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Title:
Modeling planktonic and biofilm growth of a monoculture (*P. fluorescens*) in drinking water

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Summary

A simple monoculture-and-single-substrate approach was used for understanding and modeling planktonic and biofilm growth of bacteria in drinking water treatment and distribution systems. For the monoculture, *Pseudomonas fluorescens* P-17 was chosen, and for the single substrate, acetate was chosen. Planktonic growth was followed in batch cultures at different temperatures and different substrate concentrations, using fluorescent staining and flow cytometric enumeration of the grown cells. Biofilm growth was studied in a Propella™ biofilm reactor using fluorescent staining and epi-fluorescence microscopy for enumeration of the grown cells. The data from these two separate sets of experiments were subsequently combined in a model developed by RTU. Initial runs with the model did not manage to predict accurately the experimental results which were observed. One of the main conclusions was that the yield for biofilm growth should be lower than that for planktonic growth. However, specific and consistent data on the differences between planktonic and biofilm growth in drinking water is not generally available.

Contents

Abbreviations

AOC	assimilable organic carbon without addition of inorganic nutrients
BDOC	biologically degradable organic carbon
DAPI	4′,6-Diamidino-2-Phenylindole
DOC	dissolved organic carbon (< 0.45 µm filtrate)
EAWAG	Swiss Federal Institute for Aquatic Science and Technology
HPC	heterotrophic plate count
HS	humic substances
IMSL	software library used in computer programming language FORTRAN
PMWR	perfectly mixed with water recycling
RTU	Riga Technical University
TOC	total organic carbon
TBN	total bacterial number
TM	trade mark

Introduction

Although there are several multi-species bacterial regrowth models available, of which the two best known are the SANCHO model (Servais et al, 1995) and the PICCOBIO model (Dukan et al, 1996), they are not widely used by the water industry. One of the reasons is that they are proprietary objects, which implies that they are not useful for further improvements by third parties. Secondly, there is urgent necessity for a powerful hydraulic model which can be operated due to diurnal variations in water demand. Thirdly, there is lack of wide verification of models against empirical research data. There have been several attempts to verify the above-mentioned models against full-scale data (Laurent et al 2005; Piriou et al 1998). Overall the verification of the models in full-scale is difficult to accomplish due to the influences of many factors not included the models (e.g. corrosions products, particles) and problems with sampling (e.g. obtaining biofilm samples from the buried pipes). Therefore, the constants used in the models usually are not experimentally justified. The aim of this study was to verify a mathematical model of bacterial regrowth with *Pseudomonas fluorescencs* P-17 in a laboratory-scale biofilm reactor using growth coefficients derived from batch experiments.

To simplify the approach, we have looked specifically at the growth of a single bacterial strain on easily assimilable organic carbon (AOC). The purpose was to combine planktonic growth knowledge from Eawag with the biofilm growth knowledge from RTU, in an effort to optimize modeling of bacterial growth in drinking water distribution systems.

1 Methodology

1.1 Strain and preparation

Pseudomonas fluorescens P-17, stored at -20 °C until use, was pre-cultured on solid growth media (R2A agar) at either 30 °C (Eawag) or 20°C (RTU). A higher incubation temperature (30 °C) was chosen for batch experiments (Eawag) to shorten the strain cultivation period. Liquid pre-cultures for the batch growth experiments were made in sterile water (see below).

1.2 Carbon source and concentrations

Acetate was chosen as the carbon source, based on available literature (Hammes and Egli, 2005; van der Kooj, 2002). Acetate concentrations of 0, 0.2 and 1 mg-C L^{-1} were selected for batch experiments, while the biofilm experiments employed a concentration of 1 mg-C L^{-1}. After preliminary yield experiments, it was decided to lower the maximum carbon concentration to 0.5 mg-C L^{-1} for the planktonic batch growth experiments.

1.3 Water matrix

For the planktonic growth data, Eawag has tested various combinations of synthetic growth media. However, in the end the best (and most easily reproducible) result was obtained with pasteurised (see below) commercially available bottled mineral water (Evian, Danone, France). Bottled mineral water was used due to the low organic carbon content (Servais et al., 1989). Figure 1 shows a liquid chromatography organic carbon detection (LC-OCD) fractionation of an Evian sample, with the resulting TOC measured at 80 - 100 µg L^{-1}.

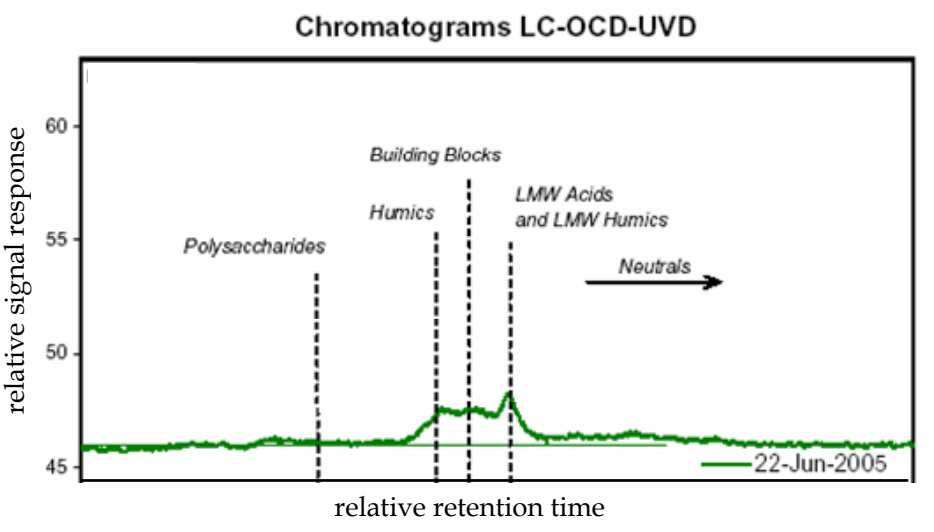

Figure 1. LC-OCD fractionation of bottled mineral water (Evian) with a TOC concentration of 80 – 100 µg L^{-1} (n = 3). (Data courtesy of Dr. S. Meylan, Eawag)

The bottled mineral water was supplemented with a buffer and trace element solution (Ihsen & Egli, 2004) which was relative to the concentrations of acetate-C to be added (Table 1).

Table 1. Composition of the buffer and trace element solution which was added to the bottled mineral water to ensure that carbon remains the growth limiting compound (adapted from Ihsen and Egli, 2004)

Buffer	**Per Liter**
$Na_2HPO_4.2H_2O$	12.8 g
KH_2PO_4	3 g
$(NH_4)_2SO_4$	1.77 g
Trace Substances	**per Liter**
$CaCO_3$	8 g
$FeCl_3.6H_2O$	7.74 g
$MnCl_2.4H_2O$	1.15 g
$CuSO_4.5H_2O$	0.146 g
$CoCl_2.6H_2O$	0.130 g
ZnO	0.400 g
H_3BO_3	0.124 g
$EDTA.Na_4.2H_2O$	79.2 g
$MgCl_2.6H_2O$	13.42 g
$Na_2MoO_4.2H_2O$	1.04 g

Note that the buffer and trace element solutions are mixed separately. Before use, mix 10 mL of buffer with 100 uL of the trace element solution (1:100). This combination was **designed (full strength) for 4 g acetate-C L^{-1}** (Ihsen and Egli, 2004). For **10 mg acetate-C L^{-1}**, add 2500 uL of the combined buffer to 1L pasteurized medium.

Figure 2 shows the need to add the buffer and trace element solution, as demonstrated with a natural community and acetate as carbon substrate. This experiment was done with a simple AOC-like batch growth test where different concentrations of acetate-carbon were added to Evian directly, and the bottles were incubated at 30 °C (Hammes and Egli, 2005). Growth of the indigenous bacteria (natural microbial community) in Evian on the acetate carbon was followed with flow cytometry (FCM). From this data it is clear that the bottled mineral water had a limiting factor (which was not carbon), which effectively restricted growth at a carbon concentration of about 200 µg L^{-1} upwards.

