrated NaOH and HCl solutions. Temperature was controlled by three heat resistances placed inside the reactor.

The *Microbacterium* sp. bacteria were inoculated into the pilot MBR, which treated full-scale MBR effluent (post-treatment) and raw municipal wastewater in respectively the first and second experiment. The operating conditions of the pilot MBR were optimized based on the results of

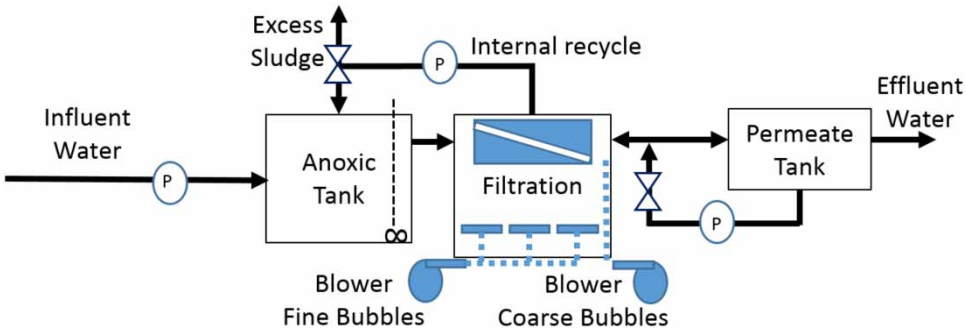

Figure 1 | Schematic representation of pilot MBR used for the experiments.

the batch tests and the limitations for practical application (see Results and discussion).

Grab samples were taken on a regular basis and these were analysed for NH_4-N, NO_2-N, NO_3-N, chemical oxygen demand (COD) and MLSS according to the American Public Health Association (1999). Both MBRs were equipped with on-line DO, nitrate (effluent), pH and temperature sensors. More details of the operating conditions of both MBRs are given in Table 1.

Micro-pollutant sampling and analysis

Influent and effluent samples of the MBRs were taken by flow composite automatic samplers in glass containers and stored at −20 °C. For SMX analysis in the water phase, samples were filtered through 0.45 μm glass-fibre filter and the pH was adjusted to 7.5. Samples were concentrated on pre-packed Oasis HLB cartridges (200 mg, 6 mL) (Waters, Eschborn, Germany) that were preconditioned with 1 × 2 mL heptane, 1 × 2 mL acetone, 3 × 2 mL methanol and 4 × 2 mL non-carbonated mineral water (pH 7.5). Percolation was performed at a constant rate of 10 mL/min. Then, cartridges were completely dried with nitrogen stream (200 mbar) for 1 hour and the eluate was diluted with 1 mL of high performance liquid chromatography (HPLC) mobile phase. The HPLC was equipped with a Zorbax SB C18 column (150 × 3.0 mm, 3.5 μm particle size, Macherey-Nagel) and operated with a flow of 0.350 mL/min, using methanol and HPLC-H_2O + 0.1% formic acid solvents. SMX-d4 was used as an internal standard.

For SMX analysis in the sludge phase, sludge water was stored at –20 °C. The analysis of the samples was carried out by a commercial laboratory (Omega Laboratory), with HPLC (Agilent 1200) on a C18 column with gradient elution and detection by tandem mass spectrometry.

RESULTS AND DISCUSSION

Preliminary batch experiments

In batch experiments B, *Microbacterium* sp. was inoculated into raw influent wastewater and the ability to grow on this medium was evaluated using FISH analysis. Apart from investigating whether the micro-organisms were able to survive, optimal temperature and minimum required inoculation ratio, defined as the ratio (weight/weight) between inoculated biomass and activated sludge, were determined. From the results, depicted in Figure 2, one can clearly see that *Microbacterium* sp. does not survive in temperatures below 20 °C. Eight days after inoculation, only negligible amounts of *Microbacterium* sp. were observed, regardless of the applied inoculation ratio. Since the temperature in a typical central European activated sludge plant fluctuates between 7 and 19 °C (winter/summer), these results indicate that *Microbacterium* sp. will not survive when inoculated into a full-scale installation treating municipal wastewater.

