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Transport of metabolic active bacteria through saturated quartz sand columns with and without substrate addition

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der Rheinisch-Westfälischen Technischen Hochschule Aachen zur Erlangung des akademischen Grades einer Doktorin der Naturwissenschaften genehmigte Dissertation

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Für Benedikt, Dominik, Matthias, Florian und Michael

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Abbreviations

CFU	colony forming unit
gfp	green fluorescent protein
IEP	isoelectric point
Lag-phase	start of growth phase
Log-Phase	logarithmic growth phase
PFA	para-formaldehyde
PV	pore volume

1 Introduction

Xenobiotics in the environment cause harm and processes of contamination are described manifold in the literature (Leeson 2001; Scott 2004). Beside chemical pollutants in the environment biological pollutants such as microorganisms and viruses also have to be considered, since they can enter the environment via human assistance: At lower concentrations, by filter plants and their sewage and in case of crop protection through biological agents like *Bacillus thuringiensis*. In normal cases disposal of sludge or manure on fields (Unc 2004) with a significantly higher concentration of microorganisms is harmless because the bacteria are not pathogenic, they are kept at the soil surface, or in the upper levels of the soil. The problem emerges when these bacteria or viruses are pathogenic and reach the saturated soil, subsequently groundwater. The passage of microorganisms may be possible when the soil is near the surface and the soil is wetted through time, i.e. it never dries. Beside transport of pathogenic bacteria themselves, pathogenic and antibiotic resistance genes can be exchanged between the infiltrated and autochthone bacteria. This can also lead to a contamination without the abundance of the pathogenic bacteria and plays a role in drinking water safety.

Further, due to bioremediation, soil-derived bacteria were applied on contaminated areas to degrade the contaminant. After successfully decontamination the remaining bacteria stay in unknown parts inside the soil. The problem may occur when the microorganisms reach the aquifer due to irrigation and passage along preferential flow paths (Bundt M. 2001).

The transport of microorganisms through variably saturated soil is simulated in laboratory experiments in different scales: soil- or sand-filled columns in lab-scale setup (Becker 2004), undisturbed soil columns (Lovins 1993), lysimeters as an intermediate setup (Hase 2001, Pitkäjärvi 2003) and field experiments as the biggest scale that can be monitored (Oyster site, Virginia, for detailed review see (Ginn 2002) and (Taylor 2004).

Although the high environmental impact of pathogenic bacteria from manure or from fields of bioremediation is known as a global topic, the specific mechanism of bacteria transport from the surface to the groundwater is not yet clearly understood. Currently, there are two basic approaches: experiments taking place under unsaturated conditions, simulating the vadose

zone, and experiments under saturated conditions mimicking the region directly above the groundwater level, the aquifer.

Up to now there is no general prediction of bacterial transport possible because of the lack of fundamental datasets to develop simulations for bacterial breakthrough and considering all features of heterogenic bacterial cell populations at the same time.

In this work experiments under saturated conditions were performed to acquire information about metabolic active microorganisms (*Pseudomonas fluorescens*) and the influence of substrate addition on the bacterial behaviour, such as age and oxygen availability. The experimental setup was established for online measuring breakthrough of *Pseudomonas fluorescens-gfp* as well as the proceeding of balancing this breakthrough in form of distribution of total cell amount in effluent and layers.

The investigations were divided into two main parts:

(1) Bacterial breakthrough under saturated conditions without substrate. Includes factors like different ages of the used cultures, morphological changes during the column passage and the influence of experimental runtime

(2) Bacterial breakthrough under saturated conditions with substrate-addition. After determining the magnitude of influence of the described factors above (without substrate), the influence of the age of the used culture under substrate addition was investigated.