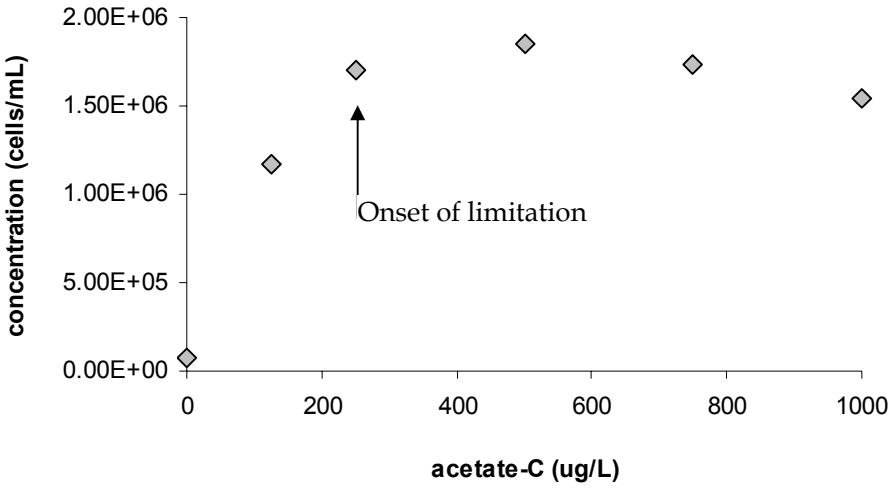

Figure 2. Net-growth of the indigenous natural microbial community of bottled mineral water (Evian) on additional acetate-carbon (0 – 1000 µg L^{-1}) (n = 3) at 30 °C after 3 days.

For the biofilm growth data, sterile ultra pure water (Elga PureLab Ultra, Veolia Water Ltd., UK) supplemented with inorganic nutrients (Miettinen et al., 1999) and 1 mg-C/L acetate was used (Table 2.). Due to the large volumes of water required for the biofilm reactors, the use of bottled mineral water (similar to the batch growth experiments) was deemed impractical.

Table 2. Composition of the trace element solution which was added to the sterile ultra pure water (Elga) to ensure that carbon remains the growth limiting compound

Buffer	Per Liter
$(NH_4)_2SO_4$	4.55 g
KH_2PO_4	0.2 g
$MgSO_4.7H_2O$	0.1 g
$CaCl_2.2H_2O$	0.1 g
NaCl	0.2 g

1.4 Sterilization procedure

Based on ongoing research at Eawag, it has been decided that filter sterilization alone cannot be used (Wang et al., 2007). For work with the monoculture, the medium was therefore pasteurized (30 min., 60 °C) in assimilable organic carbon (AOC) free glass bottles followed by sterile filtration (0.22 µm) with a syringe filter which was pre-washed with at least 150 mL nanopure water. The purpose of the filtration step was to remove any inorganic precipitates that may have formed during the pasteurization step, and which may interfere with the FCM measurements. The effectiveness of

the sterilization treatment was in all experiments controlled with a sterile non-inoculated control.

1.5 Incubation temperature

For the planktonic growth experiments, the temperature was kept at 18 °C, and for the biofilm growth experiments, the temperature was kept at 20.1°C. Eawag has furthermore opted to include and experimental set at 30 °C for comparison with other studies from this group (Hammes and Egli, 2005; Vital et al., 2007).

1.6 Cell enumeration

The total cell concentrations during the planktonic growth experiments were measured with flow cytometry (FCM) coupled with SYBR Green I staining as described previously (Hammes & Egli, 2005, Hammes et al., 2007; Vital et al., 2007). The total cell concentrations during biofilm growth experiments were measured using an epifluorescence microscope (Leica DM LB, Leica Microsystems GmbH, Wetzlar, Germany) equipped with a 50-W mercury lamp at magnification of 1000× after staining with 4',6-Diamidino-2-Phenylindole (DAPI) (Brunk, 1979).

1.7 Substrate measurements

The concentration of dissolved organic carbon (DOC) was determined using a Shimadzu 5000 A TOC analyzer (Shimadzu Corporation, Kyoto, Japan). The concentration of AOC was determined using a batch growth assay described previously (Hammes et al., 2006; Hammes & Egli, 2005). In short, 0.22 µm filtered water samples (15 mL) were inoculated with 10 µL (1×10^4 cells/mL final concentration) of a bacterial AOC-test community. These suspensions were then incubated at 30°C for 72 h (until stationary phase was reached) and the resulting growth was measured by FCM. AOC (µg/L) was estimated from cell concentrations (cells/mL) using a theoretical conversion factor (Hammes et al., 2006; Vital et al., 2007):

$$AOC\,[\mu gC/L] = \frac{(net\ grown\ cells)/L}{1\times10^7\ cells/(\mu gC)}$$

The biologically degradable organic carbon (BDOC) measurements were performed on fixed biofilm according to Servais et al. (1987), Ribas et al. (1991) and Frias et al. (1992), using two glass columns (H=29 cm, ø=2.5 cm, Chromaflex, USA) filled with 200 g of glass carrier in the spherical form (Assistant, Germany) having a diameter of 6 mm and the surface area of 3.76 cm^2/g. The sample was continuously pumped upward by a peristaltic pump (Masterflex L/S, Cole-Parmer, USA). An optimal flow of 3-5 mL/min was used, representing a compromise between the required retention time (1h/ column) and rapidness of the assay. The biofilm was cultured by incubation in a mixture of one-third river water, filtered through 1.2 µm pore diameter membrane filters and two-thirds drinking water (after biofiltration). To adapt the microorganisms, the carriers were stored for 16 weeks at 21±2°C in the dark (weekly water exchange was applied). The BDOC value was calculated as the difference (ΔDOC) between the inlet DOC and the outlet DOC of the second column (after 2h).

1.8 Experimental device and sample collection: Batch experiments

The planktonic batch growth experiments were done in 20 mL AOC-free glass vials with complete sealing Teflon coated caps, as previously described (Vital et al., 2007). The medium was inoculated at a final cell concentration of 5 x 10^3 cells/mL and incubated at the chosen temperature (18 °C and 30 °C) without shaking. Samples (1 mL) were taken at regular intervals (1 – 3h) during the growth curve and analysed with flow cytometry as described above.

1.9 Experimental device and sample collection: Biofilm experiments

The biofilm growth was performed in the Propella™ reactor (Xenard, Mechanique de Precision, Seichamps, France) at controlled conditions (temperature, 20.1±1.0°C; water retention time, 24 h; flow rate, 0.25 m s^{-1}; no chlorine) to simulate the drinking water distribution system. Propella™ is a perfectly mixed reactor with water recycling (PMWR) which is made of a 500 mm long and 100 mm in diameter water distribution pipe made of stainless steel with approximate volume of 2.23 L and inner surface area of 1604 cm^2. The reactor was continuously supplied with synthetic water (see above) and inoculum. To exclude bacteriological growth glass bottle containing inoculum was kept in the refrigerator (4°C). The water velocity in reactor was controlled by a marine propeller forcing the water along the inner cylinder and producing a flow parallel to the pipe wall. Into the inner surface of the pipe 20 coupons made of stainless steel (1.7 cm^2) were inserted, which were then used for analyzing of the biofilm on the pipe wall. The coupons and bulk water sample from the outlet were collected daily. For the biofilm analyses, coupons were aseptically removed from the reactor and put in 30 mL of sterile ultra pure water. Adhered cells were removed by a gentle sonication (Cole Parmer, USA, 2 min., 20 µA, 22 kHz, diameter 3 mm) and the total volume of bacterial suspension was filtered as a duplicate sample. The total bacteria number (TBN) was determined by counting at lest all DAPI stained cells with in randomly chosen microscopic viewing fields delineated by the eyepiece micrometer. Minimum of 300 bacteria were counted or as many viewing fields as were required to provide the coefficient of variation of <30%. For image capture a video camera (Leica DC 100) was used and the data was processed using a software (Image Pro Plus 4.5.1, Media Cybernetic, Inc., Silver Spring, USA).