Table 1 | Operational parameters in full-scale and pilot MBR

Operating parameter	Full-scale MBR	Pilot MBR 2° treatment	Pilot MBR post-treatment
Hydraulic retention time [h]	7	22	48
Influent COD concentrations [mg COD/L]	196 ± 55	196 ± 55	22 ± 8
Inflow [m^3/h]	220 ± 10	0.045 ± 0.005	0.020 ± 0.005
Total volume [m^3]	1,200	1	1
Anoxic to aerobic volume ratio [L/L]	500/650	550/450	550/450
pH	7.9 ± 0.4	7.0 ± 0.2	7.1 ± 0.3
T [°C]	8–19	22.9 ± 1.4	22.6 ± 1.9
Dry solids concentration range [g/L]	9.75 ± 0.75	3.1 ± 0.9	0.75 ± 0.3
Recirculation/influent flow ratio	6:1	6:1	6:1
Inoculated weight per MLSS weight [%]	0	1	45
Membrane type	GE ZW 500	GE ZW 10	GE ZW 10
Membrane surface [m^2]	10000	2	2
Membrane pore size [μm]	0.04	0.04	0.04

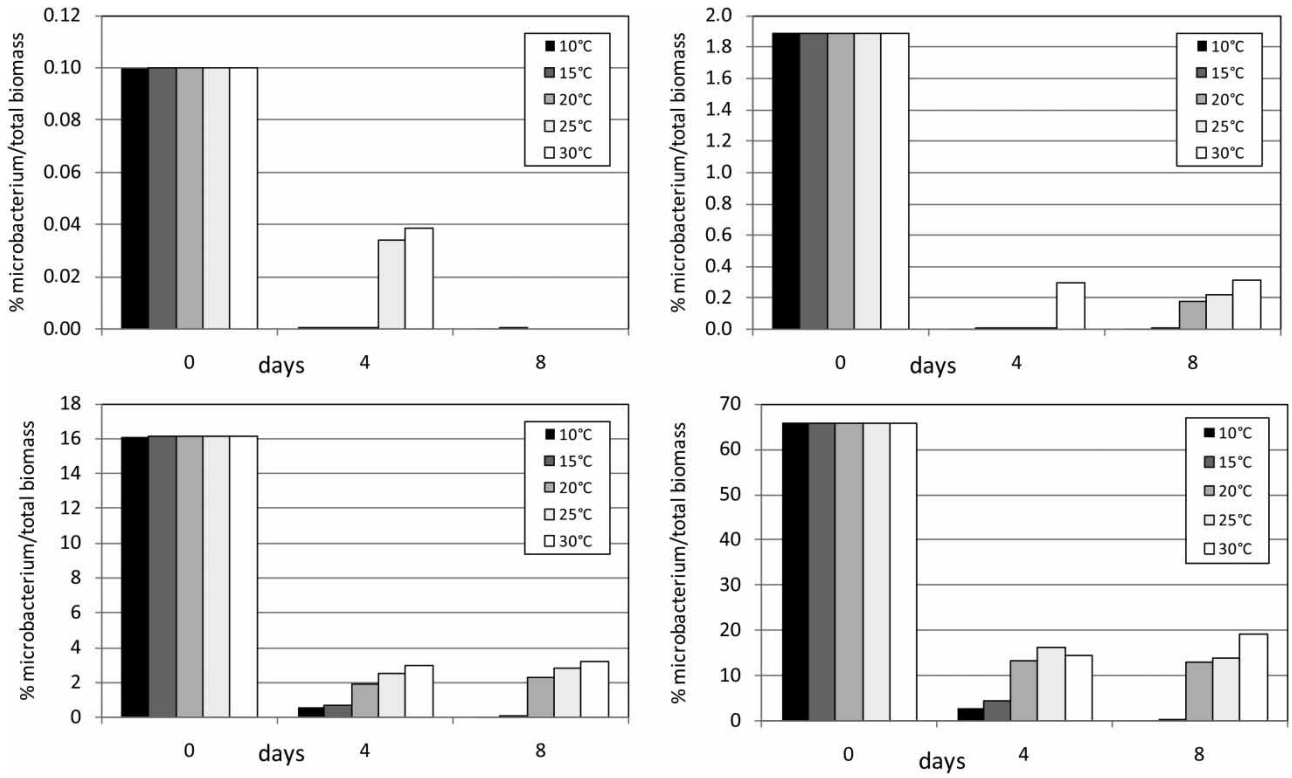

Figure 2 | Results of the batch tests where the effect of temperature and inoculum ratio was investigated. *Microbacterium* and total (viable + dead) biomass (DAPI) are measured by FISH analysis.

