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Biofilm population dynamics in a trickle-bed bioreactor used for the biodegradation of aromatic hydrocarbons from waste gas under transient conditions

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Abstract

The dynamics of a multispecies biofilm population in a laboratory-scale trickle-bed bioreactor for the treatment of waste gas was examined. The model pollutant was a VOC-mixture of polyalkylated benzenes called Solvesso 100®. Fluorescence in-situ hybridization (FISH) was applied in order to characterise the population composition. The bioreactor was operated under transient conditions by applying pollutant concentration shifts and a starvation phase. Only about 10% of the biofilm mass were cells, the rest consisted of extracellular polymeric substances (EPS). The average fraction of Solvesso 100®-degrading cells during pollutant supply periods was less than 10%. About 60% of the cells were saprophytes and about 30% were inactive cells. During pollutant concentration shift experiments, the bioreactor performance adapted within a few hours. The biofilm population exhibited a dependency upon the direction of the shifts. The population reacted within days after a shift-down and within weeks after a shift-up. The pollutant-degraders reacted significantly faster compared to the other cells. During the long-term starvation phase, a shift of the population composition took place. However, this change of composition as well as the degree of metabolic activity was completely reversible. A direct correlation between the biodegradation rate of the bioreactor and the number of pollutant-degrading cells present in the biofilm could not be obtained due to insufficient experimental evidence.

Introduction

Trickle-bed bioreactors have proven to be effective systems for waste gas treatment. This is especially the case for relatively low pollutant concentration levels of around 1 g C_XH_Y per m^3 of waste gas. The biodegradation of the organic pollutants takes place mainly via aerobic oxidation by a mixed population of microorganisms. These microorganisms are immobilised in a biofilm which covers the surface of a packing material of the trickle-bed column. Previous studies have confirmed the suitability of trickle-bed bioreactors for the treatment of volatile organic compounds (VOC's) such as dichloromethane (Diks & Ottengraf 1991), toluene (Arcangeli & Arvin 1992; Møller et al. 1996; Pedersen et al. 1997), and polyalkylated benzenes (Hekmat & Vortmeyer 1994). Further work was performed with regard to the biodegradation dynamics, since the industrial process under real conditions is usually unsteady (Deshusses et al. 1996). This is because on one hand, the pollutant source is not constant over time and on the other hand, the bacterial culture exhibits an internal dynamic behavior depending upon variable physiological conditions. Therefore, the start-up behavior of trickle-bed bioreactors and the effect of feed pollutant concentration cycling was investigated (Hekmat et al. 1997). For simplification, however, the biofilm was regarded as a homogeneous mass in most studies. Thus, the properties of individual species was ignored (Siebel & Characklis

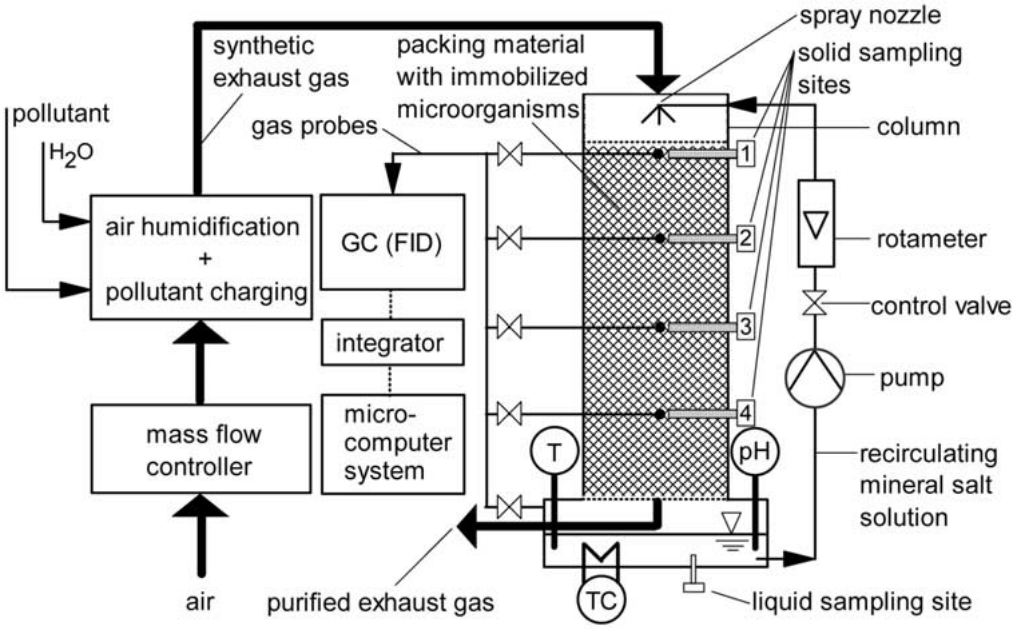

Figure 1. Schematic diagram of the experimental set-up of the trickle-bed bioreactor (GC (FID) = gas chromatograph with flame ionization detector).

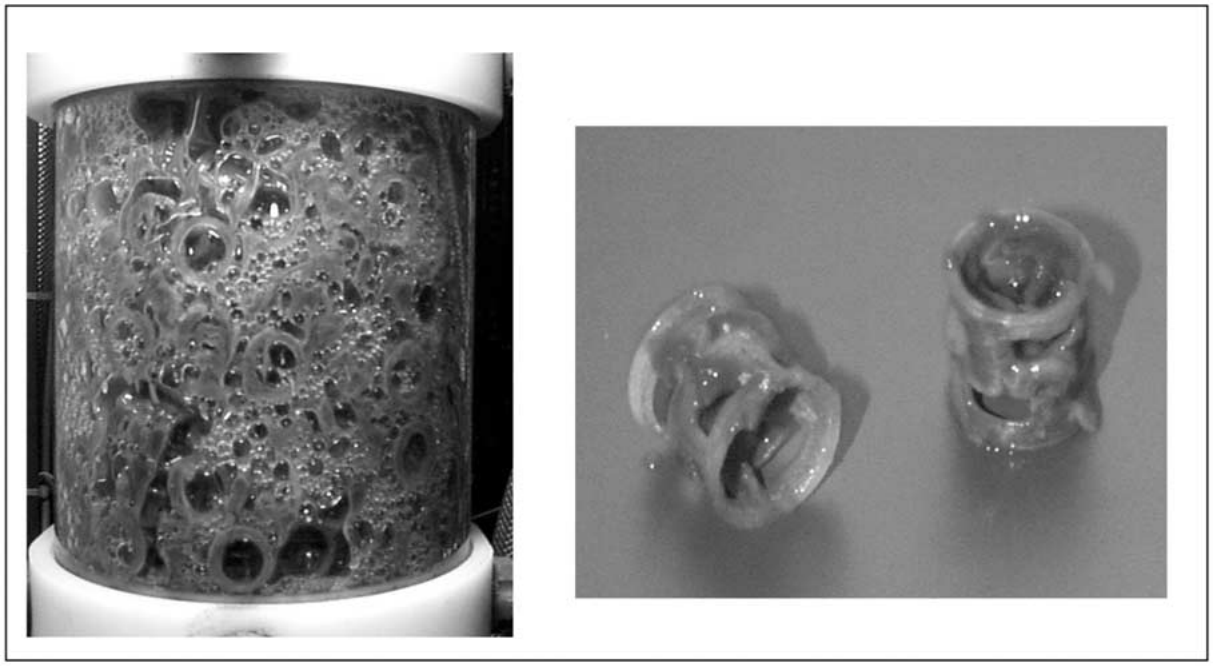

Figure 2. Left: segment of the packed column during continuous long-term operation in the trickle regime. Right: hydrophilised polypropylene Ralu®-ring packing material. The carrier material is covered with a thick biofilm.