1.10 Mathematical model

Biofilm regrowth in a mathematical model was modelled using the main assumptions, equations and coefficients proposed by Zhang et al. (2004) for straight and sufficiently long pipes. In this model it was assumed that calculated concentrations of bacteria are measured as TBN while Zhang et al. (2004) used the approach that bacterial concentrations are measured as HPC. To verify the coefficients in lab-scale conditions, a mathematical model using exactly the same equations (see below) was written for the biofilm reactor Propella™.

A system of equations subject to initial and boundary conditions was solved numerically by means of IMSL routine MOLCH, which solves a system of partial differential equations using the method of lines. The solution is represented by cubic Hermite polynomials.

The model developed by Zhang et al. (2004) is valid for straight and long pipes (or straight and long pipe segments) where the size of the pipe in the radial direction is much smaller than the size of the pipe in the longitudinal direction. As a result, Zhang et al. (2004) neglected the dependence of the solution on the radial coordinate. There are two major differences between Zhang's model and the model used for the Propella™. First, the characteristic size of the Propella™ reactor in the radial direction is comparable to that in the longitudinal direction. Second, water is circulating in the PropellaTM reactor while, in Zhang's model, water passes only once through the pipe (or system of pipes). The modelling approach used for Propella™ is briefly described below.

It was clear that the inclusion of terms depending on the radial coordinate would certainly improve Zhang's model. However, keeping in mind that (1) there are many coefficients in the Zhang's model with only approximately known values and (2) the addition of the radial coordinate would increase the number of coefficients whose values may be not known at all, we decided to neglect the dependence of the flow characteristics on the radial coordinate. Thus, a balance was sought between the complexity of a model and its ability to reproduce experimental data with minimum required empirical information. Of course, the validity of this assumption can only be tested when more advanced models become available.

In order to take into account water recirculation in the Propella™ reactor, boundary and initial conditions were modified. Suppose that the length of the Propella™ reactor is L so that the longitudinal coordinate varies from $x = 0$ to $x = L$. During the first run the boundary conditions at $x = 0$ are fixed, all the functions were specified at certain level. The boundary conditions from Zhang's model were used. The derivative of all functions at $x = L$ is assumed to be zero. The initial conditions have also been specified by Zhang et al. (2004). After the first run the solution at all grid points becomes available. This solution is considered as initial condition for the next run. In addition, the conditions at $x = 0$ for the next run are assumed to be equal to the conditions at $x = L$ from the previous run. The procedure is repeated many times (this is how re-circulation is modelled for Propella™) to get water quality variables renewed every 24 hours while water detention time in the reactor could be changed.

The input values for bacterial regrowth simulation were water temperature, duration time of the simulation, constant velocity, constant diameter of pipe, TBN in bulk water (cells/mL) and in biofilm (cells/cm^2), BDOC, mg/L and the residual chlorine concentration (mg/L).

To describe growth dynamic of bulk and biofilm bacteria Monod kinetic is used (Vadstein, 2000). The system of four equations where water temperature, concentration of chlorine and BDOC are assumed to control growth rate, is as follows:

$$\mu_b = \begin{cases} \mu_{\max,b}\left(\frac{S}{S+K_s}\right)\exp\left[-\frac{Cl_2 - Cl_{2,tb}}{Cl_{2,c}}\right]\exp\left[-\left(\frac{T_{opt}-T}{T_{opt}-T_i}\right)^2\right], if\_Cl_2 > Cl_{2,tb} \\ \mu_{\max,b}\left(\frac{S}{S+K_s}\right)\exp\left[-\left(\frac{T_{opt}-T}{T_{opt}-T_i}\right)^2\right], if\_Cl_2 \le Cl_{2,tb} \end{cases}$$

$$\mu_a = \begin{cases} \mu_{\max,a}\left(\frac{S}{S+K_s}\right)\exp\left[-\frac{Cl_2 - Cl_{2,ta}}{Cl_{2,c}}\right]\exp\left[-\left(\frac{T_{opt}-T}{T_{opt}-T_i}\right)^2\right], if\_Cl_2 > Cl_{2,ta} \\ \mu_{\max,a}\left(\frac{S}{S+K_s}\right)\exp\left[-\left(\frac{T_{opt}-T}{T_{opt}-T_i}\right)^2\right], if\_Cl_2 \le Cl_{2,ta} \end{cases}$$

where $\mu_{max,\ b}$ = maximum growth rate of planktonic bacteria; $\mu_{max,\ a}$ = maximum growth rate of bacteria in biofilm; S = substrate concentration measured as BDOC; K_s = half-saturation constant of substrate uptake (substrate concentration where growth rate is half of maximum); T_{opt} = optimal temperature for bacterial activity; T_i = temperature dependent shape parameter, which is assumed to be a significant in bacteria growth; T = in situ temperature of water; $Cl_{2,t}$ = threshold above which chlorine affects bacterial activity ($Cl_{2,ta}$ will be greater than $Cl_{2,tb}$ because biofilms are more resistant to inactivation by chlorine); $Cl_{2,c}$ = characteristic chlorine concentration defining the rate of decreases of bacterial activity with increasing chlorine concentration (when $Cl_2 > Cl_{2,t}$, μ_b decrease exponentially due to chlorine concentration, but when $Cl_2 \le Cl_{2,t}$, μ_b was not depend on concentration of chlorine); Cl_2 = in situ residual chlorine concentration.

The material balance for planktonic bacteria (X_b) is described by the equation:

$$\frac{\partial X_b}{\partial t} = -v\frac{\partial X_b}{\partial X} + D\frac{\partial^2 X_b}{\partial X^2} + \mu_b X_b + K_{det} X_a \frac{v}{R_h} - k_d X_b - k_{dep} X_b \qquad (1)$$

where v = flow velocity, D = dispersion coefficient, k_{det} = first-order kinetic constant for detachment, R_h = hydraulic radius, k_d = bacterial mortality rate, k_{dep} = first-order kinetic constant for deposition, X_a = biofilm bacteria.

Unlike planktonic bacteria, the material balance to describe biofilm bacteria does not depend on transport of water in the bulk flow. The descriptions of growth rate and mortality rate of biofilm bacteria are similar to those of planktonic bacteria, except $Cl_{2,t}$ (see above). The material balance for biofilm bacteria (X_a) is described as follows:

$$\frac{\partial X_a}{\partial t} = \mu_a X_a - k_{det} v X_a - k_d X_a + k_{dep} R_h X_b \qquad (2)$$

The material balance to account for loss of substrate from bulk water includes substrate utilization by both the planktonic and biofilm bacteria expressed as:

$$\frac{\partial S}{\partial t} = -v\frac{\partial S}{\partial X} + D\frac{\partial^2 S}{\partial X^2} - \frac{1}{Y_g \beta}\left(\mu_a \frac{X_a}{R_h} + \mu_b X_b\right), \qquad (3)$$

where Y_g = growth yield coefficient of bacteria; β = number of bacteria that are produced for each milligram of organic carbon in cell biomass.

The resulting material balance for chlorine is not described there because during this experiment we didn't use chlorine.