Figure 2 also shows that even for higher temperatures, significant amounts of *Microbacterium* sp. can only be maintained in the activated sludge community when high inoculation ratios are applied. However, because the growth rate of *Microbacterium* is very low, it is not realistic to produce *Microbacterium* sp. cultures in quantities that allow such high inoculation ratios for full-scale applications.

Removal of sulfamethoxazole by full-scale MBR activated sludge

Prior to discussing the results obtained with the pilot MBR, the removal efficiencies of the full-scale MBR were determined by measuring the SMX concentration in the influent and effluent during a sampling campaign of 4 months (Figure 3, right). The average concentrations of SMX in the full-scale MBR influent and permeate were 120 ± 62 ng/L and 58 ± 54 ng/L, respectively, and an average efficiency of approximately 52% was observed. One can also see that the removal efficiencies fluctuate considerably, often dropping to very low values. This variation could not be fully explained, but dilution of the influent and the occurrence of peak loads (so-called first-flush phenomenon) during rain events, may have strongly influenced the observed removal rates. Nevertheless, these observations provide a reference to compare the removal efficiencies obtained when *Microbacterium* sp. is inoculated (see further).

The observed removal efficiencies are in agreement with literature. Göbel *et al.* (2007) investigated the performance of a conventional activated sludge system and observed removal efficiencies of 50 to 90%, whereby less than 5% could be attributed to sludge sorption processes. Suàrez *et al.* (2010) observed 20% removal and no sorption onto the sludge for an activated sludge system with sludge retention time of 20 days. Also in our case, no SMX was measured on the activated sludge phase.

The removal of SMX was also evaluated in batch experiments (A) using activated sludge from the full-scale MBR. These experiments confirmed the results obtained from the full-scale MBR, i.e. that SMX can be degraded by the activated sludge community. Indeed, SMX was removed from the liquid phase with an efficiency of $92.5 \pm 2.5\%$. The higher removal efficiency can be explained by the fact that the hydraulic retention time (HRT) (here, equal to the duration of the experiment) was 4 days, whereas the average HRT in the full-scale MBR was about 7 hours.