Prior to the breakthrough experiments the *gfp*-mutant of *P. fluorescens* had to be developed and selected. The amount of cells, that were injected by short (1ml, 0.02 PV) pulse, was not higher than 10^9 per ml to prevent that the cell amount within the column reaches levels that are not present in natural areas (not more than 10^4 per ml) (Uhlman 1986), i.e. no continuous pulse application was performed. The qualitative detection of bacterial breakthrough occurred by monitoring of the fluorescence signal of the *gfp*-expression as well as UV/VIS-absorption to determine bacterial and other colloidal breakthrough. The breakthrough curves had to be compared to ones of the conservative tracer D_2O and to particles (melanin microspheres, carboxylate coated) of the same size as bacteria under the same experimental conditions.

The balancing of cell amount in effluent and column was studied by digital image analysis of cells stained with fluorescence dyes (according to Weinbauer et al. (1998)). By means of data base queries the morphology of recovered cells could be determined and compared to cells of used inoculum.

In comparison to batch- and chemostat-experiments the effect of oxygen starvation during the breakthrough should be explained. In this work the influence of substrate addition had to be investigated with prior knowledge of bacterial behaviour inside a saturated column and the role of a limiting amount of oxygen. In these substrate experiments the columns were equilibrated with the substrate solution so an overall availability of substrate excess was established. With the subsequent pulse of bacteria the growth effects with morphological changes and increase of cell amount were determined - in contrast to works in literature (Murphy 1997, 2000; Jordan 2004) where a simple bioassay was investigated by detection and calculation of degradation of limited substrate in a bacteria-saturated column.

2 Bacterial transport and breakthrough through porous media

Particle transport has been investigated in detail, and results are available that allow modelling of transport under saturated and unsaturated conditions (Bradford 2002; 2003; 2006). However, investigations on bacterial transport are still matter of research interest in either saturated or unsaturated porous media.

Bacteria are exposed to convective transport as particulates species that move forward with the pore-water, but some strains (amongst others *Pseudomonas*), are able to move actively throughout the aqueous solution by flagella-mediated forward movement (Schlegel 1992). The factors which affect bacterial convective transport and retardation of bacteria are diverse (Stevik 2004) and not only of physical and physico-chemical but also of biological nature:

Flow rate: Increasing water flow leads to an increased transport of biomass (Klauth et al. 2006) whereas the increase of the ionic strength inside the eluant leads to a decrease of bacterial breakthrough (Tan 1994)

Grain (and pore) and particle size: increasing grain size leads to an increased breakthrough of the same particles, until the pore size reached a definite value, where particles are trapped (straining, see below). Increasing particle size leads to an increased breakthrough (size exclusion, filtering), too, until the particle size reaches a level where the particles are retained on the top of the column (McGechan 2002).

Particle concentration: increasing the particle concentration leads to increased breakthrough and to increasing straining of particles. This is one of the factors leading to blocking and ripening (McGechan 2002).

Surface characteristics of matrix and microorganism: the resultant hydrophilic/hydrophobic interactions between microorganism and matrix surfaces can enhance or retard the transport. These interactions are influenced by ionic strength, solved organic compounds and the age of the microorganism (Becker 2004; Foppen 2005)

Growth/decay/chemotaxis/motility: each culture is a dynamic system of growth and decay of cells. Chemotaxis allows bacteria to enter a preferred medium on their own, in some cases this movement is supported by flagella motion, as in the case of *Pseudomonas fluorescens*.

The factors mentioned above are responsible for the resulting retardation or breakthrough of microorganisms through porous media. The mechanisms for transport and retardation are:

Straining indicates the physical trapping of any particle in small pores and throats within the matrix and is influenced 1st by the particle to mean grain size ratio, and 2nd by hydraulic characteristics like flow rate and ionic strength. The breakthrough curves which show straining are non-symmetric with a broad shoulder behind the maximum (Corapcioglu 1984).