The initial and boundary conditions are:

$$X_a(0,t) = 0\ cells/cm^2,\ \ \frac{\partial X_a(1,t)}{\partial x} = 0,\ \ X_a(x,0) = 0$$

$$X_b(0,t) = 28600\ cells/mL,\ \ \frac{\partial X_b(1,t)}{\partial x} = 0,\ \ X_b(x,0) = 0$$

$$Cl_2(0,t) = 0\ mg/L,\ \ \frac{\partial Cl_2(1,t)}{\partial x} = 0,\ \ Cl_2(x,0) = 0$$

$$S(0,t) = 1.164\ mg/L,\ \ \frac{\partial S(1,t)}{\partial x} = 0,\ \ S(x,0) = 0$$

Values of all coefficients used in the bacterial regrowth model simulations are listed in Zhang et al. (2004).

After biofilm growth simulation in the model, biofilm and planktonic bacteria concentration within the time scale continuously were recorded in MS Excel worksheet as output data. The simulation generated 12 data points every hour, from which the average value of 1 day was calculated and plotted in figure.

Computer simulations were done both using coefficients derived from the batch experiments and those previously reported by Zhang et al. (2004) in order to fit for experimental data in Propella™ reactor. The experimental results from the batch experiments were used to calculate Monod half saturation coefficient K_s of *P. fluorescens* P17 with BiotechSIM software (http://www.biotechlab.net).

1.11 Statistical analyses

The data from experimental devices and data of biofilm regrowth model simulations were tested with the Wilcoxon test for matched pairs (Fowler et al., 2004).

2 Results and Discussion

2.1 Effect of buffer and trace element solution on the growth of bacteria in bottled mineral water

In a first experiment, it was investigated whether the bottled mineral water (Evian) is limited in terms of any inorganic nutrients (N, P) or trace elements. We have demonstrated that growth limitation occurs at carbon concentrations in the range of 200 mg-C L^{-1} (Figure 2). We therefore compiled a mineral buffer and trace element solution as described in Ihssen and Egli (2004) (Table 1), and applied it to the water in a working solution relative to 10 mg-C L^{-1}. We henceforth performed a simple AOC-like batch growth test where different concentrations of acetate-carbon was added to amended Evian directly, and the bottles were incubated at 30 °C (Hammes and Egli, 2005). Growth of the indigenous bacteria in Evian on the acetate carbon was followed with flow cytometry (FCM).

The results showed that the addition of mineral buffer and trace elements indeed had the desired impact, in that no restriction on the growth was further observed (outside of carbon limitation). The natural bacteria produced a crop proportional to the different carbon concentrations which were added to the water (Figure 3). The natural community displayed a yield of about 1 x 10^7 cells per ug C, similar to the theoretically proposed yield of natural bacteria on AOC (Hammes et al., 2006; 2007; Vital et al., 2007; Van der Kooij, 2002).

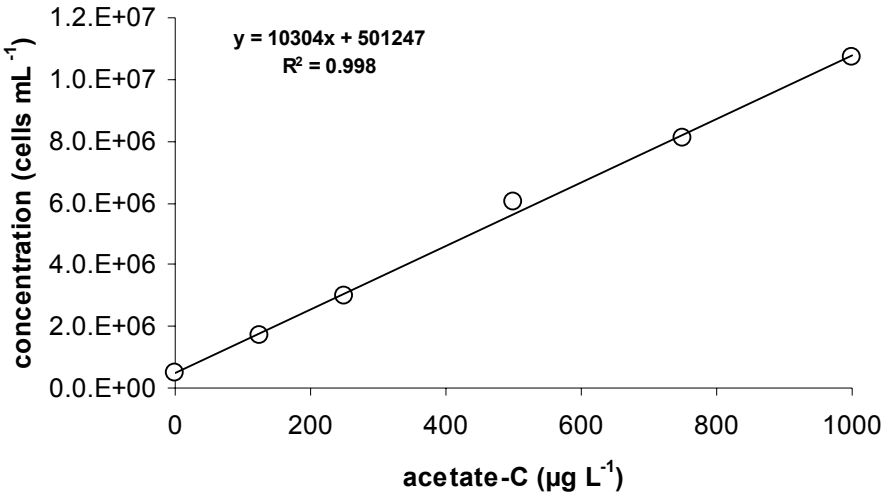

Figure 3. Net-growth of the indigenous natural microbial community of bottled mineral water (Evian) amended with a buffer and trace element solution on additional acetate-carbon (0 – 1000 µg L^{-1}) at 30 °C after 3 days.

2.2 Yield of P17 on acetate at 30 °C relative to a natural microbial community

We hence devised a sterilization (pasteurization) protocol (see Methodology section) and subsequently tested the yield of *P. fluorescens* P-17 compared to that of a natural community (AOC inoculum, see Vital et al. (2007)) when grown on acetate carbon in pasteurized Evian amended with the buffer and trace element solution (Figure 4 and 5).

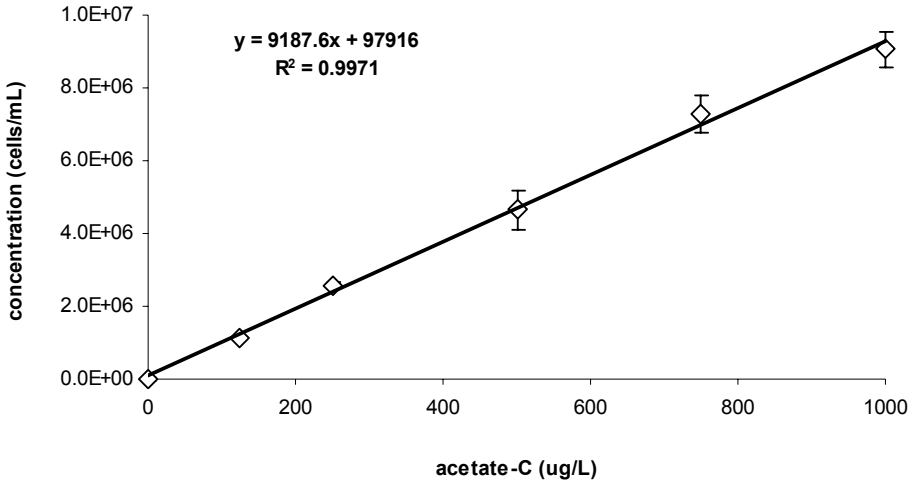

Figure 4. Net-growth of the indigenous natural microbial community AOC-test inoculum (Hammes and Egli, 2005) in pasteurized Evian amended with a buffer and trace element solution on additional acetate-carbon (0 – 1000 µg L^{-1}) at 30 °C after 3 days.

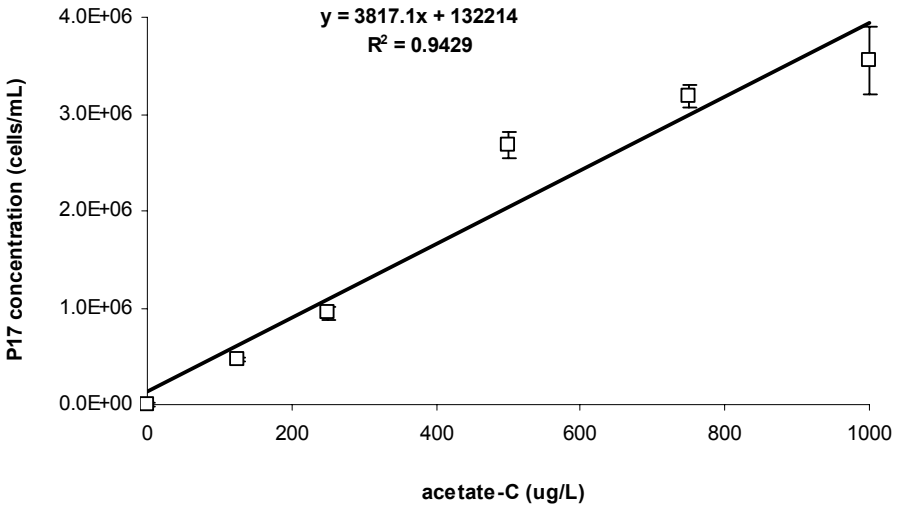

Figure 5. Net-growth of *P. fluorescens* P17 in pasteurized mineral water (Evian) amended with a buffer and trace element solution on additional acetate-carbon (0 – 1000 µg L^{-1}) at 30 °C for 3 days.