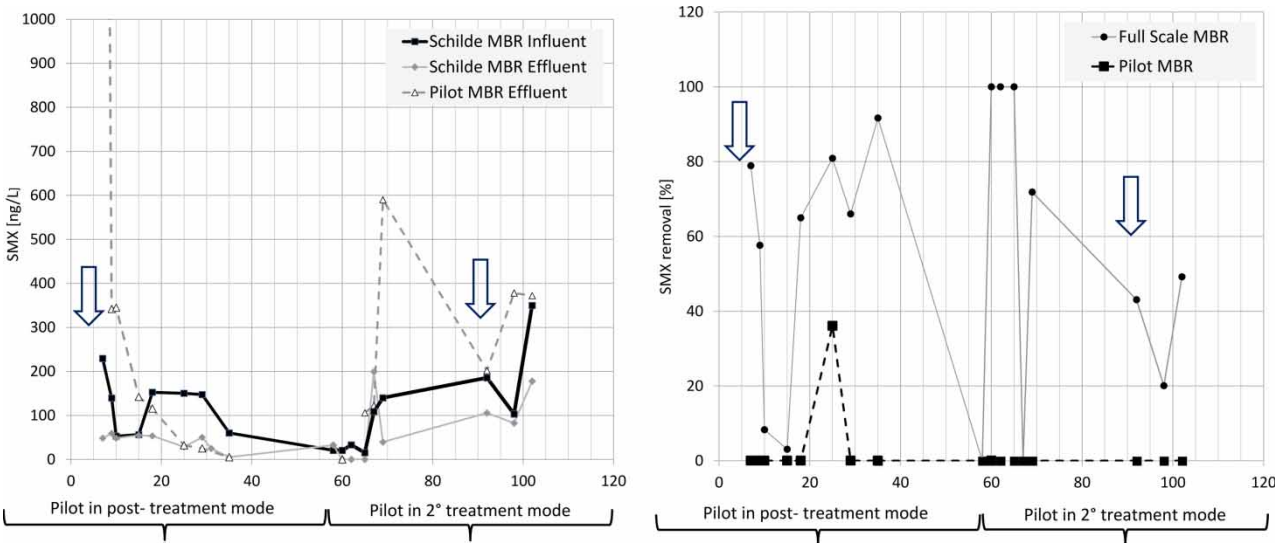

Figure 3 | On the *y*-axis full-scale MBR influent, full-scale MBR effluent, pilot-scale MBR effluent concentrations and removals of SMX. The arrows represent the inoculation of *Microbacterium* in the pilot MBR waters. On the *x*-axis, the days of operations. In post-treatment configuration, the pilot MBR receives full-scale permeate. In secondary treatment configuration the pilot MBR receives full-scale influent waters.

Removal of sulfamethoxazole in pilot MBR treating full-scale MBR permeate

Based on the results of the batch experiments, one could easily conclude that the operating conditions of the full-scale MBR did not allow survival of *Microbacterium* sp. when inoculated. Therefore, it was decided to operate the pilot MBR under more favourable, yet practically realistic, conditions as to promote *Microbacterium* sp. survival (see Table 1). As expected given the low COD content of the permeate, the MLSS concentration remained very low during the experiment (Table 1) and the application of a high inoculation ratio was feasible.

Prior to the experiments, two elements were thought to favour *Microbacterium* sp. survival after inoculation when the pilot MBR was operated as a post-treatment. First, the amount of micro-organisms in ultra-filtered MBR permeate is very low, so the competition with other micro-organisms will be limited (Hirani *et al.* 2013). Second, the COD in the permeate consists mainly of recalcitrant soluble microbial products (Fenu *et al.* 2011), which was confirmed in the 2 months prior to the inoculation experiments where no biological growth was observed.

Nevertheless, the very low SMX concentrations in full-scale MBR permeate could be limiting for the inoculated biomass. In the experiment, the removal of SMX at higher concentrations was investigated as well, by looking at the period immediately following the inoculation. Indeed, *Microbacterium* sp. was grown in a medium that contains nutrients and relatively high concentrations of SMX.

The COD and NH_4 concentrations of the permeate during the experiment were 22 ± 8 mg/L and 0.2 ± 0.1 mg/L, respectively. Elevated concentrations of COD and NH_4 were observed in the effluent for approximately 9 days after inoculation, due to the nutrients present in growth media that was added when inoculating. The measurements depicted in Figure 4 show that the DO concentration dropped from 9 to 1 mg/L immediately after inoculation, indicating an increased biomass respiration, and reached saturation after approximately 13 days.

After inoculation, the concentrations of SMX were significantly higher than the average concentrations in raw municipal wastewater (120 ± 62 ng/L) and MBR permeate (see Figure 4). To investigate the removal of SMX by *Microbacterium* sp., one has to distinguish between dilution and removal or breakdown. For this, a simple degradation model was built based on mass balances. In Figure 4, the measured SMX concentrations in the pilot MBR are compared to the ones predicted without degradation (only dilution). These results confirm that SMX is removed at high SMX concentrations. Ricken *et al.* (2013) concluded that high, non-inhibitory SMX concentrations might still drive selection for utilization as carbon and energy source, but in their work the removal efficiency of *Microbacterium* sp. was evaluated for higher concentrations (100 μM sulfonamides) that were significantly higher than the municipal range concentrations. The present experiment suggests that the actual challenge of this strain is to have it working in the municipal SMX range.

