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Modelling the growth of a methanotrophic biofilm: Estimation of parameters and variability

Jean-Pierre Arcangeli* & Erik Arvin
*Department of Environmental Science and Engineering, The Technical University of Denmark, Building 115, DK-2800 Lyngby, Denmark (*author for correspondence: Membrane Extraction Technology Ltd. Department of Chemical Engineering, Imperial College, London SW7 2BY, UK)*

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Abstract

This article discusses the growth of methanotrophic biofilms. Several independent biofilm growths scenarios involving different inocula were examined. Biofilm growth, substrate removal and product formation were monitored throughout the experiments. Based on the oxygen consumption it was concluded that heterotrophs and nitrifiers co-existed with methanotrophs in the biofilm. Heterotrophic biomass grew on soluble polymers formed by the hydrolysis of dead biomass entrapped in the biofilm. Nitrifier populations developed because of the presence of ammonia in the mineral medium. Based on these experimental results, the computer program AQUASIM was used to develop a biological model involving methanotrophs, heterotrophs and nitrifiers. The modelling of six independent growth experiments showed that stoichiometric and kinetic parameters were within the same order of magnitude. Parameter estimation yielded an average maximum growth rate for methanotrophs, μ_m, of 1.5 ± 0.5 d^{-1}, at 20 °C, a decay rate, b_m, of 0.24 ± 0.1 d^{-1}, a half saturation constant, $K_{S(CH_4)}$, of 0.06 ± 0.05 mg CH_4/L, and a yield coefficient, Y_{CH_4}, of 0.57 ± 0.04 g X/g CH_4. In addition, a sensitivity analysis was performed on this model. It indicated that the most influential parameters were those related to the biofilm (i.e. density; solid-volume fraction; thickness). This suggests that in order to improve the model, further research regarding the biofilm structure and composition is needed.

Introduction

Methanotrophic bacteria are of interest for the following reasons: Their ability to regulate the methane concentration in the atmosphere (Ojima et al. 1993); their potential to produce single cell proteins; and their ability to co-metabolize chlorinated aliphatic hydrocarbons (CAH's) (Tsien et al. 1989; Wilson & Wilson 1985). The third property of methanotrophs initiated this work. CAH's are widely used in industries as solvents for degreasing and dry cleaning and, thus, they are often found at hazardous waste sites and in contaminated groundwater. Consequently, knowledge of the growth kinetics of these bacteria is essential to the design of treatment processes for CAH-contaminated water. Although the growth kinetics of methanotrophic bacteria with suspended cultures are well established (e.g., Alvarez-Cohen & McCarty 1991; Broholm et al. 1992; Oldenhuis et al. 1991), comparatively little is known about the growth kinetics of these bacteria in biofilm systems (Anderson & McCarty 1994; Bilbo et al. 1992). Therefore, the purpose of this study was twofold: to present a biological model which is able to simulate the growth of a methanotrophic biofilm, and to discuss the variability of the parameter estimates. Preliminary experimental results have been discussed elsewhere (Arcangeli and Arvin, 1997).

Materials and methods

Experimental set-up and operating procedures

Six independent biofilm growth experiments (runs A–F) involving two different inocula were examined during this study. Runs B, D, E and F were inoculated with an enriched, mixed culture of methanotrophs originating from the top-soil of a landfill contaminated with chlorinated hydrocarbons and from the sediment of Copenhagen harbour (Denmark). Runs A and C were inoculated from a mixed culture obtained from a water works treating groundwater containing methane (Nykøbing Sjælland, Denmark).

The experimental system for runs D, E and F is shown in Figure 1. The biofilm reactor is a so-called Biodrum reactor system consisting of a drum rotating inside another drum (Kristensen & Janssen 1980). The biofilm grows on the surface of both the stator and the rotator. The volume of the reactor is approx. 0.96 litre and the surface area is 0.16 m^2. The rotation (200 rpm) and the recycling system (50 L/h) ensures nearly total mixing of the bulk liquid, and consequently promote relatively uniform biofilm growth in the reactor. Methane and oxygen were dissolved in the inflow stream within two separate bubble columns, and thereafter supplied to the reactor (Figure 1, Items 2 and 4). The inoculation was accomplished by injecting 100 ml of the mixed culture into the reactor in order to permit the biomass to attach to the walls of the rotator and the stator. Feeding of the reactor was initiated after two days. The biofilm grew under aerobic, non-sterile conditions with methane as the sole carbon source. Run A-C were performed under methane limiting conditions. The temperature was maintained at about 19–22 °C, and the pH was in the range of 7–7.4 during these growth experiments. The reactor was protected from light in order to prevent growth of photosynthetic organisms. Information regarding the experimental set-up for the other runs, may be found in Arvin (1991) (runs A and C) and Arcangeli et al. (1996) (run B).

Substrate

Methane and oxygen concentration in the bioreactor inlet are provided in Table 3. The local tapwater from Lyngby (Denmark) contains most of the macro- and micro-ions required for microbial growth, with the exception of phosphorus, iron, and nitrogen, which were added. The final concentrations of dissolved minerals in the inflow water were (mg/L): NH_4NO_3, 8.6;

Table 1. Matrix representation of the biological model. (The nomenclature is in the appendix)

Component (i) →	1	2	3	4	5	6	7	8	9	10	Process rate
Process (j) ↓	X_I	X_S	X_H	X_N	X_M	CH_4	O_2	S_P	NH_4	NO_3	
1 Methanotrophs growth	α				1	$-\frac{1}{Y_{CH_4}}-\alpha-\gamma$	$-\frac{1-Y_{CH_4}}{Y_{CH_4}}$	γ	$-\alpha_{NH_4}$		$\mu_m \frac{CH_4}{CH_4+K_{S(CH_4)}\left[1+\frac{NH_4}{K_{S(NH_4)}}\right]} \frac{O_2}{O_2+K_{S(O_2)}} X_m$
2 Methanotrophs Nitrification					1		$-\frac{4.57-Y_{NH_4}}{Y_{NH_4}}$		$-\frac{1}{Y_{NH_4}}-0.086$	$\frac{1}{Y_{NH_4}}$	$\mu_{Nm} \frac{NH_4}{NH_4+K_{S(NH_4)}\left[1+\frac{CH_4}{K_{S(CH_4)}}\right]} \frac{O_2}{O_2+K_{S(O_2\_aut)}} X_m$
3 Methanotrophs decay	β	$1-\beta$			-1						$b_m X_m$
4 Nitrifiers growth				1			$-\frac{4.57-Y_{NH_4}}{Y_{NH_4}}$		$-\frac{1}{Y_{NH_4}}-0.086$	$\frac{1}{Y_{NH_4}}$	$\mu_N \frac{NH_4}{NH_4+K_{S(NH_4)}} \frac{O_2}{O_2+K_{S(O_2\_aut)}} X_N$
5 Nitrifiers decay	β	$1-\beta$		-1							$b_N X_N$
6 Heterotrophs growth			1				$-\frac{1-Y_H}{Y_H}$	$-\frac{1}{Y_H}$			$\mu_H \frac{S_P}{S_P+K_{S(Sp)}} \frac{O_2}{O_2+K_{S(O_2)}} X_H$
7 Heterotrophs decay	β	$1-\beta$	-1								$b_H X_H$
8 Hydrolysis		-1						1			$k_H X_S$