Note that we opted on purpose for a pasteurization protocol since it has been shown in our laboratory (Eawag) that a large fraction of indigenous drinking water bacteria can easily pass through sterile grade 0.1 – 0.22 µm pore size filters (Wang et al., 2007). The purpose of using the natural community was to see whether the pasteurization step had any adverse influence on the yield (compared to Figure 3). The natural community displayed the same yield in the pasteurized water as in the non-pasteurized water (Figure 3 and 4), suggesting no dramatic impact from the sterilization treatment. The pure culture produced a significant lower yield than the natural community (c.a. 4 x 10^6 cells per µg C) which is slightly below the expected value of 4.6 x 10^6 cells per µg (Greenberg et al., 1993) (Figure 5). The latter may, however, have been influenced by the high incubation temperature (30 °C), since *P. fluorescens* P-17 grows presumably optimal at 22 °C (LeChevallier et al., 1993). It has been shown before that higher specific growth rates could lead to higher cell yields (Vital et al., 2007). Due to the feeling that *P. fluorescens* P-17 might show some kinetic limitation at carbon concentrations above 500 µg L^{-1}, we decided to keep this as the maximum carbon concentration for the growth curve experiments. Anyhow, 500 µg L^{-1} easily available organic carbon, such as acetate, is extremely high in the context of drinking water.

2.3 Batch growth curves of P17 at 30 °C on acetate

Four batch growth curves were done at 2 different temperatures (18 °C and 30 °C) and 2 different carbon concentrations (200 and 500 µg L^{-1}) using acetate-carbon as substrate and *P. fluorescens* P-17 as the test organism. At 30 °C, a considerable lag phase was recorded before exponential growth phase started (Figure 6). A maximum specific growth rate of 0.14 h^{-1} was recorded for both carbon concentrations (Figure 7A and 7B). Notably, the yield in this experiment was a bit lower than the yield recorded previously at 30 °C.

Note that the maximum specific growth rate (μ_{max}) was maintained up until the moment when stationary phase is reached. This is typical for bacteria growing in batch culture on a single substrate with only carbon limitation. When the culture is limited in inorganic nutrients or when a complex substrate (e.g. natural organic matter) is used, one can reasonably expect the growth rate to decline gradually towards the onset of stationary phase (Berney et al., 2006).

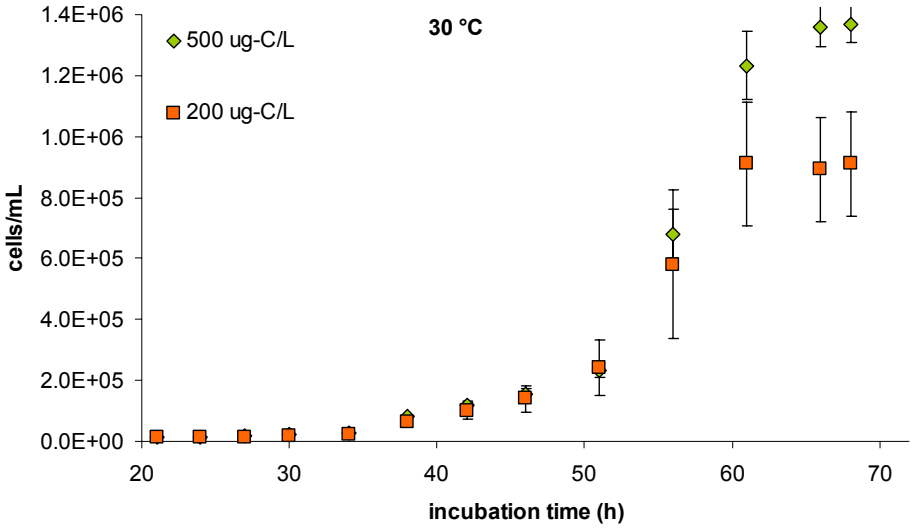

Figure 6. Batch growth curve of *P. fluorescens* P17 on 2 different concentrations of acetate at 30 °C using sterile mineral water (Evian) amended with a buffer and trace element solution as the water matrix. All data points represent average values of triplicate vials. The cell concentration was measured with flow cytometry coupled with SYBR Green I staining (see Hammes and Egli, 2005).

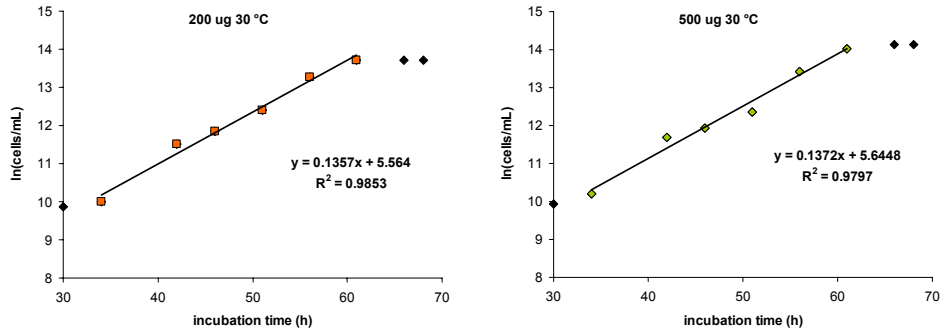

Figure 7. Growth rates determined from the natural logarithm of the cell concentrations for 200 µg L^{-1} (A) and 500 µg L^{-1} (B) acetate-C.

2.4 Batch growth curves of P17 at 18 °C on acetate

At 18 °C a similar initial lag phase was observed as seen at 30 °C (Figure 8). Exponential growth resulted in faster maximum specific growth rates (0.21 – 0.24 h^{-1}) than seen at 30 °C (Figure 9A and 9B), showing the effect of temperature clearly. This seems to coincide with the data reported by LeChevallier et al. (1993), which suggested that the optimal temperature of this bacterium is rather low (c.a. 22 °C). Higher yields were recorded in this experiment compared to 30 °C, which corroborates the earlier suggestion that higher growth rates would lead to higher cell yields.

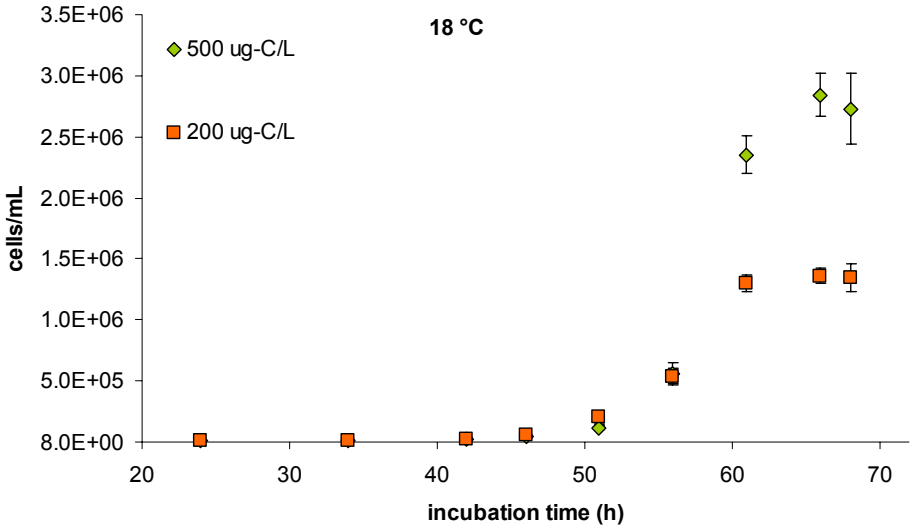

Figure 8. Batch growth curve of *P. fluorescens* P17 on 2 different concentrations of acetate at 18 °C using sterile mineral water (Evian) amended with a buffer and trace element solution as the water matrix. All data points represent average values of triplicate vials. The cell concentration was measured with flow cytometry coupled with SYBR Green I staining (see Hammes and Egli, 2005).