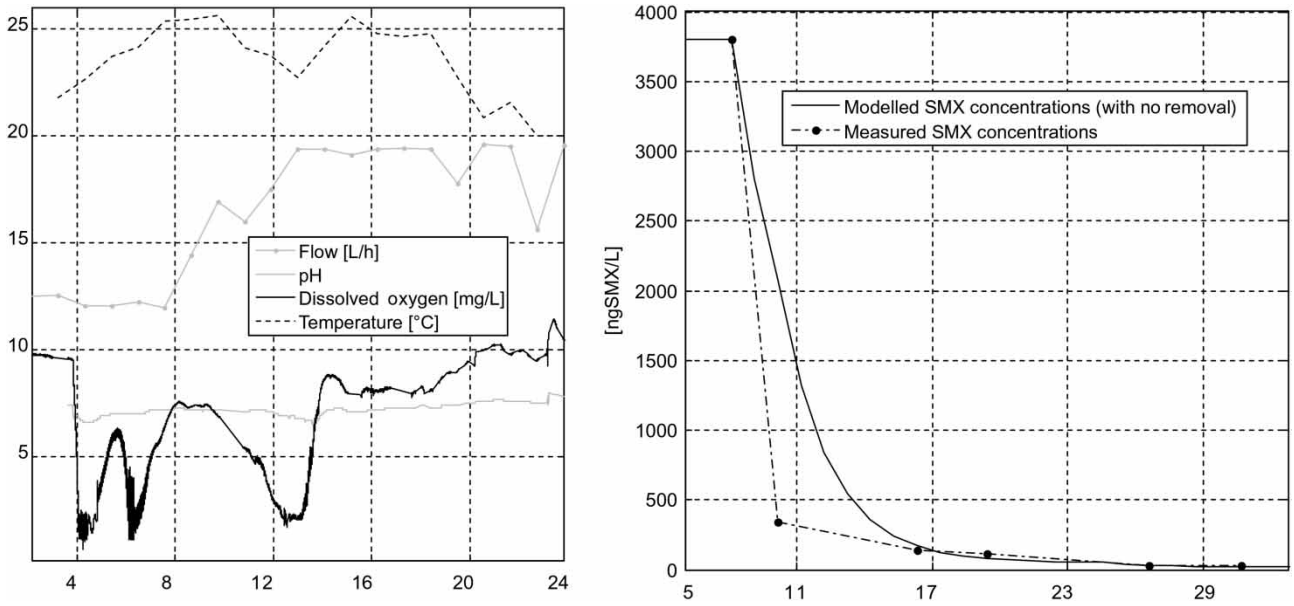

Figure 4 | Left: on-line sensors data during pilot MBR treatment of the full-scale permeate waters. On the *x*-axis, the days of pilot operations. Right: predicted (assuming no removal) and measured SMX concentrations of pilot MBR during post-treatment of full-scale permeate waters. On the *x*-axis, the days of pilot operations.

These results are also confirmed by FISH analysis. The concentration of both viable biomass and *Microbacterium* sp. dropped with time after inoculation, but the latter dropped faster (Figure 5, left). The observed drop in the viable biomass concentration can be explained by the low COD load and the fact that it consists mostly of recalcitrant soluble microbial products. One can also see that the percentage of *Microbacterium* sp. on total cells dropped to about 1–3% in 20 days. This indicates that *Microbacterium* sp. could not cope with the autochthonous bacterial growth, and full-scale application as a post-treatment of MBR permeate is practically not feasible.

Removal of sulfamethoxazole in pilot MBR treating raw municipal wastewater

In this section, the results obtained when the pilot MBR was used to treat raw influent wastewater are discussed.