Attachment and detachment are interactions between microorganisms and the matrix within a column based on the electrostatic and van-der-Waals forces. The interactions between microorganisms can result in filtering (Murphy 1997) and subsequently blocking and ripening (described further in this section). During unsaturated conditions the system becomes more complex due to the presence of air enclosed in the matrix and therefore an additional interface becomes available which is known to be a favourable site for bacterial attachment. Jewett et al. (1999) found that fewer than 100% saturation – no air-water or air-matrix surfaces are present - the breakthrough of bacteria reaches a maximum. With decreasing saturation the breakthrough decreases (and the retardation increases, respectively) due to resulting attachment of microorganisms at the air-water-interface (Schaefer 1998)

Filtering is the removal of any particle from solution by collision with the porous media and deposition on it.

Size exclusion results in detected breakthrough times of bacteria that are shorter than those of conservative tracers. Assuming that large cells are released to the effluent by means of size exclusion (Corapcioglu 1984) implicates additionally a dependence from the age and nutrient condition due to size shrinkage occurs with increasing age and under starvation (Sanin 2003).

Blocking occurs when first injected particles attach to the matrix and occupy preferential attachment sites. Subsequently following particles are then prevented from attaching to the matrix surface and are eluated.

Ripening occurs when prior attached particles serve as new preferred attachment sites for subsequently following particles.

The greatest part of bacterial transport in an aqueous system is facilitated by the advective flow (Dong 2002). Several works are presented in literature where bacterial and particle transport are compared (Pang 1998; Becker 2004). Becker et al. recorded breakthrough curves of inactive bacteria, particles (polystyrene, 1 µm) and bromide through raw material (glass beads of 3 mm diameter).

Biological factors vary with age and metabolic state of the regarding culture. A culture consists of different morphological species under certain conditions and the surface characteristics change during a cell-cycle (van Loosdrecht 1990). Depending on the age of a cell this cell can interact with surfaces to start culture-growing or is able to leave the surface to rest in aqueous solution for finding new binding regions. Surfaces become preferred areas for metabolic active cells because during exponential growth of bacteria cell wall hydrophobicity is increasing and therefore adhesion is increasing, too. Adhesion is necessary as well for colonization and afterwards developing a biofilm as for colonization and afterwards leaking into the surrounding aqueous solution. Attached cells are metabolic mor active than suspended cells (Ellwood 1982).

The surface characteristics can be determined by physico-chemical parameters (IR-data of bacterial cell surface (Chen 2001), ζ-potential, isoelectric point IEP, and contact angle Θ (Rijnaarts 1995)). The hydrophobicity-index measured by MATH test according to (van der Mei 1995) gives information about the hydrophobicity (and hydrophilicity, respectively) of bacteria in a defined hexadecane-water-system which allows comparison of different strains. The influence of the surface characteristics of different soils and different bacteria is important. I.e. hydrophobic soil compartments can be occupied by hydrophobic cells, which may start culture growing and therefore serve as a depot for leaching bacteria into the subsurface, especially when there are microchannels available where the leaching may be enhanced. The same is valid for hydrophilic soil (sand for example) and hydrophilic bacterial strains.

Beside the surface characteristics of bacteria and the matrix, bacterial features like flagella can also affect the transport (Camper 1993, Gannon 1991). Flagella allow bacteria like *P. fluorescens* to enter aqueous medium independently. This movement can reach velocities up to 4 mm/h.

For visualization of bacterial transport different methods were used in the past. As well as the online-detection of radio-labelled cells (Jewett 1999), the fluorescence-detection of genetic modified cells after *gfp*- or *lux*-transformation was used (Dunn 2005; Oates 2005).

The greatest part of investigations concerning about the microbial transport has been carried out as either field or column experiments with inactive bacteria or viruses (Mallen 2005). Hekman et al. (1995) balanced the breakthrough of bacteria only with regard to the effluent and did not consider the age of a used culture. All living organisms which should pass a column or even a field were held inactive because of the problems occurring when simulating bacterial breakthrough with transport-models. Foppen et al. (2005) came to this conclusion as they modelled breakthrough of an *E. coli*-strain assuming heterogenic cells and the results did not match the experimental data. To allow for the heterogeneity of bacterial suspensions, Tufenkji et al. (2003) developed a model where the retention profile of cells within a column was adapted to observed experimental data based on different transport theories: the colloid filtration theory, microbial deposition patterns and steady-state filtration with no dispersion. All different theories are able to describe one part of bacterial transport, which is always simplified as a particle transport. Boundary conditions were inactivation of the used microorganisms, short residence-times of microorganisms inside the column, constant depositions rate coefficients.