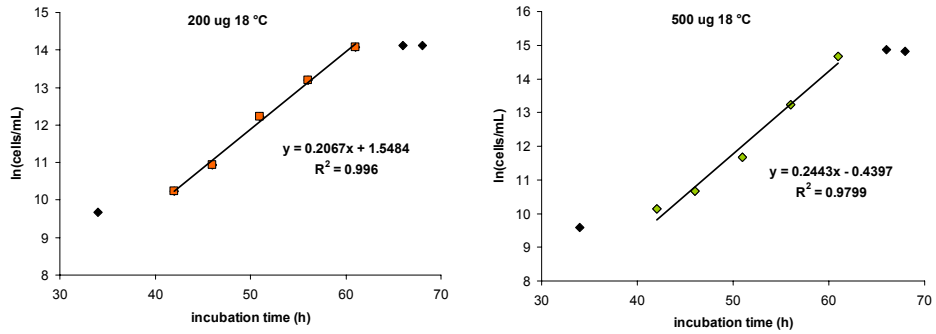

Figure 9 Growth rates determined from the natural logarithm of the cell concentrations for 200 µg L^{-1} (A) and 500 µg L^{-1} (B) acetate-C.

2.5 Estimation of the Ks value using BiotechSIM software

We subsequently modeled the growth of *P. fluorescens* P17 at 18 °C (Figure 8) with BiotechSIM software (www.biotechLAB.net). The modeling is based on Monod-kinetics. The numerical cell yield and the maximum specific growth rate on acetate-C, as determined above, was entered as fixed parameters to the model. Different values of Ks were tested in order to fit the simulation curve to the experimentally obtained values (Figure 10). From this simulation a Ks value of c.a. 25 µg acetate-C per liter was observed as best-fit for the experimental data.

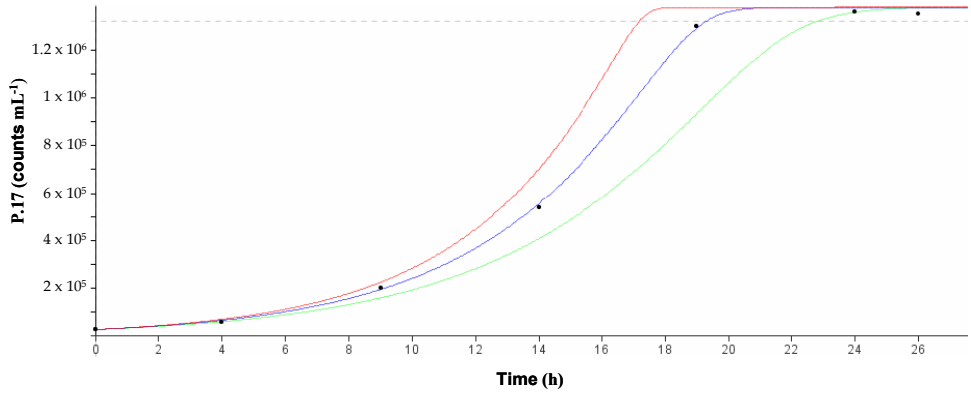

Figure 10 Simulation of growth of P17 on 200 ug L-1 acetate-C at 18 °C using BiotechSIM software. Only the exponential growth phase was considered for the model. Green: Ks = 50 ug L^{-1}; Blue: Ks = 25 ug L^{-1}; Red: Ks = 10 ug L^{-1}.

2.6 Planktonic growth in reactor and model simulations

The experimental results using P. *fluorescens* P17 in Propella™ reactor showed that a steady state concentration of bacteria at the reactor outlet was reached within 70 hours and consisted of 7.0×10^6 cells/mL (Fig. 11). In the batch experiments (above), a concentration of 2.7×10^6 cells/mL was measured at 20 °C. It should be noted that inoculum concentration in Propella™ experiment and in batch experiments were 2.5×10^4($\pm1.5\times10^4$; n=19) cells/mL and 1.0×10^4 cells/mL, respectively. The Propella™ experiment was run using 1.164 mg-C L^{-1} which was two times higher than in batch growth experiment (500 µg/L acetate-C). The comparative planktonic yields from the two different approaches are therefore rather similar: 5.4×10^6 cells/µg-C (batch experiments) and 6.01×10^6 cells/µg-C (Propella™ experiments). There was also a slight temperature difference between the 2 experimental set-ups, which may have contributed to the differences in yields (see Materials and Methods).

The first simulations in a mathematical model were made to reproduce growth according assumptions suggested by Zhang et al. (2004) and the input values were set the same than what was used in Propella™ reactor experiment (see above). The simulation results showed a constant bacterial concentration increase in the outlet water from the reactor during the entire simulation time and after 480 h the concentration reached 2.6×10^6 cells/mL. The results of the simulation, where the same yield for both suspended and biofilm bacteria was used, indicated too high bacterial concentrations in the outlet water (Figure 11). Unlike to other mathematical models, we opted to include the case where the yield differs between planktonic and biofilm bacteria and some of our results are showed in Figure 11.

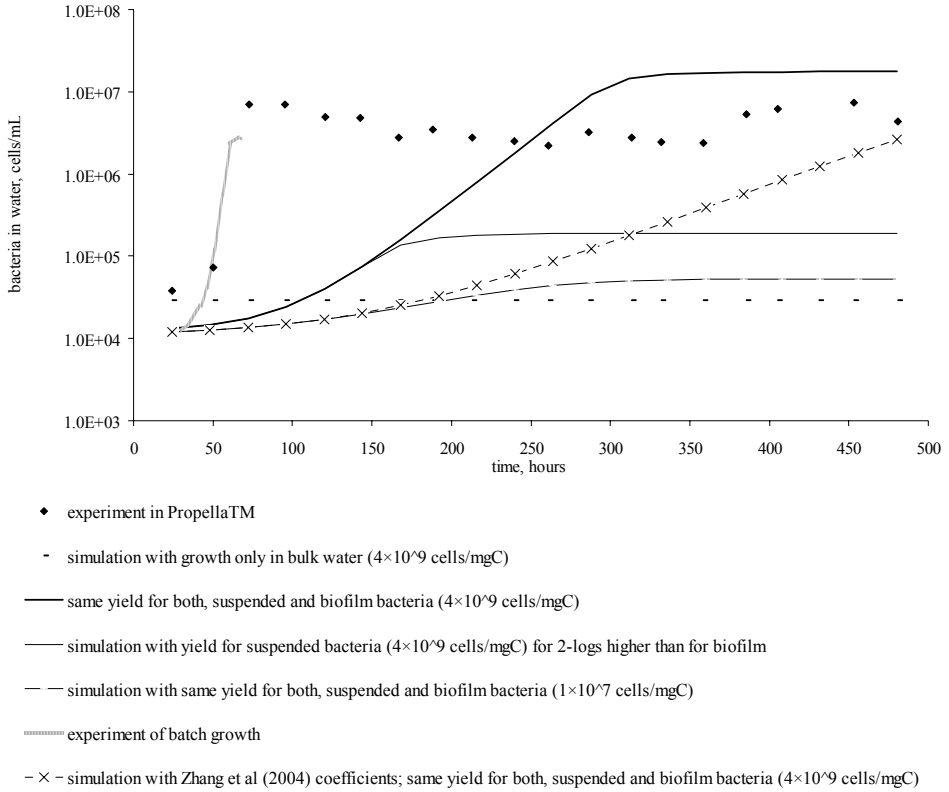

Figure 11. Growth curve of *P. fluorescens* P17 in the outlet water from biofilm reactor PropellaTM, batch scale experiments and simulation data. The substrate concentration (measured with BDOC method) in the inlet water was 1.164 mg-C/L at 20.1±1.0°C (n=72) using synthetic drinking water, except batch scale experiments where *P. fluorescens* P-17 on 0.5 mg-C/L measured with AOC and grown at 18°C using Evian water amended with buffer and trace elements. The data points from PropellaTM and batch scale experiments represent average values, n=2 and n=3, respectively. The cell concentration in Propella™ was measured using epifluorescence microscopy applying DAPI staining method (Brunk, 1979); in batch scale - with flow cytometry coupled with SYBR Green I staining (Hammes & Egli, 2005)