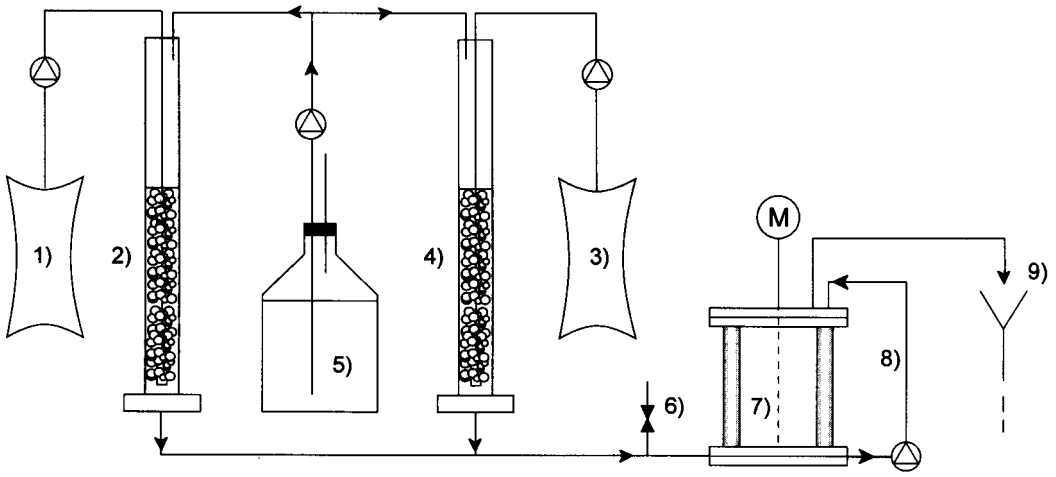

Figure 1. Experimental set-up. (1) methane supply (®Tedlar bag); (2) methane bubble column; (3) oxygen supply (®Tedlar bag); (4) oxygen bubble column; (5) mineral medium; (6) inlet sampling port; (7) biofilm reactor; (8) recirculation loop. (9) outlet.

NaH_2PO_4, H_2O, 4.9; $FeSO_4$, $7H_2O$, 2.5; EDTA, 2.0. (EDTA was used to avoid precipitation of metal phosphate in the mineral medium). The concentration of Cu^{2+} was measured to be < 0.005 mg/L. In one of the growth experiments (run C), the nitrogen source consisted of $NaNO_3$ (7.4 mg N-NO_3/L) and NH_4Cl (0.14 mg N-NH_4/L).

Analytical procedures

Oxygen and methane were monitored by membrane inlet mass spectrometry, MIMS (Lauritsen 1990). This technique involves a membrane which is attached directly to the inlet port of the mass spectrometer. An aqueous sample flows across the membrane's external surface (flow 60 ml/h) whereby a small fraction of the aqueous compounds diffused through the membrane into the source of the mass-spectrometer source, where it is detected. A considerable enrichment of hydrophobic and volatile compounds, relative to water, can be achieved using a silicone membrane (thickness: 125 μm; SILTEC sheeting, Technical Products Inc., GA). The membrane inlet system was made of stainless steel: the surface area of the membrane was approximately 7 mm^2 (Lauritsen 1990). It was connected to a mass spectrometer (MS QMG 421-1, Balzers, Liechtenstein), mass range up to 200 amu, equipped with a turbomolecular pump, a electron multiplier, and a gastight electron impact (70 eV) ion source. The ratio mass/charge (m/z) used in the mass-spectrometer was 15 amu for methane and 32 amu for oxygen. A computer equipped with the software QUASTAR 421 (Balzers, Liechtenstein) communicated with the MIMS and acquired all data. The concentration of methane and oxygen were determined by comparison with a standard curve made in water. Using this measurement technique, the concentration of the dissolved compounds in the bulk liquid could be measured every 20 seconds, allowing a real-time control of the reactor performance.

For runs A, B, and C, the increase in biofilm thickness was measured by monitoring the reduction in the mass of the water in the reactor via a standardized emptying procedure, as described by Arvin (1991). This method permitted estimation of the average biofilm thickness for the entire reactor. The biofilm thickness in runs D, E and F was measured on removable slides (located horizontally in the reactor stator wall) with a stereo-microscope equipped with a micro-ruler. The objective lens was lowered to the edge of the slide and focused on the cross-section of the biofilm. Comparison of the biofilm cross-section with the micro-ruler gave the biofilm thickness. In addition to this, a small area of the biofilm was scraped off, in order to measure the biofilm dry-weight and the protein content. The biofilm dry-weight content was calculated as the attached dry biomass per unit of wet biofilm volume. The chemical oxygen demand (COD) of the biofilm was determined according to Standard methods (APHA 1989). Proteins were assayed according to the method described by Lowry et al. (1951). The detachment of the biofilm was quantified by determination of proteins in the effluent. Dissolved polymers were determined according to the colorimet-

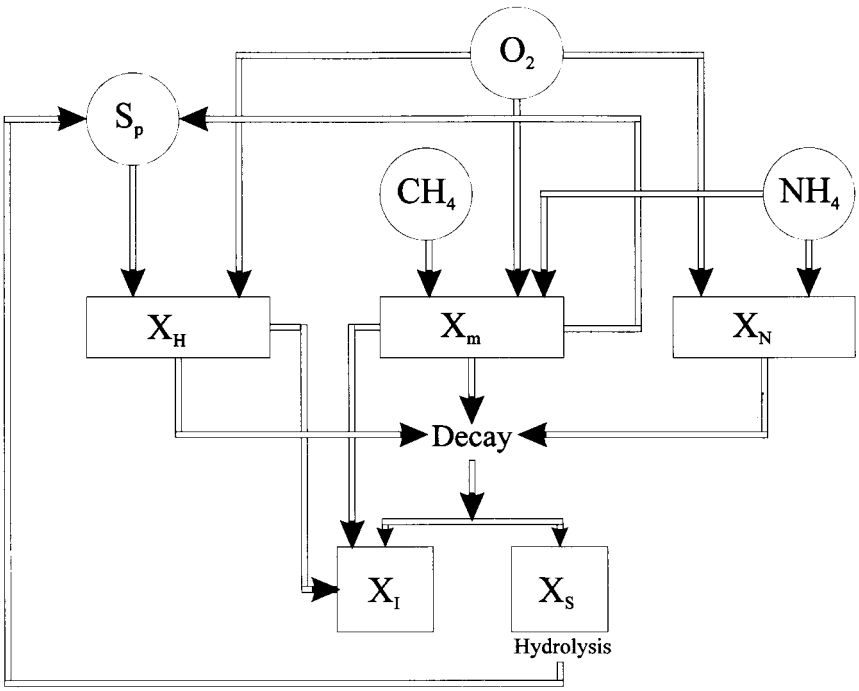

Figure 2. Biological model. O_2: dissolved oxygen; CH_4: methane; NH_4: ammonia; S_p biodegradable-soluble polymers; X_H: heterotrophic biomass; X_m: methanotrophic biomass; X_N: nitrifiers; X_S: biodegradable biomass; X_I: inert biomass.

ric method developed by Dubois, et al. (1956) for polysaccharides.