1991) and the biofilm was treated as a black box. However, it is well known that in any open environment biological system, multispecies biofilms exist. In these biofilms, different bacterial populations interact with each other. These interactions influence the structure and the physiology of the biofilm as it develops (James et al. 1995). The evidence of direct metabolic interactions between community members was reported by Møller et al. (1998) who studied a binary population biofilm of *Pseudomonas putida* and *Acinetobacter* sp. degrading toluene. In a later work, Christensen et al. (2002) analysed the metabolic interactions of the above mentioned two-species microbial consortium degrading benzyl alcohol. It was observed that the two organisms exhibited competition and/or commensal interactions depending on their relative physical positioning in the biofilm. Thus, it was shown that multispecies biofilms represent quite differentiated systems with various complex processes taking place simultaneously.

In order to identify microorganisms and to determine their quantity in biofilms, the fluorescence in-situ hybridization (FISH) method was developed (Amann et al. 1990) and has been successfully applied in order to detect individual microbial cells without cultivation (Amann et al. 1995). The FISH-method was used to characterise the microbial populations of a trickle-bed bioreactor degrading polyalkylated benzenes from synthetic waste gas (Hekmat et al. 1998; Stoffels et al. 1998). In these studies, the population dynamics during the start-up phase of the trickle-bed bioreactor was examined. However, no data of the long-term operation was obtained.

Due to the above mentioned complexity, current understanding of biofilm systems for waste gas treatment is limited. Only little work exists on the description of the population dynamics in a multispecies biofilm of trickle-bed bioreactors for the treatment of waste gas. In fact, very few examinations of the microbial population behavior during long-term operation under transient conditions of such systems have been carried out. Therefore, the aim of the present study was to perform laboratory experiments under transient conditions with a trickle-bed bioreactor using polyalkylated benzenes as a model pollutant. It was planned to determine the population dynamics during pollutant concentration shift experiments and during a long-term starvation period.

Description of model pollutant and bioreactor system

The model pollutant was a mixture of hydrophobic polyalkylated benzenes (Solvesso 100®, Deutsche Exxon Chemical GmbH, Cologne, Germany). This product is commonly used in industrial applications as a solvent. Solvesso 100® contains mainly aromatic hydrocarbons such as trimethylbenzenes or ethyltoluenes (composition given in Hekmat et al. 1997). The inoculum of the bioreactor originated from the wastewater of a car painting facility (BMW AG, Munich, Germany). The experiments were performed in a laboratory-scale trickle-bed bioreactor under non-sterile conditions (Hekmat et al. 1997). A schematic diagram of the experimental set-up is presented in Figure 1. The synthetic waste gas entered the column continuously from the top. The recirculating mineral salt solution flowed co-currently with the gas downward through the column. The inner diameter of the column was 140 mm and the height was 0.7 m resulting in a column volume of 10.8 l. The column was packed with hydrophilised polypropylene Ralu®-rings (Raschig AG, Ludwigshafen, Germany). A segment of the trickle-bed column during continuous long-term operation and Ralu®-ring carrier material are presented in Figure 2. As can be seen, the carrier material is covered with a thick biofilm. Detailed specifications of the packing material, GC analytics, and media are given in Hekmat et al. (1997).

Preliminary experiments were performed in order to examine the component-specific biodegradation of Solvesso 100®. It was shown that the standard deviation of the degree of conversion of six major C_9-components of Solvesso 100® with fractions higher than 5% was about 30%. However for reasons of simplicity, the pollutant was treated as one single carbon source during the subsequent investigations.

Description of analytical methods, isolation of Solvesso 100®-degrading strains, and design of Solvesso 100®-specific oligonucleotide probes

The fixation of liquid samples for FISH measurements was performed as reported by Wagner et al. (1993). Solid samples were obtained from biofilms grown on glass coverslips and Ralu®-ring carriers. Solid sampling ports existed at four positions along the reactor height as indicated in Figure 1. From each port, glass coverslips or Ralu®-rings being distributed

along the reactor cross section were removed. The biofilm of the carriers was removed and disintegrated in 0.9 M phosphate buffered NaCl solution (pH 7.4) by a combination of scraping and vigorous shaking, subsequent vortexing for 5 min, and final treatment in an ultrasonic bath for 2 min. The biofilm suspension was cooled in an ice bath inbetween the steps. In order to be able to differentiate the biomass into cells and extracellular polymeric substances (EPS), a quantitative EPS extraction method using a cation exchanger resin (Dowex 50X8, 20–50 mesh, Fluka, Neu-Ulm, Germany) was developed (Linn 1999). The biofilm of the carriers was removed and disintegrated using the above mentioned procedure. 60 ml of biofilm suspension was then separated in a Sorvall centrifuge (Kendro Laboratory Products, Langenselbold, Germany) at 12000 min^{-1} for 15 min at 4 °C. 20 ml phosphate buffered extraction solution (pH 7) containing 30 g Dowex was added to the pellet. After shaking this solution for 3 h at 4 °C, the centrifugation step was repeated. The pellet was then separated from the residual Dowex and the weight of the pellet was determined gravimetrically. Two parallel samples from 10 carriers were treated every time. The standard deviation was ±4.5%.

Total cell counts (active and inactive cells) from homogenized biofilm samples from three carriers were determined by a combination of membrane filtration and staining with 4′,6-diamidino-2-phenylindole (DAPI) as described by Wagner et al. (1993). Depending on the cell concentration, 5–20 μl sample solution was incubated in the dark with 50 μl of a 1 μg ml^{-1} DAPI solution in 100 μl purified H_2O for 15 min at room temperature. Vacuum filtration under sterile conditions in the dark for 5 min was performed using a 0.2 μm GTTP-polycarbonate filter (Millipore, Schwalbach, Germany). After drying in the dark for 5 min, the filter was embedded in Citifluor solution (Citifluor Ltd., London, UK) and examined with an Axioplan microscope (Carl Zeiss, Oberkochen, Germany) using the filter set 01. For each sample, 25 randomly chosen microscopic fields were counted. Mean values and standard deviations were determined. Color photomicrographs were taken with Kodak Ektachrome P1600x films. For the application of the FISH-method, the biofilm suspension samples were fixed with paraformaldehyde. This fixation protocol was adequate since almost no Gram-positive bacteria were present in the bioreactor system (Linn 1999). Combined FISH and DAPI measurements of fixed samples from three carriers immobilised on glass slides were obtained by hybridization and subsequent DAPI staining using the above mentioned microscopic procedure with filter sets 01, 09, and 15. The sequences, hybridization conditions, and references for the oligonucleotide probes used in this study are given in Table 1. The oligonucleotides were synthesized and labeled at the 5′ terminus with fluorescein, CY3 or CY5 (Interactiva, Ulm, Germany).