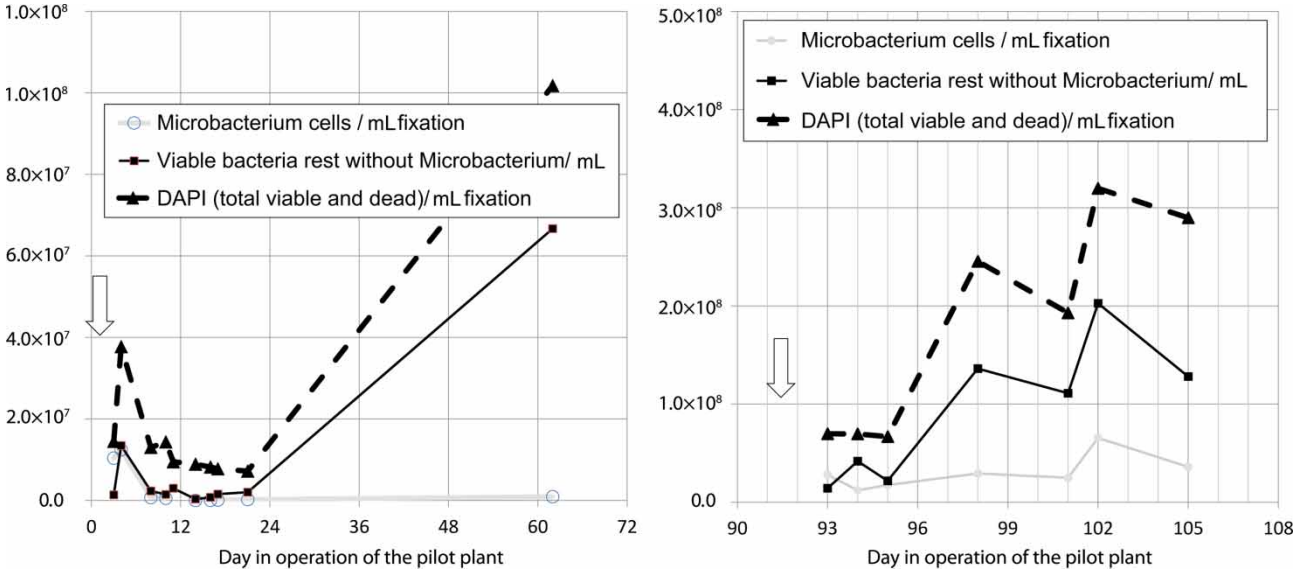

Figure 5 | Monitoring of biomass according to FISH techniques. The arrows represent the day *Microbacterium* sp. was inoculated. At the left side figure, the pilot treats full-scale permeate waters. At the right side, the pilot treats full-scale influent waters.

Although the composition of the wastewater is the same as for the full-scale MBR, a comparison between both systems is difficult because, as discussed above, the operating conditions of the pilot MBR (Table 1) were chosen such as to promote survival of *Microbacterium* sp., after batch experiments showed that survival in the conditions at which the full-scale MBR was operated was very unlikely.

The measurements performed during this inoculation experiment are shown in Figure 4. The COD concentration of the influent wastewater was 196 ± 55 mg/L and a COD removal of 55% was established by the pilot MBR. Ammonia was fully removed and the nitrate concentration in the effluent fluctuated between 1 and 4 mgNO_3-N/L, resulting in an overall nitrogen removal of approximately 93%.

During the time of the experiment, the operation of the full-scale MBR was monitored as well (see 'Removal of sulfamethoxazole by full-scale MBR activated sludge' section) and it was found that SMX was removed by the activated sludge (Figure 3). From the measurements in the pilot MBR, conversely, one could conclude that no removal of SMX occurred in the pilot MBR since the effluent concentrations were not significantly different from the influent concentrations (Figure 3). The sludge phase of the pilot MBR was also analysed and no SMX was detected, meaning that no significant sorption onto sludge occurred.

An explanation for the inability of the inoculated *Microbacterium* sp. bacteria to survive is found in the fact that they cannot compete with micro-organisms present in activated sludge. This is confirmed by the results of the FISH analysis. In the inoculation medium, the viable *Microbacterium* sp. cells accounted for 40% of the total number of viable cells. However, after inoculation, the viable bacteria in the activated sludge increased from 1.4×10^7 cell/mL to 1.3×10^8 cell/mL, whereas *Microbacterium* sp. only increased from 2.8×10^7 cell/mL to 3.6×10^7 cell/mL (29% increase in 14 days). In other words, the autochthonous cells grew approximately 15 times faster than the inoculated *Microbacterium* sp. cells. As a result, the fraction of *Microbacterium* sp. rapidly dropped to 5–10% of the total cells, despite the fact that SMX was non-limiting.

The experimental doubling time of *Microbacterium* sp. in the pilot reactor proved to be exceptionally long, i.e. around 45 days. Since municipal MBRs are operated with sludge retention times in the range of 10–25 days, *Microbacterium* sp. would have no chance of survival in realistic applications. An adjustment of municipal MBR design to allow the survival of *Microbacterium* sp. would involve either filtration operations at very high MLSS concentrations or increased design volumes. In both cases, operational and financial feasibility of such adaptation is a clear objection.

CONCLUSIONS

In this study a pilot MBR was inoculated with *Microbacterium* sp. strain BR1 to investigate the potential for SMX removal in full-scale applications. From the results, one can conclude that high wastewater temperatures were necessary to increase the chance of *Microbacterium* sp. survival in raw municipal wastewater. When fed with full-scale MBR permeate, SMX removal was only observed for concentrations that were several times higher than typically found in municipal wastewater. When fed with raw influent wastewater, no SMX removal was observed either. The doubling time of *Microbacterium* sp. was found to be far higher than the sludge retention times of municipal MBRs. Application of bio-augmentation with *Microbacterium* sp. in full-scale applications is unrealistic.

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