Biological model

The following assumption are made: (i) the biofilm is homogeneous and the liquid film diffusion is negligible; (ii) the transport of soluble components into the biofilm takes place by diffusion only; and (iii) the biofilm reactor is modelled as a non steady state CSTR. The proposed biological model is illustrated in Figure 2 and in Table 1. Three groups of active bacteria are considered: methanotrophs, X_{m}, heterotrophs, X_{H}, and nitrifiers, X_{N}. These active biomasses are growing on methane, soluble polymers, S_{P}, and ammonia, respectively. These active bacteria decay into inert biomass, X_i, and biodegradable biomass, X_{S}. The biodegradable biomass hydrolyse into soluble polymers which are degraded by heterotrophs. In addition, extracellular polymeric substances (EPS) which hold the bacteria together are produced by active bacteria. These EPS's are assumed to have a composition similar to the inert biomass formed by biomass decay. Therefore, EPS's do not constitute a separate variable in the model. Soluble polymers are also formed by methanotrophs. Nitrification is also carried out by methanotrophs: a cross-competitive inhibition governs the biodegradation of methane and ammonia. The inhibition coefficient of the competitive inhibitor is approximated by its single-substrate half-saturation coefficient. Finally, the biofilm detachment process is specified by the detachment velocity, which depend on various biofilm and system parameters. In this work, it is proportional to the biofilm thickness-growth velocity. The biofilm detachment leads to suspended solids formation in the bulk. This process can be described by the following expression:

$$r_{FXi} = u_{\mathrm{de}} \cdot X_{F,Xi}$$

with:

$$u_{\mathrm{de}} = \mathrm{det} \cdot u_F$$

where r_{FXi} is the interfacial mass flux of suspended solids, X_i, at the biofilm side of the biofilm-bulk fluid interface ($\mathrm{ML}^{-2}\ \mathrm{t}^{-1}$), u_{de} is the velocity by which particulate components are detached from the biofilm to the bulk fluid (Lt^{-1}), u_F is the velocity by which the biofilm solid matrix is expanding (Lt^{-1}) and det is detachment constant (−). These equations originate

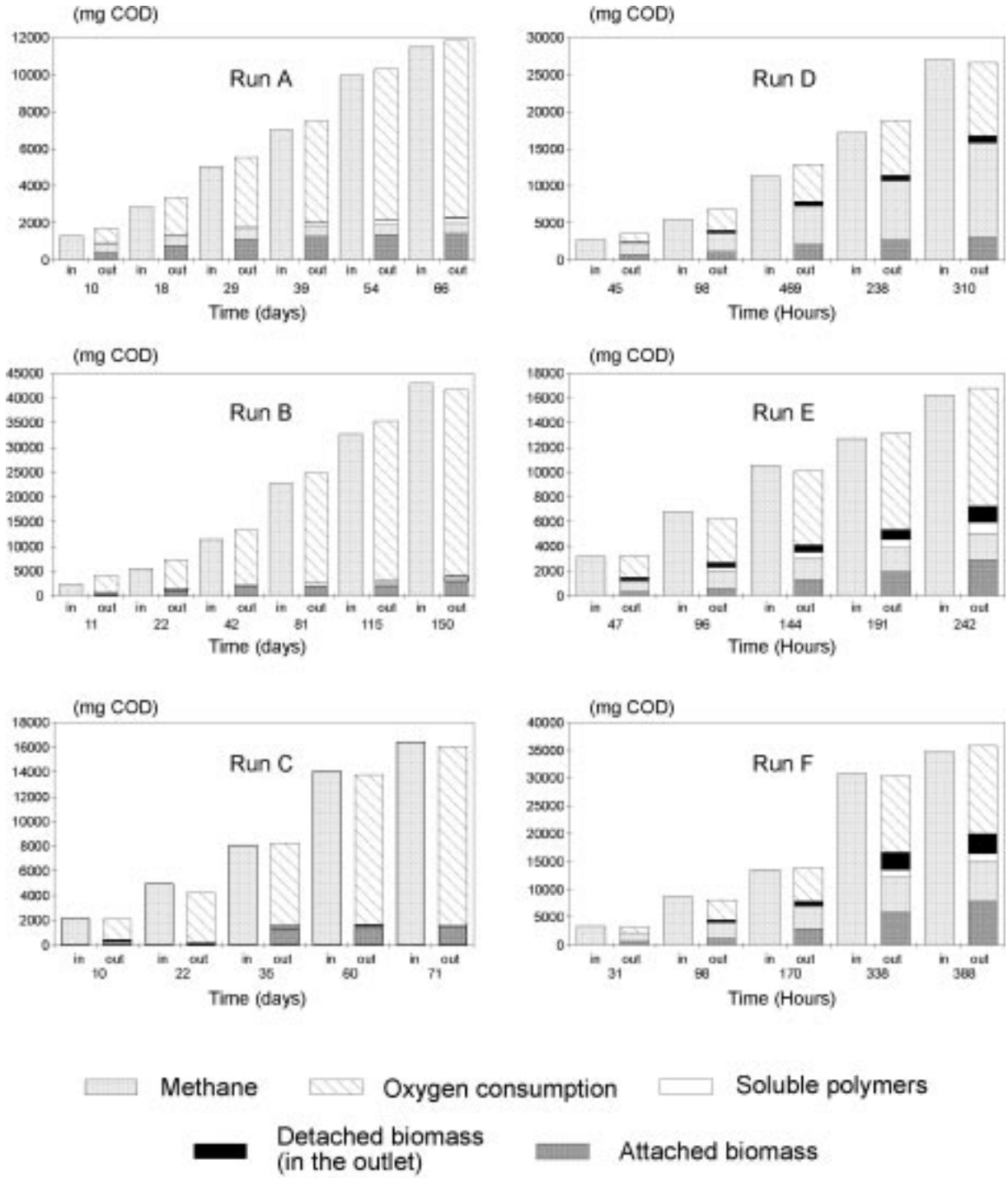

Figure 3. Cumulative COD balance for runs A–F. For each time step two histograms are indicated: the first gives the cumulated methane input in the reactor, the second gives the cumulated output.