The isolation procedure of Solvesso 100®-degrading bacteria was as follows: serial solutions in the range of 10^{-1} to 10^{-10} of biofilm suspension and circulation liquid were plated in duplicate on mineral medium (Hekmat et al. 1997). A filter paper soaked with Solvesso 100® was placed at the bottom of an inverted glass petri dish and served as the sole carbon source. Then, the dishes were incubated in an air-tight metal box at 30 °C. The filter paper was replenished with Solvesso 100® every 3–4 days. Pure cultures were isolated by striking out single colonies. Two distinct new Solvesso 100®-degrading strains were isolated from a mature biofilm after about 2 months of bioreactor operation (Linn 1999). They were identified as *Pseudomonas* sp. by sequencing of the 16S rRNA gene. The new isolates were named *Pseudomonas* sp. strain w20 (accession number: Y18344) and *Pseudomonas* sp. strain g24 (accession number: Y18345). The 16S rRNA sequences were used to design two new isolate-specific oligonucleotide probes Pw20-586 and Pg24-586, both complementary to position 586-605 according to *E. coli*-numbering (Brosius et al. 1981) (see Table 1). Oligonucleotides of the selected regions were synthesized and CY3-labeled at the 5′-end. Hybridization conditions for the new oligonucleotide probes were optimised by gradually increasing the formamide concentration in the hybridization buffer as described previously (Manz et al. 1992). The newly designed probes Pw20-586 and Pg24-586 were used together with the probe Bcv13b to monitor the Solvesso 100®-degrading bacteria. The latter probe was characterised by Stoffels et al. (1998) to be specific for *Burkholderia vietnamensis* and *Burkholderia cepacia*, both representing Solvesso 100® degrading bacteria. The probes BET42a and GAM42a were used with competitor oligonucleotides as described earlier (Manz et al. 1992).

During preliminary experiments, the gradients of biofilm density/composition along the reactor height were examined. However, no significant gradients were observed. Thus, it was deduced that the biomass distribution and the microbial community composition were practically homogeneous and non-variant

Table 1. Oligonucleotide probes used in this study

Probe	Specificity	Target site,[1] rRNA position (probe sequence 5′ to 3′)	% FA in-situ[2]	Reference
ALF968	Alpha subclass of *Proteobacteria*	16S, 968–985	20%	Neef 1997
Bcv13b	*Burkholderia vietnamensis*,[3] *Burkholderia cepacia*[3]	23S, 255–277	30%	Stoffels et al. 1998
BET42a	Beta subclass of *Proteobacteria*	23S, 1027–1043	35%	Manz et al. 1992
EUB338	Bacteria	16S, 338–355	0%	Amann et al. 1990
GAM42a	Gamma-subclass of *Proteobacteria*	23S, 1027–1043	35%	Manz et al. 1992
Pw20-586	*Pseudomonas* sp. w20[3]	16S, 586–605 (ACATCCAACTTGCTGA ACC)	40%	This study
Pg24-586	*Pseudomonas* sp. g24[3]	16S, 586–605 (ACCTTCAACTTGCTGA ACC)	30%	This study

[1] *Escherichia coli* numbering (Brosius et al. 1981).
[2] Percent formamide (FA) in in-situ hybridization buffer.
[3] Specific for Solvesso100®-degrading isolate.

along the column height. Hence, the chosen sampling method ensured representative biofilm samples of the entire reactor. For further experiments, solid samples from only one port (port 4, see Figure 1) were examined.

Performance of the trickle-bed bioreactor during operation with pollutant concentration shifts and during a long-term starvation period

The performance of the trickle-bed bioreactor under transient conditions was examined for a period of approx. 9.5 months. During this time period, the pollutant gas inlet concentration was altered instantaneously and then kept constant for several weeks (shift experiments). The bioreactor performance is characterised by the three parameters: specific pollutant load (PL), specific elimination capacity (EC), and degree of conversion (DC). These parameters are defined as follows

$$\mathrm{PL} = c_0/\tau$$
$$\mathrm{EC} = (c_0 - c_1)/\tau$$
$$\mathrm{DC} = (c_0 - c_1)/c_0,$$

where c_0 is the gas inlet concentration, c_1 is the gas outlet concentration, and τ is the mean gas residence time. The operating conditions for the transient experiments with pollutant concentration shifts and a long-term starvation period are given in Table 2. The time courses of the specific pollutant load and the specific elimination capacity are presented in Figure 3. After a quasi-steady-state at $c_0 = 600$ mg m^{-3} had been reached for several weeks (average specific elimination capacity $\overline{\mathrm{EC}} = 80$ g m^{-3} h^{-1}), the first shift-down to $c_0 = 200$ mg m^{-3} took place on the 38th day. The gas inlet concentration was then kept constant for 3 weeks ($\overline{\mathrm{EC}} = 38$ g m^{-3} h^{-1}). The first shift-up occurred on the 55th day and the gas inlet concentration was then kept constant again at $c_0 = 600$ mg m^{-3}. It was observed that the EC rose within a few hours up to the original value of approx. 80 g m^{-3} h^{-1} and stayed relatively constant at this level. Since this observed fast response was surprising, the response times of abiotic physical mechanisms and of biological processes were compared (Roels 1983).

Table 2. Operating conditions for the transient experiments with pollutant concentration shifts and a long-term starvation period

Gas inlet concentration	1440 (peak value); 600; 200; 0 mg m^{-3}
Gas flow rate	35 l min^{-1}
Recirculating liquid flow rate	120 l h^{-1}
Average gas residence time τ	18.5 s
Trickling density	7.8 m^3 m^{-2} h^{-1}
Specific pollutant load	280 (peak value); 120; 40; 0 g m^{-3} h^{-1}
Temperature	30 °C
pH	7

The response times of relevant abiotic mechanisms (e.g. convective mass transfer, diffusion, and absorption processes) were typically in the range of seconds or minutes. On the other hand, the biological response times (e.g., growth, population dynamics) were in the order of hours or days. Therefore, it was deduced that abiotic mechanisms were not responsible for the short-term responses in the range of hours of the mixed population in the bioreactor.