The influence of substrate on bacterial breakthrough under saturated conditions has not been investigated in detail. Jordan et al. (2004) and Murphy et al. (1997, 2000) obtained for first data with an experimental setup of water- and bacteria-saturated column where a substrate-pulse was added and the degradation was monitored. But up to now no investigations were performed regarding the breakthrough of bacteria under consideration of the culture age and conditions simulating the aquifer (saturated conditions, limited amount of cells, presence of non-limited substrate, oxygen limitation). These aspects are some of the objective of this thesis and should be investigated as a function of age of a used culture, i.e. in dependence of the culture age and nutrient starvation state as well as a function of available nutrient (substrate) during the transport process. For this purpose it was important to know the normal standard behaviour of the bacterial culture, so that *Pseudomonas fluorescens* was the appropriate strain to be examined under the given conditions.

In this thesis the transport of a gfp-mutant of *Pseudomonas fluorescens* was investigated under saturated conditions as a function of the age of a used culture (duration of carbon and energy starvation) without and with substrate-addition during the breakthrough. The effect of aerotaxis as a special feature of chemotaxis was investigated and balanced and its effect on bacterial size and shape was described.

3 Materials and methods

3.1 Pseudomonas fluorescens, its cultivation and gfp-modification

P. fluorescens is a soil-derived bacterium which is taken as a model organism (next to *E. coli* and *Bacillus* or other *Pseudomonas* species) in a large number of transport experiments (Brunninger 1999; Chen 2001; Ripp 2001; Timms-Wilson 2001; Rockhold 2002; Chen 2004).

P. fluorescens is a motile, normally rod-shaped gram-negative bacterium from dimensions of 0.5-1.0 µm in diameter and 1.5-4 µm in length, depending on age and nutrient state (Bergey`s Manual of systematic Bacteriology, 1986). Cells of the logarithmic phase are lophotric (monopolar polytrich). That is, the flagella are located at one pole of the longitudinal cell and the typical flagella motion leads to velocities from 20 to 60 µm/sec, i. e. in average about 14 cm/h (Schlegel 1992). Wilhelm et al. (1998) showed size differences of physiologically distinguishable cell species as a function of cell age. Figure 3-1 shows the growth curve ("total cells") and its composition of large and small cells. The logarithmic phase (phase of exponential growth of the culture) consists mainly of large cells whereas the stationary phase and afterwards the resting cells consist of mainly small cells.

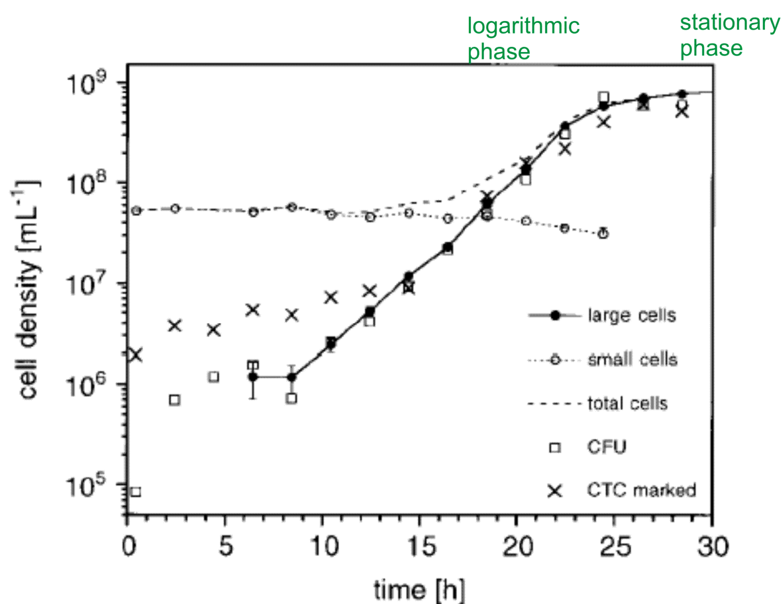

Figure 3-1 Growth curve and morphological species detected during cell-cycle from *P. fluorescens*. CFU indicated the viable and reproducible cells, CTC indicates viable cells. Modified according to Wilhelm et al. (1998)