Table 2. Initial values used in the model

	A[c]	B[d]	C[e]	D	E	F
Q_W (m^3/d)	0.0047	0.02[a]	0.0041	0.065[b]	0.088	0.09
H.R.T. (min)	294	69.1	337	21.3	15.7	15.4
L_{f_ini} (μm)	1 ± 0.5	1 ± 0.5	1 ± 0.5	20 ± 10	30 ± 10	30 ± 10
X_t (kg/m^3)	62 ± 11.1	52.4 ± 11	62.4 ± 17.4	27.2 ± 3.8	25 ± 12	25.2 ± 5.6
X_{act} (kg/m^3)	9.8 ± 2.2	14.6 ± 9	7.1 ± 2.5	9.6 ± 1.6	n.m	10.8 ± 4.5
ϵ_t	0.062 ± 0.015	0.094 ± 0.06	0.045 ± 0.015	0.061 ± 0.01	n.m	0.069 ± 0.03
ϵ_{m_ini}	0.05	0.07	0.05	0.04	0.05	0.04
ϵ_{I_ini}	0.03	0.03	0.03	0.025	0.025	0.02

[a] Q_W = 0.005 m^3/d for the first 5 days.
[b] Q_W = 0.035 m^3/d for the first day. n.m.: not measured. $\pm$: standard deviation for all runs: ϵ_{S_ini} = 0.0001; ϵ_{N_ini} = 10^{-5}; ϵ_{H_ini} = 10^{-5}; a standard deviation of 50% was assumed for each of the solid volume fractions.
[c] From Mejlhede (1993).
[d] From Arcangeli et al. (1996).
[e] From Pedersen (1991).

from a mathematical framework for modelling mixed culture biofilms which has been develop by Wanner and Reichert (1996).

The solid phase density parameter required in the model is a characteristic of the solid-phase, particulate component of the biofilm and is expressed as the mass of dry solids per unit solid phase volume. The value is estimated as 217,840 g/m^3 for the active biomass (COD units). This calculation is based on a relationship proposed by Norland, et al. (1987) which correlates the dry matter and volume of bacteria. The volume of the bacteria is determined to be 1.47 μm^3 (*Methylosinus* type II) according to the result of microscopic examination: the biovolume of this strain (Stanier et al. 1987) is in agreement with the average biovolume of the micro-organisms observed in this mixed culture. The density of the inert biomass (EPS) is estimated to be 1.4×10^6 g COD/m^3. The biofilm model assumes a total solid volume fraction, ϵ_t, ranging from 4.5 to 9.4% estimated according to the average active biomass content of the biofilm and an average dry weight of 0.23 pg/cell (Norland et al. 1987). This calculation assumes that all the cells in the biofilm have the same volume as the *Methylosinus* type II. Furthermore, the model requires an initial solid-volume fraction for each particulate component (Table 2). These values are chosen from within the range of values used in the model calibration, so that their sum is within the confidence interval of the total solid-volume fraction, ϵ_t, defined above. The diffusion coefficients of soluble components in water were estimated using the Wilke and Chang method (Perry and Green, 1987). Values of 1.6×10^{-4}, 1.73×10^{-4}, 1.7×10^{-4}, 1.5×10^{-4} and 5×10^{-5} m^2/d were found for of methane, oxygen, ammonia, nitrate and soluble polymers, respectively. The ratio of the diffusivity in biofilm by the diffusivity in water was set to 0.8 (Christensen and Characklis 1990). Each of the experimental runs was modelled with the computer programme AQUASIM developed by EAWAG in Switzerland (Reichart 1994). AQUASIM is an interactive program used to identify and simulate aquatic systems. The biofilm model used in this program is based on the formulation described by Wanner and Reichert (1996).

Parameters can be determined by the estimation routine included in AQUASIM. However, in this study, a simultaneous optimization of all kinetic and stoichiometric parameters was not possible, due to the complexity of the biological model. Instead, the following procedure was used to obtain an estimates of the parameters. First, stoichiometric and kinetic parameters for the methanotrophs, and the maximum nitrification rate constant for methanotrophs, μ_{Nm}, were fitted. Then, parameters related to biomass hydrolysis, γ and k_H, were fitted from the measured polymer production, oxygen consumption, and biomass growth data. The biofilm detachment constant, det, was fitted according to the suspended biomass production and the biomass growth. The stoichiometric coefficient for ammonia assimilation, α_{NH_4}, was estimated by comparing the ammonia removal and nitrate production. Parameters related to the growth of nitrifiers and heterotrophs were those recommended in the IAWQ activated sludge model No. 2 (Henze et al. 1994). Based on these latter parameters, the ini-

tial volume fraction of nitrifiers, ϵ_N, was estimated according to the observed nitrification rate. The initial volume fraction of heterotrophs, ϵ_H, and the biodegradable biomass, ϵ_S, were estimated according to the degradation rate of soluble polymers measured in the reactor. Finally, the parameters estimates obtained with the procedure outlined above were used as initial condition to perform a simultaneous optimization of the mains parameters (listed in Table 4). Various initial conditions were tested to assess if the global optimum was reached.

Results and discussion

COD balance

A cumulative chemical oxygen demand (COD) balance was established for the entire biofilm growth for each experimental run (Figure 3). These balances are based on a combination of experimental data and conversion factors from the literature.

The graphs were plotted using a COD value for the attached biomass of 1 mg COD/mg dry biofilm (this is an average value of five analytical test for COD performed for each run). The detached active biomass was calculated from the protein measurement using a conversion factor of 2.8 mg cell COD/mg protein based on a theoretical cell composition of $C_5H_7NO_2$ and assuming a specific COD value of 1.4 mg COD/mg dry cell (McCarty 1972). The polymers were assumed to have a specific COD value of 1.07 mg COD/mg (same as glucose) and methane 4 mg COD/mg methane (theoretical value for total oxidation). The stoichiometry of the biofilm growth is compiled in Table 3. This cumulative COD balance shows a very high specific oxygen consumption ranging from 2.2 to 3.6 mg O_2/mg CH_4 degraded. The biomass yield that includes the production of both attached and the detached biomass ranges from 0.29 to 1.6 mg biomass/mg CH_4 degraded. It should be noted that the methane loading rate (MLR), defined as the amount of methane supplied to the bioreactor per surface area and per day, has a strong influence on these stoichiometric coefficients: a lower specific oxygen consumption and a higher biomass yield coefficient were observed for biofilm growth experiments performed with a small MLR (runs A–C) compared to conducted with a high MLR (runs D–F). Since runs A–C were performed under methane-limiting conditions, the high specific oxygen consumption observed in these runs may be the result of the endogenous respiration. The following observations were made regarding the attached biomass. The dry-weight density of the biofilm was lower when the biofilm was grown with a high MLR compared to the dry-weight density associated with growth on a low MLR. Similarly, the biofilm growth was affected by the MLR. For example, the biofilm thickness was less in run B compared with run F although the two runs were performed with the same mixed culture and the same amount of methane was degraded: the thickness reached a value of ca. 200 μm in run B and 900 μm in run E after 20 g of methane was degraded in both runs. Loosdrecht et al. (1996) discussed the influence of the substrate loading rate and shear stress on the biofilm structure. They postulated that under similar shear stress conditions, a low substrate loading rate leads to a smooth and dense biofilm with a small accumulation rate. A higher substrate loading rate will, in contrast, result in a porous and heterogeneous biofilm with lower density leading to a higher linear biofilm growth phase. This is in agreement with the results reported here. The production of soluble polymers (polysaccharides, Table 3) suggests that they account for a significant fraction of the total methane degraded and, thus, they cannot be neglected. Detailed measurements of the specific oxygen consumption showed that this value increased with an increasing biofilm thickness. For example in run A, this value increase from 3.1 to 3.7 mg O_2/mg CH_4 degraded. This increase in the oxygen consumption was the result of the growth of heterotrophs and nitrifiers in the biofilm. Heterotrophic biomass grew on soluble polymers which were formed by hydrolysis of dead biomass entrapped in the biofilm. Nitrifiers populations developed due to the presence of ammonia in the mineral medium (nitrate and nitrite were detected in the outlet of the reactor). Furthermore, COD balances obtained with a low hydraulic residence time (Run A–C) the cumulative COD at the outlet is generally higher than the cumulative methane COD in the inlet (except for run C). The difference is due to the oxygen demand required for the nitrification of the effluent. The nitrogen source involved in run C was mainly nitrate, which prevented the growth of nitrifying bacteria.