The second shift-down to c_0 = 0 mg m^{-3} took place on the 112th day. From then on, the population dynamics were examined during a prolonged phase without external supply of a carbon source. Furthermore, the recovery behavior of the biofilm population at the end of the 146 days long starvation phase was studied. The second shift-up was on the 258th day and had a peak shape. The peak gas inlet concentration was c_0 = 1.44 g m^{-3} and the maximum PL was 280 g m^{-3} h^{-1}. Again, the EC rose within a few hours after the shift-up and reached a maximum of 224 g m^{-3} h^{-1}. Thus, it was shown that the population was not irreversibly damaged during the long starvation phase and was able to raise the biodegradation rate almost instantaneously after re-supply of pollutant. The EC plotted versus the PL is presented in Figure 4. The slope of the solid line represents the maximum achieved degree of conversion $DC_{max} \approx 80\%$. The average $\overline{DC}$, however, was only approx. 65%.

For further examinations of the biofilm, the immobilised biomass dry weight per single Ralu®-ring carrier (DW) and the total number of cells per carrier (TNC) via above described DAPI-measurements were determined. The results are presented in Figure 5. The pollutant concentration shifts are depicted by vertical arrows. As can be seen, both time courses rose after a shift-up and decreased after a shift-down. The DW during the pollutant supply phase (13th–112th day) was roughly between 100–140 mg/carrier. During the starvation phase, the DW dropped by 55% down to a value of approx. 60 mg/carrier. After the second shift-up at the 258th day, a relatively fast increase of the DW to about 120 mg/carrier was observed. The time course of the TNC was comparable to the one of the DW. However, the decrease to the TNC with time was more pronounced as can be seen after the first shift-down at the 38th day. Here, the TNC dropped down to approx. a third of the previous value within 8 days. On the other hand, the TNC increased relatively slow after the shift-up at day 55. In fact, it took about 8 weeks to reach the original level again. This surprising dependency of the TNC time course upon the direction of the shifts was observed during the starvation phase, too. The observed behavior suggested that endogeneous decay occurred at a higher rate than net specific growth. Other influencing factors such as hydrodynamic conditions, physical shearing, and oxygen supply rates were kept constant during the shift-experiments. Hence, these factors did not contribute to the above mentioned behavior. The TNC dropped by 77% during the starvation phase. It was observed microscopically that the size of most of the cells was reduced during the starvation phase. However, no reproducible quantitative data were obtained with regard to the degree of size reduction. Furthermore, it was observed that the rod-type shape of the cells turned into a rather coccoidal shape during the starvation phase.

The application of the above mentioned EPS extraction method using a cation exchanger resin yielded surprisingly high EPS fractions. Two independent measurements revealed EPS values of 89% and 93% of the total biomass. Thus, only a fraction of about 10% of the total biomass were actually cells (active and inactive ones).

Characterisation of the biofilm population dynamics during operation with pollutant concentration shifts and during a long-term starvation period

In a first step, the mixed population of the biofilm was characterised using the oligonucleotide probe EUB338 to detect the majority of *Proteobacteria* (Amann et al. 1990). The bacterial phyla not targeted by the EUB 338 probe set such as *Planctomycetales* and *Verrucomicrobia* (Daims et al. 1999) were assumed not to play a significant role in the present engineered system. The probes ALF968, BET42a, and GAM42a were used to detect the alpha-, beta, and

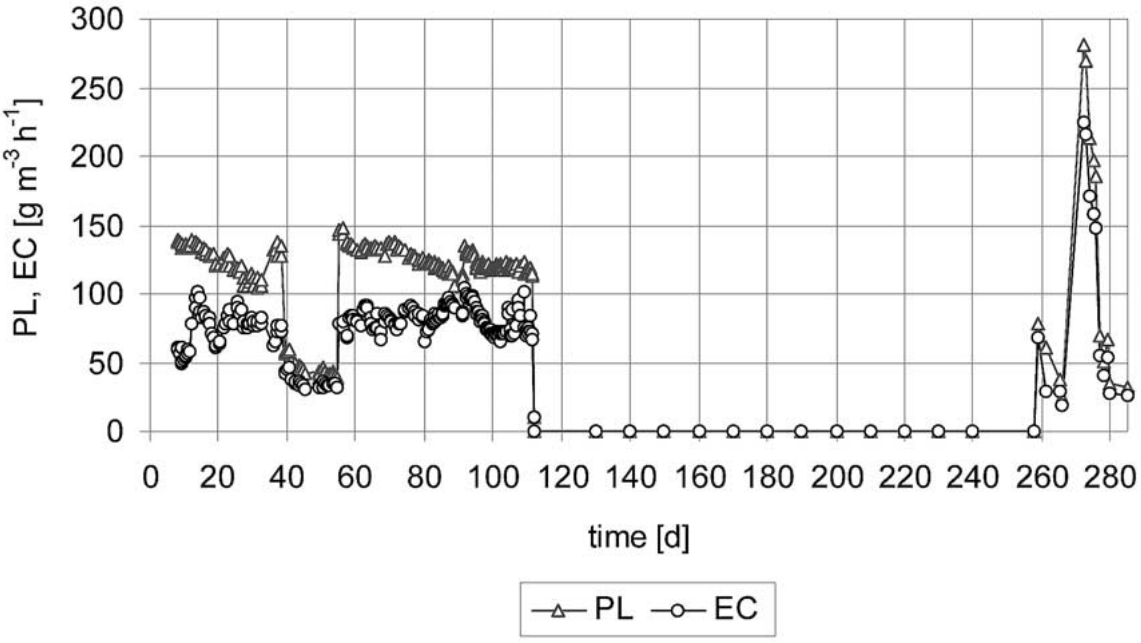

Figure 3. Time courses of the specific pollutant load (PL) and the specific elimination capacity (EC). First shift-down on the 38th day, first shift-up on the 55th day, second shift-down on the 112th day (start of starvation phase), second shift-up on the 258th day.