2.7 Biofilm growth in reactor and model simulations

The steady state of biofilm bacteria in Propella™ was reached within the first 100 hours. The bacterial concentration on the biofilm ssurface was 1.0×10^7 cells/cm^2 (Fig. 12).

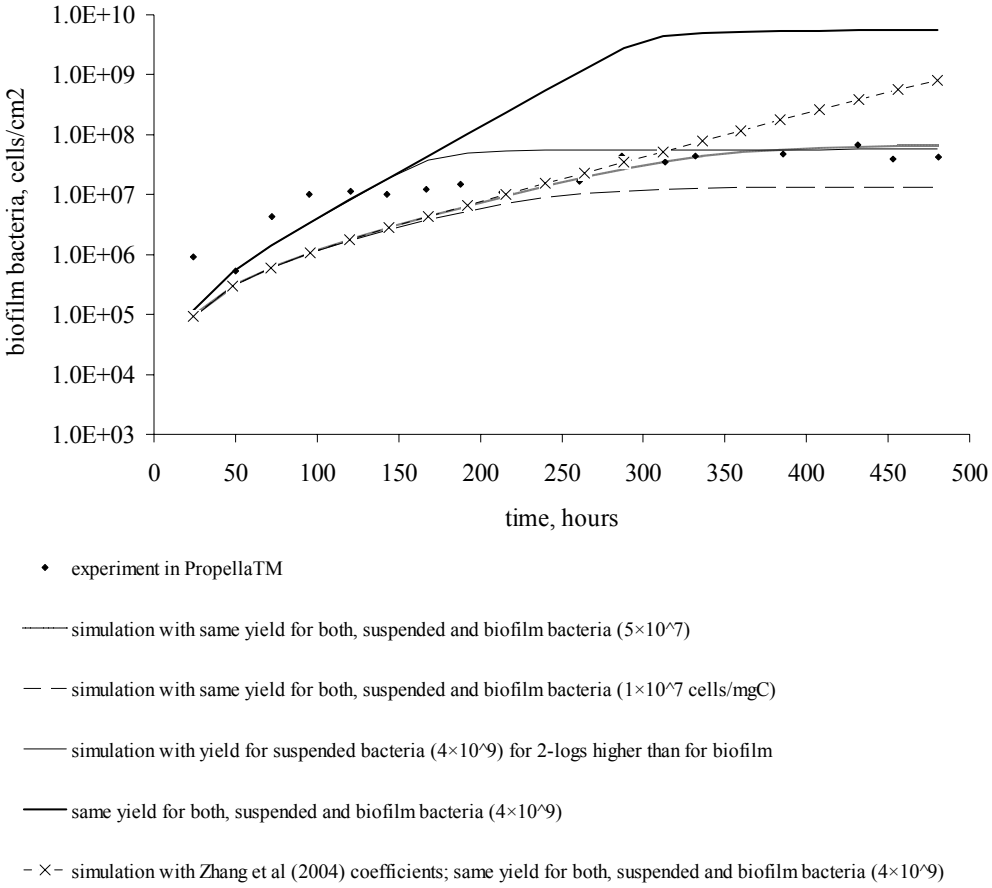

Figure 12. Growth curve of *P. fluorescencs* P17 in biofilm detected in reactor Propella™ and measured in simulation of mathematical model. The substrate concentration in inlet water measured with BDOC method was 1.164 mg-C/L at 20.1±1.0°C (n=72) using synthetic drinking water. The data points from Propella™ represent average values (n=2). The cell concentration was measured with epifluerscencs microscopy using DAPI staining method (Brunk, 1979)

This result is in range with those mentioned in the literature for typical biofilm amounts, quantified in drinking water distribution systems (Mays, 2000). The simulations in the mathematical model to reproduce growth according assumptions suggested by Zhang et al. (2004) showed bacterial concentrations in the biofilm of 5.5×10^9 cells/cm^2. The yield of 4.0×10^9 cells/mg-C are much higher than obtained in the laboratory-scale experiment with Propella™. We also observed that the mathematical model achieved steady state much later (after more than 700 h, the curve goes out of Fig. 12) in comparison with the experimental results from the Propella™ reactor. In Figure 12 we presented also simulation results with lowered yields, as well with different yields for both planktonic and biofilm bacteria. Our results were comparable with results in the pipe section from Zhang et al. (2004) hypothetical pipe network where chlorine concentration drops below the detection limit.

Statistical analysis was performed in order to compare the fit of the simulation curves with Propella™ experimental data (Tab. 3). The Wilcoxon test showed that in most cases (T≤40) the simulation results differ significantly from experimental data.

Table 3. Statistical analysis of simulation (level of significance $p < 0.05$)

Nº	simulation	parameter T
	Biofilm growth	
1	decreased yield 5×10^7 cells/mgC	56
2	yield 1×10^9 cells/mgC (Zhang's coefficients)	72
3	decreased yield 5×10^7 cells/mgC for biofilm	23
4	decreased yield 1×10^7 cells/mgC	0
5	yield 4×10^9 cells/mgC	15
	Planktonic growth	
1	yield 4×10^9 cells/mgC	41
2	decreased yield 5×10^7 cells/mgC for biofilm	0
3	decreased yield 1×10^7 cells/mgC	0
4	yield 4×10^9 cells/mgC (no growth on surface)	0
5	yield 1×10^9 cells/mgC (Zhang's coefficients)	0

Statistical analysis showed that the yield of biofilm bacteria should be lower than for planktonic bacteria. To conclude, the approach used in the model proposed by Zhang et al. (2004) is in accordance with findings by van der Wende et al. (1989) that bacterial growth in drinking water distribution systems happens almost exclusively on surfaces; cells present in the bulk water is rather due to detachment processes. Our experiments in the Propella™ reactor supplemented with the batch-growth results showed that planktonic cells can proliferate as well, and before equilibrium is reached in system, the growth of planktonic cells is comparable with that of biofilm bacteria. This finding should be kept in mind in cases when in drinking water distribution system detachment events of biofilm cells happen. The findings by Wricke et al. (2002) in distribution networks showed that a stable bacteriological situation will occur under stable preconditions in the network. These authors stated that more cells are released from the biofilm into the water when equilibrium of biofilm is disturbed or hindered. The main reasons for this are changes in nutrient concentrations, fluctuating disinfection residuals and, assumedly, temperature changes. Thus, our findings suggest that one can also expect significant regrowth of detached bacterial cells.

The assumption included in mathematical model for growth of biofilm bacteria before steady state is reached is overestimated. Our experiments showed that the existing approach about planktonic bacteria attachment in the mathematical model is correct for steady state. The first hours of biofilm development can be influenced by forces which is not included in attachment process which should be investigated in future.