Modelling and estimation of kinetic parameters

A comparison between model and experimental data is shown in Figure 4 for run A. Table 4 summarises the stoichiometric and the kinetic parameters for each

Table 3. Stoichiometric data for six experimental biofilm growth

	A	B	C	D	E	F
Inlet CH_4 concentration (gm^{-3})	12–14	3.65[a]	12–16	6–10	3–6	4–8
Inlet O_2 concentration (gm^{-3})	35[a]	32[a]	7–8	13–16	15–22	16–21
CH_4 loading rate ($gm^{-2}\ d^{-1}$)	0.38	0.46	0.35	3.1	2.5	3.3
O_2 spec. cons. (mgO_2/mg CH_4)	3.47	3.59	3.55	2.72	2.71	2.28
Biomass spec. prod. (mg$X$/mg CH_4)*	0.53	0.29	0.42	1.11	1.20	1.65
Polymers spec. prod. (mg pol./mg CH_4)	0.108	n.m.	0.104	0.066	0.24	0.217

* It includes both the attached and the detached biomass.
[a] Average values. n.m: not measured.

Table 4. Kinetic and stoichiometric parameters used in the model. All data are expressed in COD units. The temperature was approximately 20 °C. (The nomenclature is in the appendix)

	A	B	C	D	E	F	Average
μ_m (d^{-1})	1.17 ± 0.2	1.1 ± 0.2	1.4 ± 0.2	2.49 ± 0.4	1.5 ± 0.3	2 ± 0.4	1.6 ± 0.5
μ_{Nm} (d^{-1})	0.001	0.01	0.01	0.3	0.3 ± 0.1	0.0001	–
b_m (d^{-1})	0.34 ± 0.06	0.35 ± 0.1	0.25 ± 0.1	0.29 ± 0.1	0.12 ± 0.03	0.085 ± 0.03	0.24 ± 0.1
Y_{CH_4} (gX/gS)	0.21 ± 0.03	0.18 ± 0.03	0.2 ± 0.02	0.19 ± 0.2	0.2 ± 0.02	0.22 ± 0.02	0.2 ± 0.014
$K_{S(CH_4)}$ (g/m^3)	0.08 ± 0.05	0.04 ± 0.03	0.48 ± 0.2	0.49 ± 0.2	0.1 ± 0.05	0.36 ± 0.2	0.26 ± 0.21
$K_{S(Sp)}$ (g/m^3)	6	6	8	4	4	4 4.9 ± 2.2	
k_H (d^{-1})	1.4 ± 0.8	1 ± 0.8	1 ± 0.5	0.07 ± 0.04	1.5 ± 1	1.71 ± 1	1.1 ± 0.58
α_{NH_4}	–	–	0.01	–	0.08 ± 0.03	0.05 ± 0.01	–
α	0.94 ± 0.3	0.8 ± 0.3	1.2 ± 0.3	1.1 ± 0.4	1 ± 0.3	1.28 ± 0.3	1.05 ± 0.18
γ	0	0.5	0.05	0.0	0.4 ± 0.2	0.22 ± 0.1	0.19 ± 0.22
det. (–)	0.1 ± 0.02	0.1 ± 0.03	0.15 ± 0.05	0.15 ± 0.05	0.5 ± 0.2	0.64 ± 0.1	0.27 ± 0.24

±: standard deviation.

experimental run. The standard deviations were estimated by fitting the model to the experimental data for each time series, as described in the previous section.

An individual standard deviation of 20–30% was found on average for most of the model parameters. The methane yield coefficient displayed the smallest standard deviation, whereas the standard deviation reached 50% for the half saturation constants and for the coefficients controlling the hydrolysis of biodegradable biomass. However, the relative standard deviation of the average values of the model parameters (last column in Table 4) is 50–100%, except for α and Y_{CH_4}.

The parameters listed in Table 4 are within the same order of magnitude for six independent experimental runs. A comparison with literature data (Table 5) showed that μ_m and $K_{S(CH_4)}$ are the parameters with the most significant variability when compared with the other parameters in this table. However, among the values listed, only those reported by Anderson and McCarty, (1994) are related to biofilms. These latter values are within the same range as those reported in our study. The maximum specific growth rate for methanotrophs, μ_m, is generally lower in runs A–C compared with runs D–F suggesting that two group of methanotrophic bacteria were involved. This could be due to different methane concentrations in the reactor during the runs: runs A–C were grown with a very low methane concentration (less than 0.1 mg/L in the reactor), whereas the methane concentration was in the range of 3–5 mg/L during runs D–F. Such a concentration, combined with a small hydraulic residence time, might have led to the selection of fast-growing methanotrophs in runs D–F which could only survive under high methane concentration conditions.

Minor modification of the heterotrophic growth parameters values recommended by Henze et al. (1994) was necessary. The yield coefficient, Y_H, was fixed to 0.5 gX/gS for all modelled runs, whereas the half-saturation constant, $K_{,mS(Sp)}$, had to be adjusted for each run (Table 4). These adjustments improved the modelling of the biofilm thickness, the polymer

Table 5. Kinetic and stoichiometric values reported in the literature. All data are expressed in COD units. (The nomenclature is in the appendix)

	μ_m (d^{-1})	b_m (d^{-1})	$K_{S(CH_4)}$ (g/m^3)	Y_{CH_4} (gX/gS)
This study	1.1–2.49	0.085–0.35	0.04–0.48	0.18–0.22
Anderson and McCarty (1994) Model simulation.	2.17	0.1	0.12	0.245
Broholm et al. (1993)	0.344	0.12	0.8	0.07
Others literature values (taken from Broholm et al. 1993)	4.4–4.6[a]		0.96–1.68[a,b,f]	0.27–0.39[c,d,e,g]
Oldenhuis et al. (1991) *Methylosinus Trichlosporium* OB3b	4.18		0.1	0.175[h]
Heijnen and Roels (1981) Survey of different pure cultures	0.96–8.16	0.33–0.4		0.12–0.19
Pedersen (1991) Run D, early growth phase	12.9	0.96	2	0.203
Pedersen (1991) Run D, "old" biofilm	2.71	0.624	1	0.175

[a] Ferenci et al. (1975).
[b] Harrison (1973).
[c] Harwood and Pirt (1972).
[d] Morigana et al. (1979).
[e] Whittenbury et al. (1970).
[f] Wilkinson and Harrison (1973).
[g] Wilkinson et al. (1974).
[h] Value assumed by the author.

production, and the oxygen consumption. A first-order expression governed the hydrolysis of biodegradable biomass, although the results suggest that the oxygen level in the reactor controls this process. For example, oxygen was limiting during run D, whereas in runs E and F, oxygen was in excess: the estimated hydrolysis constant, k_H, was ten times greater in runs E and F than in run D. The hydrolysis models proposed in the literature for activated sludge involve saturation kinetics and take the effect of oxygen into account (Henze et al. 1987; 1995). Although a similar model has been proposed for biofilms (Characklis et al. 1989), it was found to be inappropriate for this study as it was unable to simulate the polymer production (not shown). Furthermore, not enough data were available in this study to calibrate an advanced hydrolysis model. Consequently, a simple first-order expression was used.