gamma-subclasses of the *Proteobacteria*. These three subclasses of the *Proteobacteria* include practically all relevant species to be found in the present system. The measured data are presented in Figure 6 as fractions of all detected cells (via DAPI-counts). As can be seen, alpha-subclass bacteria were dominant during the pollutant supply phases (before and after the starvation phase). However, the capability of alpha-subclass bacteria to mineralize polyalkylated benzenes was not reported. The fractions of beta- and gamma-subclass bacteria were variable during the pollutant supply phases. As already mentioned, these subclasses include genera which are able to utilize polyalkylated benzenes such as *Burkholderia* (beta-subclass) (Stoffels et al. 1998) and *Pseudomonas* (gamma-subclass) (Møller et al. 1996). Interestingly, a shift of the population composition was observed shortly after the start of the starvation phase: the fraction of alpha-subclass bacteria decreased and instead, gamma-subclass bacteria were dominant. This was somewhat contradictory since the gamma-subclass population representing pollutant-degraders was expected to be rather disadvantaged during the starvation phase. These observations led to the conclusion that a satisfactory characterisation of the mixed population was not possible using subclass-specific oligonucleotide probes. Furthermore, it was obvious that a population was present in the biofilm which did not degrade the primary carbon source, i.e. the polyalkylated benzenes. This population was believed to consist of saprophytes belonging to all three subclasses of *Proteobacteria*. These saprophytes were assumed to be able to utilize a secondary carbon source (not measured) such as metabolic intermediates and lysis products but not the polyalkylated benzenes. Based on a conceivable simplified model assumption, a different strategy was used to characterise the biofilm population: the potential pollutant-degraders were assumed to be measured as the sum of cells detected with the above mentioned isolate-specific oligonucleotide probes Bcv13b, Pw20-586, and Pg24-586. The saprophytes were then calculated from the difference of the pollutant-degraders and the cells measured with the EUB338 probe. The inactive cells were then calculated from the difference of the cells measured with the EUB338 probe and the total number of cells measured with the DAPI-method. This simplified model assumption implies that no other pollutant degraders were present in the reactor which were not detectable with the three isolate-specific probes. However, it cannot be ruled out that other strains capable to degrade Solvesso 100® existed in the reactor. Applying the above mentioned strategy yielded the results presented in Figure 7.

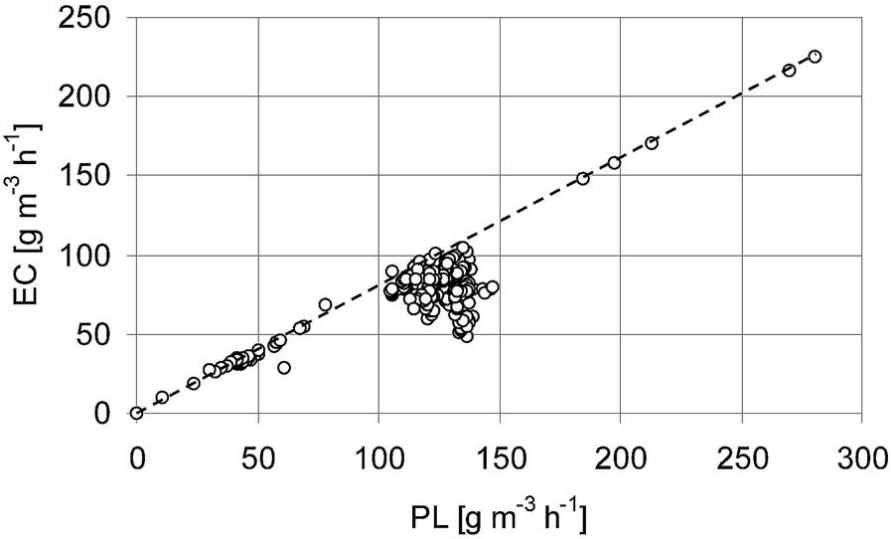

Figure 4. Specific elimination capacity (EC) plotted versus specific pollutant load (PL). The data can be regarded to be stationary with regard to abiotic physical mechanisms. The slope of the dotted line represents the maximum achieved degree of conversion $DC_{max} \approx 80\%$.

As can be seen, the fraction of pollutant-degraders was surprisingly small during the pollutant supply phases (13th–112th day and 258th–284th day). Instead, the saprophytes dominated the mixed population by far. On the average, only 8.8% of all biofilm cells during the pollutant supply phases were Solvesso 100®-degraders. During the starvation phase (113th–257th day), only 0.2% of all cells were detected by the isolate-specific probes and nearly 60% of all cells were inactive. After the starvation phase, the biodegradation rate of the bioreactor rose within a few hours and the fractions of active cells increased within several days. This led to the conclusion that the long-term starvation phase did not damage the biofilm population irreversibly. The change of cell fractions followed the direction of the pollutant shifts, i.e. a decrease of cell fractions resulted after a shift-down and vice versa. However, the dynamics of cell fraction change of pollutant-degraders and saprophytes differed strongly independent of the direction of the shifts. In order to quantify these different dynamics, the specific change of cell fractions (CCF) between two consecutive measurement points $t_i^{(n)}$ and $t_i^{(n+1)}$ was calculated from the experimental data. CCF is defined as

$$\mathrm{CCF} = \frac{1}{f_i^{(n)}} \frac{f_i^{(n+1)} - f_i^{(n)}}{t_i^{(n+1)} - t_i^{(n)}},$$

$$i = 1, 2, 3 \begin{cases} 1, & \text{degraders} \\ 2, & \text{saprophytes} \\ 3, & \text{inactive cells} \end{cases}$$

f_i is the fraction of the type i of cells of the total number of cells. The parameter CCF is a measure of the intensity of the change of cell fraction with time. The time courses of the CCF of pollutant-degraders, saprophytes, and inactive cells are given in Figure 8. As can be seen, the dynamics of the pollutant-degraders were by far the most sensitive ones. Interestingly, the degree of change of the degrader cells was higher after a shift-up than after a shift-down. Here, a dependency upon the direction of the shifts was observed again. Furthermore, it was noticed that the intensity of change of the saprophytes was lowest. Hence, the changes of the fraction of inactive cells were faster compared to the saprophytes.

Correlation of the performance of the trickle-bed bioreactor with the population composition of the biofilm

It is known that a dynamic and flexible complex community, as it was observed in the present study, could maintain an ecosystem with a stable and sustainable function (Fernández et al. 1999; Tresse et al. 2002). Therefore, a possible correlation of the function of