3 Conclusions

A mathematical model written for biofilm reactor Propella™ was verified with coefficients obtained from batch experiments with monoculture *P. fluorescens* P17 in the media of acetate as a substrate. The model was used to calculate bacterial concentration expressed as total bacteria number. To get maximum specific growth rates (μ_{max}) and yield data (cells µg-C^{-1}) the batch experiments for planktonic growth of the bacteria were performed on acetate at different temperatures and different carbon concentrations.

Specific growth rates of *P. fluorescens* P17 for planktonic growth on acetate were between 0.14 - 0.24 h^{-1}. For modeling at 18°C we have chosen a μ_{max}=0.2 h^{-1}, which agrees with Camper (1996). However, we have demonstrated the clear dependency of the growth rate on the temperature, and more work with colder temperatures typical to drinking water might be required for a definitive answer.

We found similar yield values to that reported by Van der Kooij (see Greenberg et al., 1993), and described that the yield is probably related to the specific growth rate (and thus to temperature). We have also seen the yield of the natural microbial community is at least 2.5 times higher than that of the pure culture. In this respect, *P. fluorescens* P17 show an extremely low yield on acetate-C and it should be considered to address this in the model. For modeling the yield of *P. fluorescens* P17 for planktonic growth on acetate at 18°C was set 4.0×10^9 cells/mgC. The simulations of mathematical model indicated that yield for biofilm bacteria ($\beta=1\times10^7$ cells/mgC) should be for 2 logs lower than for planktonic bacteria.

From the experimental data and from the simulation in BiotechSIM software it is obvious that the Monod half saturation coefficient (K_s) value for *P. fluorescens* P17 on acetate should be 0.025 mg acetate-C/L at 18°C, which is significantly lower than suggested by Laurent et. al. (1997). But for natural microbial communities growing on natural organic matter, this value is most likely to be significantly lower.

There is reason to believe that *P. fluorescens* P17 growing on acetate might provide some basic modeling data, but that this is of limited value to natural conditions. Eawag would like to proceed towards conditions more typical of natural environments, specifically using natural microbial communities as test organisms and natural organic matter as substrate. First experiments will again be conducted batch-wise, but eventual progress towards continuous culture experiments is foreseen.

4 References

Brunk, C.F, Jones. K.C. & James, TW. 1979. Assay for nanogram quantities of DNA in cellular homogenates. *Anal Biochem*., 92(2): 497–500.

Camper A.K. (1996). Factors limiting microbial growth in distribution systems: laboratory and pilot-scale experiments. American Water Works Research Foundation Report, Report No.90708

Dukan, S., Levy, Y., Piriou, P., Guyon, F. & Villon P. 1996. Dynamic modeling of bacterial growth in drinking water networks. *Water Research*, 30: 1991–2002.

Frias, J., Ribas, F. & Lucena, F. 1992. A method for the measurement of biodegradable organic carbon in waters. *Water Research*, 26(2): 255-258.

Fowler, J., Cohen, L. & Jarvis, P. 2004. *Practical Statistics for Field Biology*, 2nd ed., USA: John Wiley & Sons Inc.

Greenberg, A.E., Clesceri, L.S., Eaton, A.D. (eds) 1993. *Standard methods for the examination of water and wastewater*, 18th ed. Washington, D.C.: American Public Health Association.

Hammes, F., Salhi, E., Koster, O., Kaiser, H.P., Egli, T. & von Gunten, U. 2006. Mechanistic and kinetic evaluation of organic disinfection by-product and assimilable organic carbon (AOC) formation during the ozonation of drinking water. *Water Research*, 40: 2275-2286.

Hammes, F. & Egli, T. 2005. New method for assimilable organic carbon determination using flow-cytometric enumeration and a natural microbial consortium as inoculum. *Environmental Science Technology*, 39: 3289-3294.

Hammes, F., Berney, M., Wang, Y., Vital, M., Köster, O. and Egli, T. 2007. Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. *Water Research*, *In Press* (corrected proof available online)

http://www.biotechlab.net , Internet – Modelling and Simulation of Dynamic Systems – A Collection of Applied Examples.

Ihsen, J. & Egli, T. 2004. Specific growth rate and not cell density controls the general stress response in *Escherichia coli*, *Microbiology*, 150(6): 1637-1648.

Laurent, P., Barbeau, B. & Servais, P. 2005. Evaluating the impacts of treatment modification on regrowth potential in distribution systems: a new screening procedure using water quality modeling. *Urban Water Journal*, 2(2): 81-92.

Laurent P., Servais P., Prevost M., Gatel D. and Clement B. (1997). Testing the SANCHO model on distribution systems. *J.AWWA*, 89(7): 92-103

LeChevallier, M.W., Shaw, N.E., Kaplan, L.A. & Bott, T.L. 1993. Development of a rapid assimilable organic carbon method for water. *Applied and Environmental Microbiology*, 29: 1526-1531.

Mays, L.W. 2000. *Water Distribution Systems Handbook*. 1st ed., New York, USA: American Water Works Association.

Miettinen, I.T., Vartiainen, T. & Martikainen, P.J. 1999. Determination of assimilable organic carbon in humus-rich drinking waters. *Water Research*, 33(10): 2277-2282.

Piriou, P., Dukan, S. & Kiene L. 1998. Modelling bacteriological water quality in drinking water distribution systems. *Water Science and Technology*, 38(8-9): 299-307.

Ribas, F., Frias, J. & Lucena, F. 1991. A new dynamic method for the rapad determination of the biodegradable dissolved organic carbon in drinking water. *Journal of Applied Bacteriology*, 71: 371-378.

Servais, P., Laurent, P., Billen, G. & Gatel D. 1995. Development of a model of BDOC and bacterial biomass fluctuations in distribution systems. *Revue des Sciences de l'Eau*, 8: 427-464.

Servais, P., Anzil, A. & Ventresque, C. 1989. Simple method for determination of biodegrable dissolved organic carbon in water. *Applied and Environmental Microbiology*, 55(10): 2732-2734.

Servais, P., Billen, G. & Hascoet, M.C. 1987. Determination of the biodegradable fraction of dissolved organic matter in water. *Water Research*, 21: 445 -450.

Vadstein, O. 2000. Heterotrophic, planktonic bacteria and cycling of phosphorus. Phosphorus requirements, competitive ability, and food web interactions. *Advance in Microbial Ecology*, 16: 115-167.

van der Kooij, D. 2002. *Assimilable organic carbon (AOC) in treated water: determination and significance*. In Encyclopedia of Environmental Microbiology, Bitton, G. (Ed.), Hoboken, NJ, USA: John Wiley & Sons.

van der Wende, E., Characklis, W.G. & Smith, D.B. 1989. Biofilms and bacterial drinking water quality. *Water Research*, 23: 1313-1322.

Vital M., Füchslin H., Hammes F. A. and Egli T. (2007) Growth of *Vibrio cholerae* O1 Ogawa Eltor in freshwater. Microbiology SGM, 153, 1993–2001.

Wang, Y., Hammes, F., Boon, N. and Egli, T. 2007. Quantification of the filterability of freshwater bacteria through 0.45, 0.22 and 0.1 µm pore size filters and shape dependent enrichment of filterable bacterial communities. *Environmental Science Technology*, In Press.

Wricke, B., Korth, A., Petzoldt, H., & Krüger, M. 2002. Change of bacterial water quality in a drinking water distribution system working with or without low chlorine residual. *Water Science and Technology: Water Supply*, 2(3): 275-281.

Zhang, W., Miller, C.T. & DiGiano, F.A. 2004. Bacterial regrowth model for water distribution systems incorporating alternation split-operator solution technique. *Journal of Environmental Engineering*, 130(9): 932-941.