Both nitrifiers and methanotrophs are responsible for nitrification in the biofilm. Model simulations indicated that nitrifiers populations developed in the biofilm several weeks after the reactor was inoculated (Figure 5). Consequently, runs D, E, and F were likely unable to support nitrifiers because the biofilm was grown for a maximum period of two weeks. In these cases, nitrification was accomplished by methanotrophs (Figures 4g and 4h). It is probably a cometabolic reaction resulting from the broad substrate specificity of methane monooxygenase (Bedard & Knowles 1989). It was observed, however, in agreement with other works (i.e., Whittenbury et al. 1970; Bedard & Knowles 1989; Hanson & Hanson 1996), that ammonia competitively inhibited the methane oxidation. The maximum nitrification rate constant for the methanotrophs, μ_{Nm}, was estimated to 0.3 d^{-1} in run A, and the half saturation constant and the yield coefficient were set to those values proposed for nitrifiers: Y_{NH_4} = 0.24 gX/gNH_4–N, $Ks_{(NH_4)}$ = 1 mg NH_4–N/L. The estimated μ_{Nm} is high because, according to Bedard and Knowles (1989), the maximum nitrification rate determined for methanotrophs is more than 20 times lower than one of the lowest rates reported for an ammonia oxidizer. The substrate inhibition constant was assumed to be the same as the ammonia half saturation constant, $Ks_{(NH_4)}$, namely 1 mg NH_4–N/L. Studies on ammonia inhibition with *Methyllococcus capsulatus* (Bath) showed that $Ks_{(NH4)}$ is pH dependant (Carlsen et al. 1991): an increase in pH from 6.2 to 7.7 leads to a decrease in the inhibition constant, K_I from 94 to 8 mg NH_4–N/L. Recently, Dunfield

and Knowles (1995) reported a value of K_I on the order of 0.11 mg NH_4–N/L in soil. They also reported that the inhibitory effect of ammonia was not only competitive, but could also be the result of the nitrite production. It should be noted that only runs E and F could provide a reasonable estimation of μ_{Nm} because ammonia consumption and nitrate production were monitored thoroughly in these experiments. Finally, the small μ_{Nm} observed in run F is probably the result of an inhibitory effect of *cis*-DCE which was supplied to the reactor during the entire biofilm growth. It is interesting to note that, despite the presence of *cis*-DCE (ca. 600 μg/L), neither the biofilm growth, nor the methane removal appear to be inhibited: only nitrification was inhibited.

Variability analysis

A sensitivity test was performed on the biological model. This involves altering various model parameters independently to assess how much these variations affect the modelled curves. Such a task can be performed automatically by AQUASIM provided that the standard deviation of each parameters is given. The parameters of interest were kinetic and stoichiometric parameters, substrate diffusivity, dry-weigh density, initial volume fraction of the methanotrophs, and the inert biomass in the biofilm. The standard deviations used for this test were those listed in Tables 2 and 4. A relative standard deviation of 20% was assumed for the biomass density, the diffusivity of the soluble components, the effective diffusivity of the substrates, as well as for the for the kinetic and stoichiometric parameters related to the nitrifiers and the heterotrophs. However, a variability of 50% was assumed for the half-saturation constant. The results, which are summarised in Table 6, indicate that the most crucial parameters are the biofilm characteristics, namely the biomass density, the initial biomass volume fractions, the detachment, and the initial biofilm thickness. The kinetic- and the stoichiometric parameters related to the methanotrophic bacteria also have a strong effect on the variability of the model, especially on the simulation of the methane and the oxygen consumption. The parameters controlling the hydrolysis of biodegradable biomass and the growth of heterotrophs strongly affect the modelling of the polymer production. The diffusivity of soluble components has a very small impact on the variability of the model.

The variability range of the model is shown in Figure 4. The envelope defined by dashed lines describes the maximum variation of the model using the parameters and their respective standard deviation. On average, the variability of the model ranges from 10 to 20% for modelling of methane degradation, oxygen consumption, and nitrification. However, the variability of the model is high with respect to the biofilm growth, as well as for the polymer production and the detachment.

Validation of the model

The model discussed in this paper was calibrated on six independent time series. However, before this model may be utilized to design treatment processes, it is important to assess its accuracy and its application range. For that purpose, the calibrated model was compared with another biofilm growth experiment found in the literature (Arvin 1991; Bilbo et al. 1992). Parameters were the average of those listed in Table 4. Only input parameters such as the liquid flow, the initial biofilm thickness, and the methane and the oxygen concentration at the reactor inlet were adjusted to match the experimental conditions of the mentioned biofilm growth experiment. Figure 6 shows the results of this comparison. The agreement of our model with the experimental data from the other study is fairly good. The biofilm thickness is overestimated. However, the experimental data are within the confidence interval defined by the model. Furthermore, the trend of the model is in agreement with the experimental data. Unfortunately, the model could not be validated with respect to the hydrolysis process and the detachment since the suspended solids and the polymer production were not reported in the other papers. However, these two components do not represent a significant fraction of the COD balance (Figure 3).

The huge confidence interval generated for the biofilm thickness denotes the lack of reliable data on the biofilm composition and structure, and the uncertainty regarding how this information should be integrated in the model. This is a subject of further research (Bishop and Rittmann 1995).