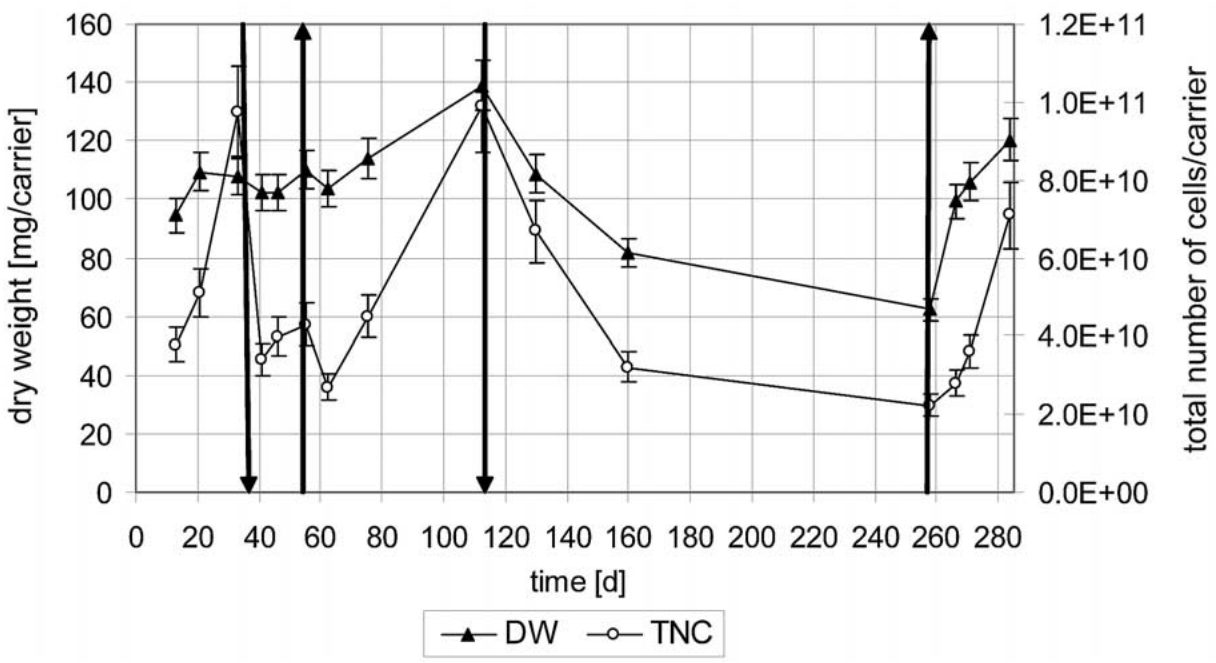

Figure 5. Time courses of the biomass dry weight per carrier (DW) and the total number of cells per carrier (TNC). The pollutant concentration shifts are depicted by vertical arrows (standard deviation was 6% for DW and 12% for TNC).

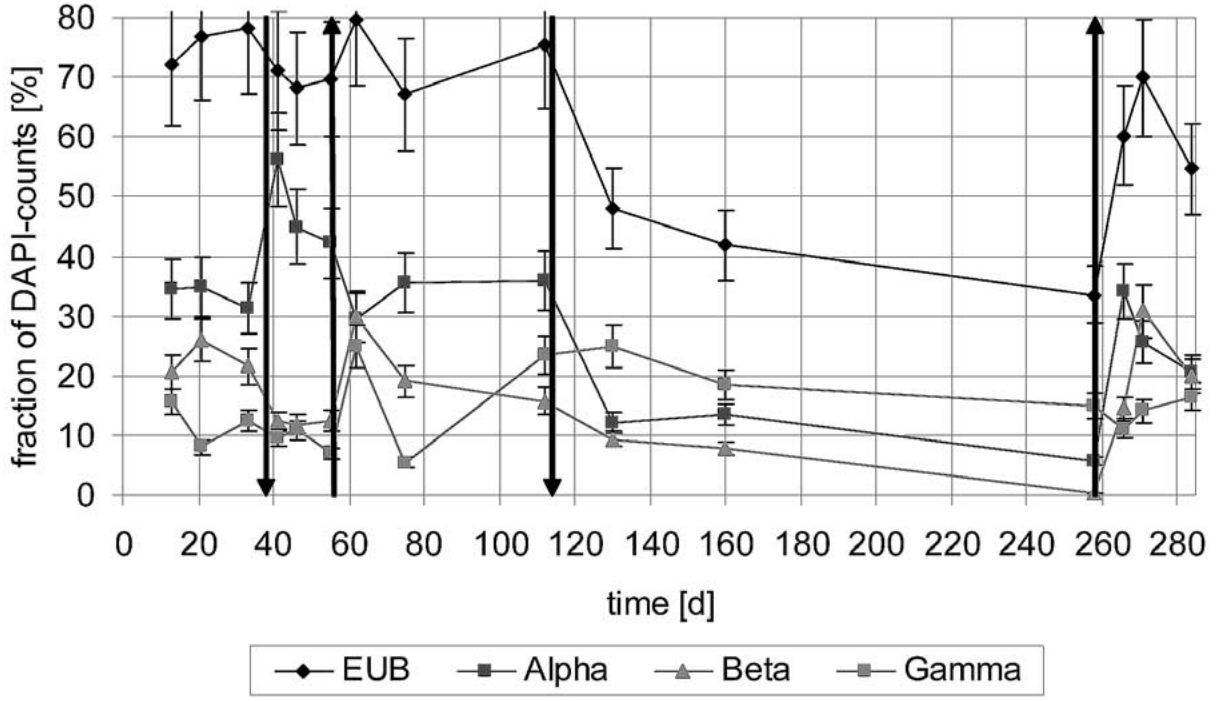

Figure 6. Time courses of *Proteobacteria* and the alpha-, beta-, and gamma-subclasses of *Proteobacteria* during operation with pollutant concentration shifts and a long-term starvation period. The pollutant concentration shifts are depicted by vertical arrows (standard deviation was 14%).

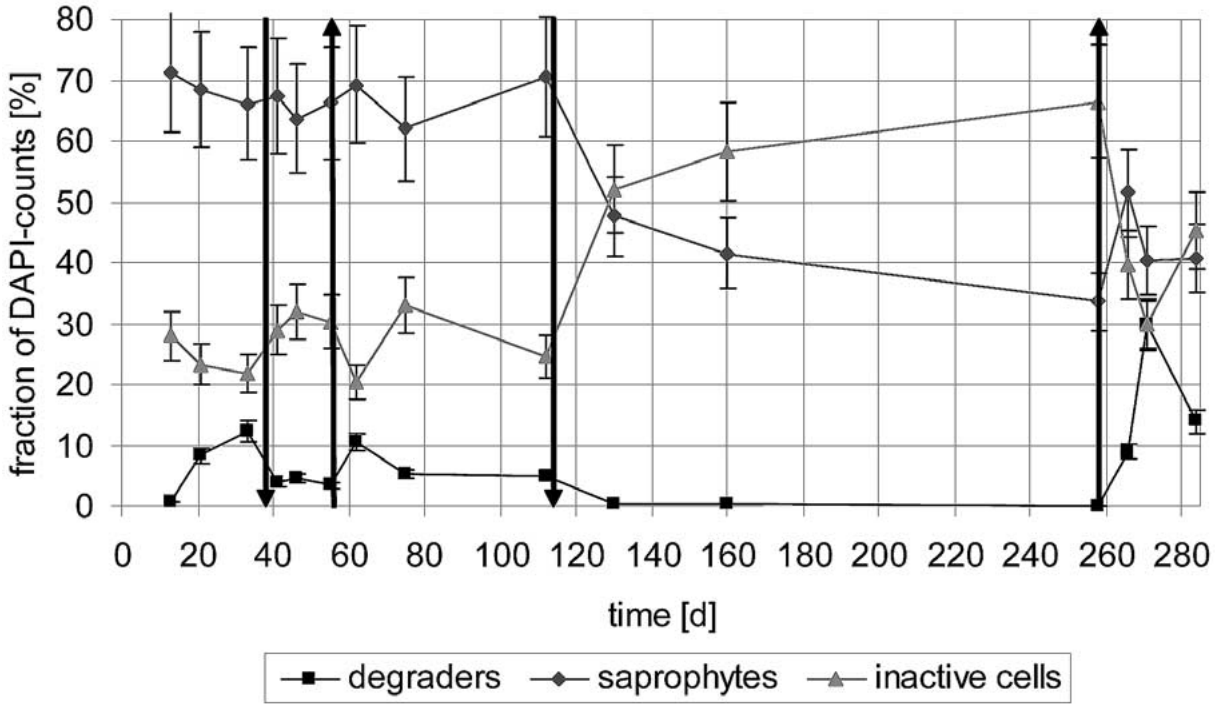

Figure 7. Time courses of pollutant-degraders, saprophytes, and inactive cells during operation with pollutant concentration shifts and a long-term starvation period. The pollutant concentration shifts are depicted by vertical arrows (standard deviation was 14%).