Depending on the detection and enumeration method of cell sizes and shapes (and therefore the measurement of factors that influence directly the magnitude of these parameters) age and nutrient state have to be monitored. Agranovski et al. (2003) came to the conclusion that for *P. fluorescens* case no size distribution could not be obtained via UV/APS (UV-aerodynamic particle sizer). In the same paper stress factors for *P. fluorescens* were not obtained although for other bacteria these factors played an important role and bacteria showed differences in their performance.

ζ-potential [mV]*	-19.13	Chen and Strevett (2001)
IEP [I=0.01M]	3.6	Rijnaarts et al. (1995)
U	-1.03	Rijnaarts et al. (1995)
contact angle Θ [deg]	25	Rijnaarts et al. (1995)

Table 1 Physico-chemical characteristics of *P. fluorescens*. * average value from all stages (logarithmic, stationary, decay)

Table 1 shows the physico-chemical parameters of *P. fluorescens* from literature.

The ζ-potential was determined to be independent from the age of the culture -19 mV. IR-data (Chen and Strevett 2001) indicated that the surface composition changed with increasing age from polar to apolar, the rate of carbonyl and amide-groups was decreasing whereas the rate of apolar ethenyl-groups was increasing with progressing age (Chen 2001).

(Fouchard 2005) found that different growth conditions led to varying membrane compositions. Several results have been achieved about the effect of nutrient starvation (carbon, nitrogen; (Sanin 2003)), oxygen and heat stress, and their effects on other bacteria (Smeulders 1999; Ojha 2000) and *Pseudomonas putida* (Givskov 1994).

The used strain was *Pseudomonas fluorescens* that was genetically modified for gfp-expression: the gfp-gene was inserted into the genome by conjugation of *E. coli*-S17λpir carrying the donor-plasmid pAG408 (provided by D. Pieper, GBF Braunschweig) as mini-transposon vector and the wild type *Pseudomonas fluorescens* as recipient. The conjugation was conducted as described before (de Lorenzo 1990). During the insertion a kanamycin-resistance was also inserted into the genome. The cells were plated on kanamycin-agar and separated after detection of the green fluorescent colonies. For the breakthrough experiments

single colonies were grown in BC minimal-medium (Sambanis 1985) or Medium 462 with glucose (0.5 g/l). The inoculum of cells (about 10^9 per ml) for each column was taken out directly from the culture-flasks, an aliquot was taken for cell counting (see below). For fresh cultures of less than 18h (experiment “4h- without substrate addition” and “5h- with substrate addition”) the amount of cells was about 10^8 per ml. To concentrate the cell suspension, a centrifugation step was done, but this centrifugation altered the cells dimensions and therefore the morphological appearance. This effect is described by Smets et al.(1999), too. Therefore for fresh cells the inoculated cell amount was a magnitude lower than for the older cells. The morphological appearance should be investigated in dependence from the age of a culture.

3.2 Columns and buffer for breakthrough

The transport of gfp-labelled bacteria was conducted under saturated conditions in columns of quartz sand (TECO-SIL, CE minerals, -50 +100). The dimensions of the columns were 3 cm inner diameter and 12 cm length.

The used quartz sand (CE minerals, density 2.2 g/cm^3, porosity 0.5, diameter ±150µm) was directly used, without sterilization. The cell background and the substrate availability were determined once as 10^4 cells per 5 g sand and no cell enhancement after 24h under standard conditions, shaker, air access.