Implication of the results

Simulation of the profile of particulate components in the biofilm (Figure 5) shows that the proportion of methanotrophs is larger in a biofilm that grew for 10 days than in a biofilm 70 days old. This suggests that in practice, a bioreactor with a young biofilm

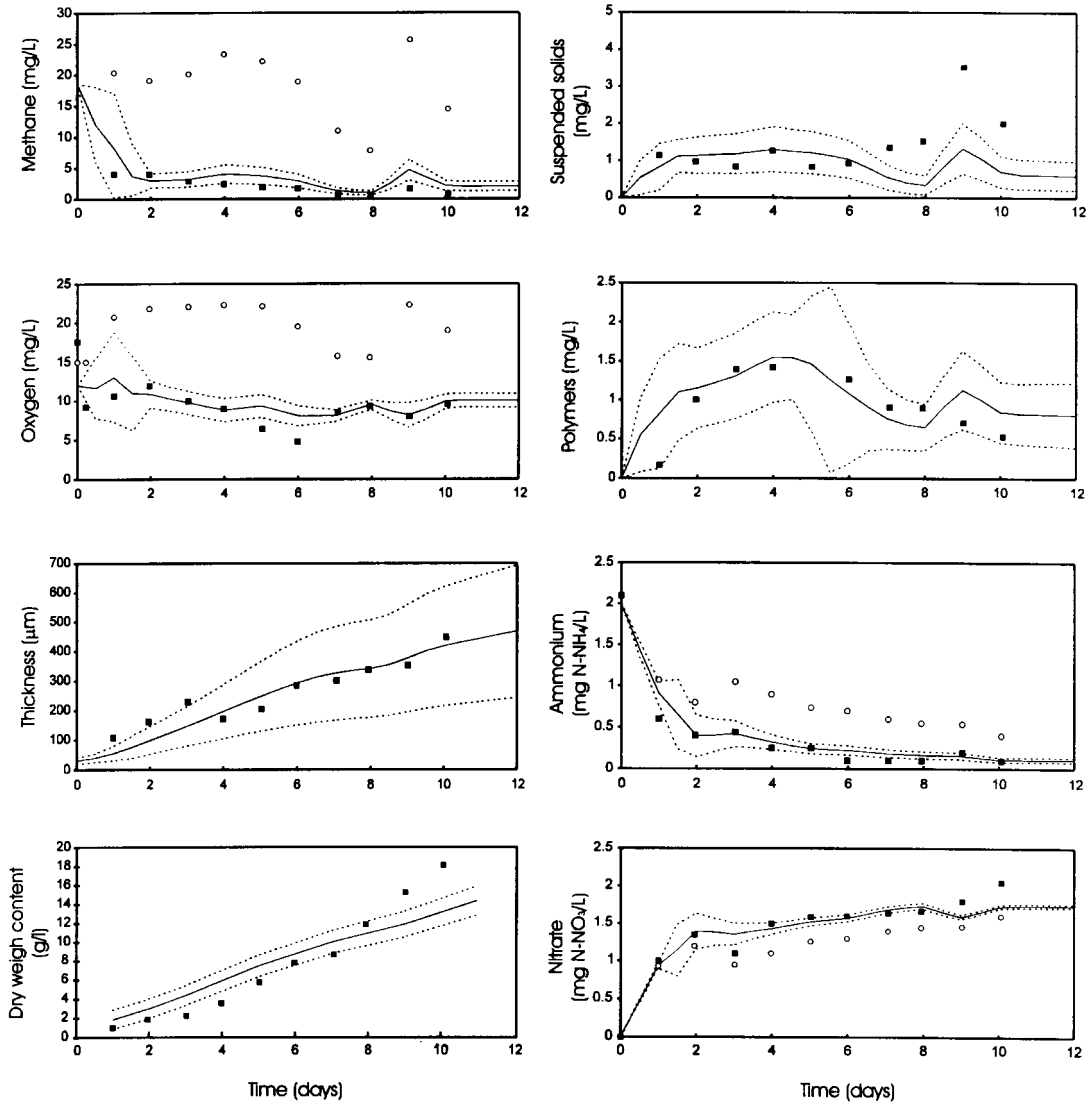

Figure 4. Comparison between model and experimental data (run E). All concentration units are expressed in COD units. Solid line: model. Dashed line: variability range of the model. Inlet concentration (○); outlet concentration (■).

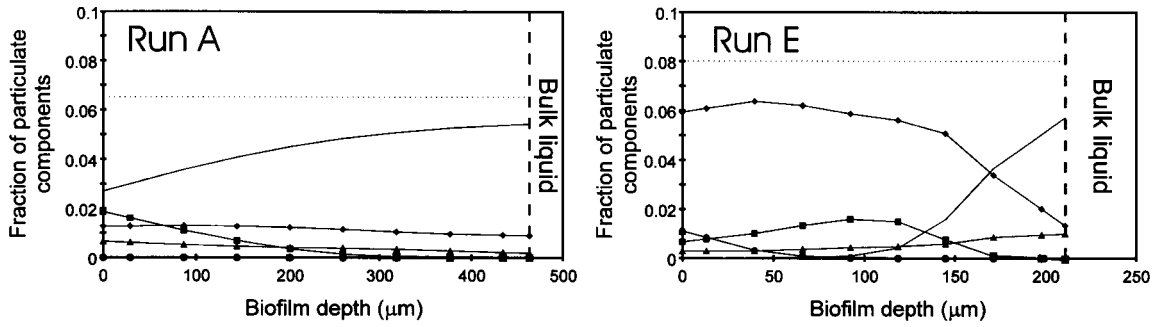

Figure 5. Profile of particulate component in runs A and E. Model simulation after 16 days for run A and after 70 days for runs E. (–): X_m; (●): X_N; (■): X_H; (▲): X_S; (♦): X_I; (. . .): X_t.

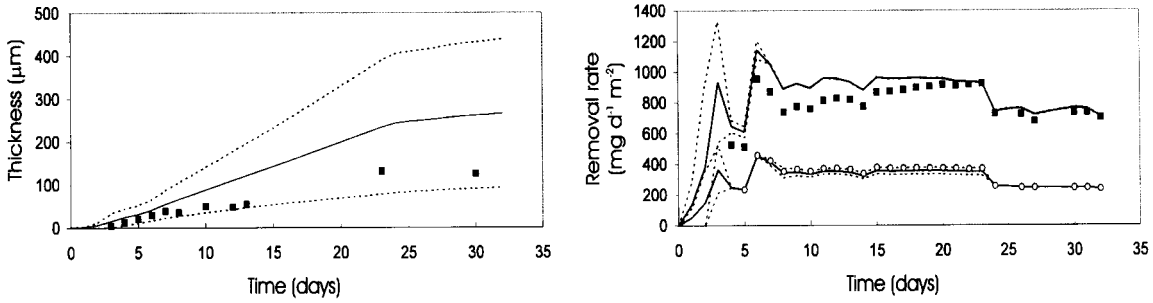

Figure 6. Verification of the model. Solid line: model. Dashed line: variability range of the model. Biofilm thickness (■) (left); methane (○) and oxygen (■) consumption rate (right). Methane Inlet concentration: 6–9 mg/L; Oxygen Inlet concentration: 32 mg/l; Flow 4.7 l/h. Experimental data from Arvin (1991).

will be more active (in terms of methane oxidation) than an old one. Also, comparing the specific oxygen consumption for runs A–C (runs carried out for 2–3 months) and runs D–E (runs carried out for 10–15 days) it is clear that the oxygen requirement is more significant in old biofilms than in new ones. This is the result of nitrifying and heterotrophic biomass development in the biofilm.

Consequently, providing an intermittent backflush to prevent biofilm ageing would not only improve bioreactor performance but would also optimized oxygen requirement in the bioreactor. Obviously, this is of practical interest for the treatment of CAHs-contaminated effluent because optimizing bioreactor aeration would minimize CAH emission into the atmosphere.