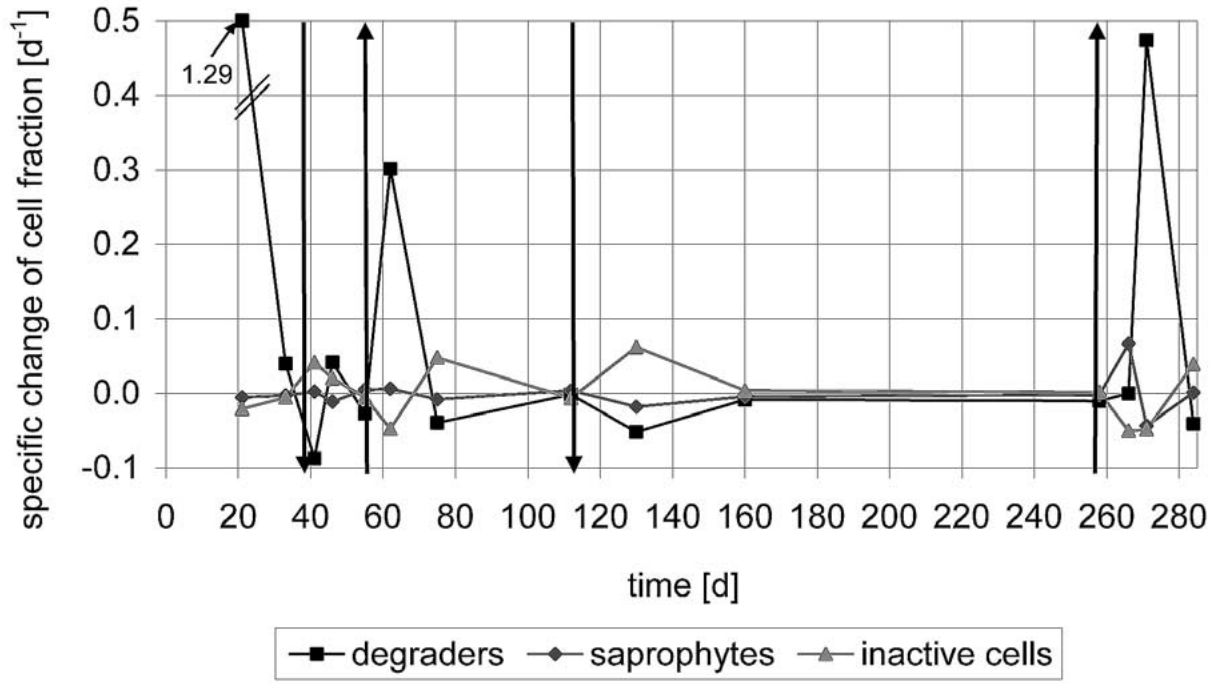

Figure 8. Time courses of the specific change of cell fractions (CCF) of pollutant-degraders, saprophytes, and inactive cells during operation with pollutant concentration shifts and a long-term starvation period. The pollutant concentration shifts are depicted by vertical arrows.

the microbial system with the community composition was examined. This was a difficult task due to the following facts: (i) obviously, not only the number of cells but also the individual cell-specific physiological activity determined the biodegradation activity. However, in the present study, only cell numbers were measured without evaluating the physiological activity; (ii) the removal rate of pollutant from the gas phase was not only determined by the biodegradation rate but also depended upon various simultaneous mass transfer processes. The rates of these mass transfer processes were variable depending upon factors such as the amount of rhamnolipid biosurfactants present in the biofilm (Vandyke et al. 1993); (iii) moreover, not all Solvesso 100®-degrading bacteria might have been detected using the three above mentioned isolate-specifc oligonucleotide probes. In addition, metabolic interactions between community members could have altered the biodegradation rate (Møller et al. 1998). Hence, it was expected that a direct correlation of bioreactor performance and community composition was not evident. As a matter of fact, the existing experimental evidence was not sufficient to provide a direct correlation. Therefore, further experiments have to be performed in the future using improved techniques.

Conclusions

The cultivation-independent fluorescence in-situ hybridization (FISH) method was applied in order to analyse the mixed population of a biofilm from experiments using a laboratory scale trickle-bed bioreactor. The model pollutant was a VOC-mixture of polyalkylated benzenes called Solvesso 100®. Two new types of Solvesso 100®-degrading bacteria were isolated from the mature biofilm and specific oligonucleotide probes for FISH measurements were designed. Both isolates belonged to the gamma-subclass of the *Proteobacteria* and showed a strong sequence similarity to the genus *Pseudomonas*. It is well known that these types of microorganisms are able to mineralize aromatic hydrocarbons and to produce large amounts of EPS in order to facilitate immobilisation and subsequent formation of biofilms. Furthermore, these EPS contain rhamnolipid biosurfactants which enhance the availability of hydrophobic carbon sources (Vandyke et al. 1993). A major experimental result was that the fraction of Solvesso 100®-degrading bacteria in the biofilm was low. About 90% of the biofilm was EPS, hence, only about 10% of the biomass were cells. The average fraction of Solvesso 100®-degrading cells during the pollutant supply periods was less than 10% of all cells. About 60% of the cells were saprophytes and about 30% were inactive cells.

It was observed during the transient shift-experiments that the bioreactor performance adapted to abrupt changes of the pollutant gas inlet concentration within a few hours. Even after the prolonged starvation phase, the biodegradation rate rose rapidly a few hours after re-supply of pollutant. Thus, it was shown that a robust biofilm population existed. The pollutant-degraders were capable to utilize the primary carbon source (the pollutant) as well as the secondary carbon source consisting of metabolic intermediates and lysis products. During the starvation phase, so called 'cryptic growth' took place (Hamer 1985). Besides the intermediates and lysis products, other endogeneous energy reserves might have been metabolized such as polyhydroxy fatty acids stored in the biofilm EPS-matrix (Freeman & Lock 1995). Hence, the EPS-matrix played a role as a buffer against changing organic substrate supply. The measured biomass dry weight per Ralu®-ring carrier dropped during the starvation phase. However, it is known that the mixed population was not likely to be capable to utilize the abundant EPS as a carbon source and/or energy source (Grinberg et al. 1991). On the other hand, microscopic observations showed that the cells during the starvation phase were smaller and their shape had turned into coccoidal cells. This finding confirmed similar observations by Egli (1995). The investigations of the biofilm population dynamics revealed that a directional dependency existed with regard to the pollutant shifts. The population reacted within days after a shift-down. On the other hand, it took several weeks for the population to reach previous conditions after a shift-up. Another dependency on the direction of the shifts was observed with regard to the intensity of the change with time of the Solvesso 100®-degrading cell fraction after shifts. The degree of change was higher after a shift-up than after a shift-down. A clear shift of the population composition occurred during the starvation phase. However, this change of composition as well as the degree of metabolic activity was completely reversible. After the shifts, the pollutant-degraders reacted significantly faster compared to the other cells. A direct correlation between the biodegradation rate of the bioreactor and the number of pollutant-degrading cells present in the biofilm could not be obtained due to insufficient experimental evidence.

It can be concluded that the above findings lead to a further understanding of the microbial community composition and the population dynamics of a multispecies biofilm-reactor operated under transient conditions.

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References

Amann R, Krumholz L & Stahl DA (1990) Fluorescence-oligonucleotide probing of whole cells for determination, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172: 762–770

Amann R, Ludwig W & Schleifer KH (1995) Phylogenetic identification and in-situ detection of individual microbial cells without cultivation. Microbial Rev. 59: 143–169

Arcangeli JP & Arvin E (1992) Toluene biodegradation and biofilm growth in an aerobic fixed-film reactor. Appl. Microbiol. Biotechnol. 37: 510–517

Brosius J, Dull TL, Sleeter DD & Noller HF (1981) Gene organization and primary structure of a ribosomal RNS operon from Escherichia coli. J. Molec. Biol. 148: 107–127

Christensen BB, Haagensen JAJ, Heydorn A & Molin S (2002) Metabolic commensalism and competition in a two-species microbial consortium. Appl. Environ. Microbiol. 68: 2495–2502

Daims H, Brühl A, Amann R, Schleifer K-H & Wagner M (1999) The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. System. Appl. Microbiol. 22: 434–444

Deshusses MA, Hamer G & Dunn IJ (1996) Transient-state behavior of a biofilter removing mixtures of vapors of MEK and MIBK from air. Biotechnol. Bioeng. 49: 587–598

Diks RMM & Ottengraf SPP (1991) Verification studies of a simplified model for the removal of dichloromethane from waste gases using a biological trickling filter. Bioprocess Eng. 6: 93–99 (Part I), 131–140 (Part II)

Egli T (1995) The ecological and physiological significance of the growth of heterotrophic microorganisms with mixtures of substrates. Adv. Microb. Ecol. 14: 305–386

Fernández A, Huang S, Seston S, Xing J, Hickey R, Criddle C & Tiedje J (1999) How stable is stable? Function versus community composition. Appl. Environ. Microbiol. 65: 3697–3704

Freeman C & Lock MA (1995) The biofilm polysaccharide matrix: A buffer against changing organic substrate supply? Limnol. Oceanogr. 40: 273–278

Grinberg TA, Pirog TP, Buklova VN & Malashenko YR (1991) Interrelationships of microorganisms in an exopolisaccharide-forming mixed culture. Microbiology 59: 542–548

Hamer G (1985) Lysis and cryptic growth in wastewater and sludge treatment processes. Acta Biotechnol. 5: 117–127

Hekmat D & Vortmeyer D (1994) Modelling of biodegradation processes in trickle-bed bioreactors. Chem. Eng. Sci. 49: 4327–4345

Hekmat D, Linn A, Stephan M & Vortmeyer D (1997) Biodegradation dynamics of aromatic compounds from waste air in a trickle-bed reactor. Appl. Microbiol. Biotechnol. 48: 129–134

Hekmat D, Amann R, Linn A, Stephan M, Stoffels M & Vortmeyer D (1998) Biofilm population dynamics in a trickle-bed bioreactor. Wat. Sci. Technol. 37: 167–170

James GA, Beaudette L & Costerton JW (1995) Interspecies bacterial interactions in biofilms. J. Ind. Microbiol. 15: 169–175

Linn A (1999) Populationsdynamik und Abbauverhalten einer immobilisierten mikrobiellen Mischkultur im Rieselbettreaktor zur biologischen Abluftreinigung. Dissertation, Technische Universität München

Manz W, Amann R, Ludwig W, Wagner M & Schleifer KH (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. Syst. Appl. Microbiol. 15: 593–600

Manz W, Wendt-Potthoff K, Neu TR, Szewzyk U & Lawrence JR (1999) Phylogenetic composition, spatial structure, and dynamics of lotic bacterial biofilms investigated by fluorescence in-situ hybridization and confocal laser scanning microscopy. Microb. Ecol. 37: 225–237

Møller S, Pedersen AR, Poulsen LK, Arvin E & Molin S (1996) Activity and three-dimensional distribution of toluene-degrading *Pseudomonas putida* in a multispecies biofilm assessed by quantitative in-situ hybridization and scanning confocal laser microscopy. Appl. Environ. Microbiol. 62: 4632–4640

Møller S, Sternberg C, Andersen JB, Christensen BB, Ramos JL, Givskov M & Molin S (1998) In-situ gene expression in mixed-culture biofilms: evidence of metabolic interactions between community members. Appl. Environ. Microbiol. 64: 721–732

Neef A (1997) Anwendung der in-situ Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen. Dissertation, Technische Universität München

Pedersen AR, Møller S, Molin S & Arvin E (1997) Activity of toluene-degrading *Pseudomonas putida* in the early growth phase of a biofilm for waste gas treatment. Biotechnol. Bioeng. 54: 131–141

Roels JA (1983) Energetics and Kinetics in Biotechnology. Elsevier Biomedical Press, Amsterdam

Siebel MA & Characklis WG (1991) Observations of binary population biofilms. Biotechnol. Bioeng. 37: 778–789

Stoffels M, Amann R, Ludwig W, Hekmat D & Schleifer KH (1998) Bacterial community dynamics during start-up of a trickle-bed bioreactor degrading aromatic compounds. Appl. Environ. Microbiol. 64: 930–939

Tresse O, Lorrain MJ & Rho D (2002) Population dynamics of free-floating and attached bacteria in a styrene-degrading biotrickling filter analysed by denaturing gradient gel electrophoresis. Appl. Microbiol. Biotechnol. 59: 585–590

Vandyke MI, Couture P, Brauer M, Lee H & Trevors JT (1993) *Pseudomonas aeruginosa* UG2 rhamnolipid biosurfactants – structural characterization and their use in removing hydrophobic compounds from soil. Can. J. Microbiol. 39: 1071–1078

Wagner M, Amann R, Lemmer H & Schleifer KH (1993) Probing activated sludge with proteobacteria-specific oligonucleotides: Inadequacy of culture-dependent methods for describing microbial community structure. Appl. Environ. Microbiol. 59: 1520–1525