Despite the uncertainties regarding the biofilm structure and composition, this biological model is an useful tool for the design of on-site treatment processes for the biorestoration of CAH-contaminated aquifers. However, it should be born in mind that this model was calibrated using a laboratory scale bioreactor. In practice, a fixed-film bioreactor with high specific area such as a packed bed reactor would be used. This implies a different hydraulic behaviour which may affect the biofilm development. Nevertheless, the intrinsic biological reaction described in this work is expected to remain the same. For this reason, future work should concentrate on carrying out a similar study on a full scale fixed film methanotrophic bioreactor that treats CAH contaminated groundwater.

Conclusion

The analysis of six independent methanotrophic-biofilm growth campaigns indicates that nitrifiers and heterotrophic biomass develop in the biofilm although methane is used as the sole carbon source. The development of these ‘active biomasses’ and, thus, their influence on biofilm growth can be controlled by the composition of the mineral medium and the methane loading rate. Based on this study, a biological model is presented which can predict the biofilm growth, as well as the consumption rate of methane and oxygen by a methanotrophic biofilm. This is of practical interest with regard to the on-site treatment of CAH-contaminated groundwater. There exist, however, uncertainties regarding biofilm growth. This indicates that characterization of the biofilm (i.e., composition, biomass density, solid-volume fraction) is required in order to improve the accuracy of the model.

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Appendix

(Values in parenthesis are those recommended by Henze et al. (1995). All data except are expressed in COD units, unless stated otherwise.

Table 6. Sensitivity analysis. Effect of some parameters on the simulation of: the biofilm thickness (a); methane removal (b); oxygen consumption (c); polymers production (d); detachment (e) and nitrification (f). –: insignificant effect; + moderate effect; ++ significant effect; +++: very strong effect

	Thickness[a]	CH_4[b]	O_2[c]	Poly.[d]	Detach.[e]	Nitrif.[f]
μ_m (d^{-1})	++	+++	++/+++	++	+++	+++
μ_{Nm} (d^{-1})	+	-	+	–	–	+++
b_m(d^{-1})	+	+	+	+	+	+/++
Y_{CH_4} (gX/gS)	+	++	++	+	++	++
$KS(CH_4)$ (g/m^3)	–	+/++	–	–	+	+
k_H (d^{-1})	+	–	–	+	–	–
αNH_4	–	–	–	–	–	+++
α	+	+/++	+	+	+	+
γ	–	+/++	+	++	+	-
ρX_{act}	++	+++	++	++	++	+++
ρX_{in}	+	–	–	–	–	–
f	+	++	+	+	+	++
D_f	(1)+	(2)++	(2)+	(3)+	–	(4)++
ϵ_{I_ini}	++	++	+/–	–	–	+
ϵ_{m_ini}	+++	+++	+++	+++	+++	+++
L_{f_ini}	++	+++	++/+++	++	++	+++
det.	+++	+++	++/+++	++	+++	+++
Nitrif. Par.	+	+	+	-	+	++
Hetero. Par.	+	+	+	+++	+	+

(1) D_f for CH_4 and polymers only; (2): D_f for CH_4 only; (3) D_f for polymers; (4): D_f for NH_4 only; the nomenclature is in the appendix. Nitrifi. Par and Hetero. Par: kinetic and stoichiometric parameters related to nitrifiers and heterotrophs, respectively.

Soluble components:

CH_4: methane (mg/L)
O_2: oxygen (mg/L)
NH_4: ammonia (mg N/L)
NO_3: nitrate (mg N/L)
S_P: soluble polymers (mg/L)

Other parameters:

Q_W: water flow (m^3/d)
L_{f-ini}: initial biofilm thickness (μm)
D_f: diffusion coefficient of soluble components in water (m^2/d)
f: ratio of diffusivity in the biofilm and in water (0.8)
det: detachment constant (–)

Particulate components:

X_m: methanotrophs (g/m^3)
X_N: nitrifiers (g/m^3)
X_H: heterotrophs (g/m^3)
X_S: biodegradable biomass (g/m^3)
X_I: inert biomass (g/m^3)
X_t: biofilm dry weight density; (g/m^3 of wet biofilm)

$$X_t = X_m + X_N + X_H + X_S + X_I$$

X_{act}: active biomass dry weight density; (g/m^3 of wet biofilm)

$$X_{act} = X_m + X_N + X_H + X_S$$

ρX_{act}: active biomass density (g/m^3);

$$\rho X_{act} = \frac{X_{act}}{\epsilon_t}$$

ρX_{in}: inactive biomass density (g/m^3)

$$\rho X_{\text{in}} = \frac{Xi}{\epsilon_t}$$

ϵ_t: total solid volume fraction (–)

ϵ_{m}, ϵ_{N}, ϵ_{H}, ϵ_{I}, ϵ_{S}: solid volume fraction of methanotroph, nitrifiers, heterotroph, inert and biodegradable in the biofilm (–)

$\epsilon_{\text{m\_ini}}$, $\epsilon_{\text{N\_ini}}$, $\epsilon_{\text{H\_ini}}$, $\epsilon_{\text{I\_ini}}$, $\epsilon_{\text{S\_ini}}$: initial solid volume fraction of methanotroph, nitrifiers, heterotroph, inert and biodegradable in the biofilm (–)

Kinetic parameters:

μ_{m}: maximum specific growth rate for methanotrophs (d^{-1})

μ_{Nm}: maximum specific nitrification rate for methanotrophs (d^{-1})

μ_{H}: maximum specific growth rate for heterotrophs (6 d^{-1})

μ_{N}: maximum specific growth rate for nitrifiers (1 d^{-1})

b_{m}: maximum decay rate for methanotrophs (d^{-1})

b_{H}: maximum decay rate for heterotrophs (0.4 d^{-1})

b_{N}: maximum decay rate for nitrifiers (0.15 d^{-1})

k_{H}: hydrolysis rate for X_S (d^{-1})

$K_{\text{S(CH}_4\text{)}}$ methane half-saturation constant (mg/L)

$K_{\text{S(NH}_4\text{)}}$ ammonia half-saturation constant (1 mg NH4-N/L)

$K_{\text{S(O}_2\text{)}}$ oxygen half-saturation constant (0.2 mg/L)

$K_{\text{S(O}_2-\text{aut)}}$ oxygen half-saturation constant for nitrifiers (0.5 mg/L)

$K_{\text{S(Sp)}}$ polymers half-saturation constant (mg/L)

Stoichiometric parameters:

Y_{CH_4}: Yield for methanotrophs (gX_{m}/gCH$_4$)

Y_{NH_4}: Yield for nitrifiers and for methanotrophs growing on NH$_4$ (0.24 gX_{N}/gNH$_4$–N)

Y_{H}: Yield for heterotrophs (0.5 gX_{H}/gS_{P})

α: EPS formation coefficient (–)

α_{NH_4}: mass of nitrogen per mass of COD in biomass (–)

β: fraction of biomass leading to inert biomass (0.1)

γ: S_{P} formation coefficient (–)

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