The columns (Figure 3-2) were equipped with PE-mesh (0.5 mm, Bückmann) at the inlet and the outlet for evenly distribution of buffer, teflon-mesh (pore size 10 µm, Bückmann) towards the sand for filtering bigger particles and to prevent occlusion of the detector cells. The columns were sealed with Teflon® O-rings. The package of the column was carried head first, so that the filling direction was the same as the later flow direction.

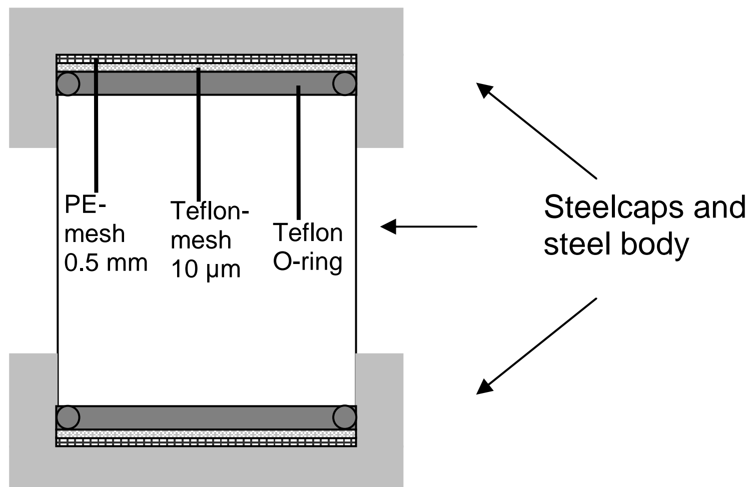

Figure 3-2 Column setup. Inner diameter 3 cm, length 12 cm.

To avoid enclosure of air bubbles during the filling process the column body was first filled 25 ml of buffer before the sand was poured in. Permanent knocking ensured that the matrix inside was stratified uniformly. Alternative pouring of buffer and sand was repeated till the sand surface established a bending that fitted the distance to the mesh of the top (thickness of the O-ring).

3.3 Column experiments and experimental setup

Several methods are available for determination of cell amount in solution. Direct methods are cell counter, flow-cytometry and digital image analysis after fluorescence staining LIT. Indirect methods like UV/VIS-absorption of cell suspensions are too imprecise and only give information on the sum of cells. The determination of CFU indicates only the viable and reproducible cells. However, the UV/VIS-absorption method was used for the qualitative determination of bacterial breakthrough additional to fluorescence detection. Methods of cell counting by means of cell counters are not able to detect the differences between morphological species of one strain, as seen in other works (Agranovski 2003), and lead to a false estimation of bacterial behaviour.

During the experimental part of this work the method of fluorescence staining of cells, microscopic detection, and subsequent digital image analysis to acquire datasets for each single cell and evaluation by data base queries was operated, though it was time consuming, but it gave the most information not only about the amount of cells but also about cell characteristics.

A scheme of the experimental setup is given in Figure 3-3. The column was loaded by a HPLC-pump (Merck-Hitachi, L-6200) with flow direction from bottom to top, to maintain saturated conditions.

The effluent passed a fluorescence- (Merck-Hitachi, F-1050) and UV/VIS-detector (Merck-Hitachi, L-4200), a refractometer (Merck, differential refractometer RI-71) and was collected in a fraction collector (ISCO, retriever 500) at least. For calibration with D_2O the effluent was not collected. All detectors were controlled by PC (Merck-Hitachi software HSM-7000). For controlling the complete setup a sample valve (rheodyne 6-valve) was installed before the series of detectors. This valve and a controlling pump (Merck-Hitachi, L-7100) were PC-controlled, too.

Stainless steel columns of 3 cm diameter and 12 (or 10) cm length were used. The column was loaded with buffer (BC minimal medium without C-source) by HPLC-pump with constant flow rate of 0.1 ml/min, simulating natural conditions of flow velocity of 1 m/d.

The effluent passed successively the fluorescence-detector, the UV/VIS-detector and the refractometer before it was collected by auto sampler.

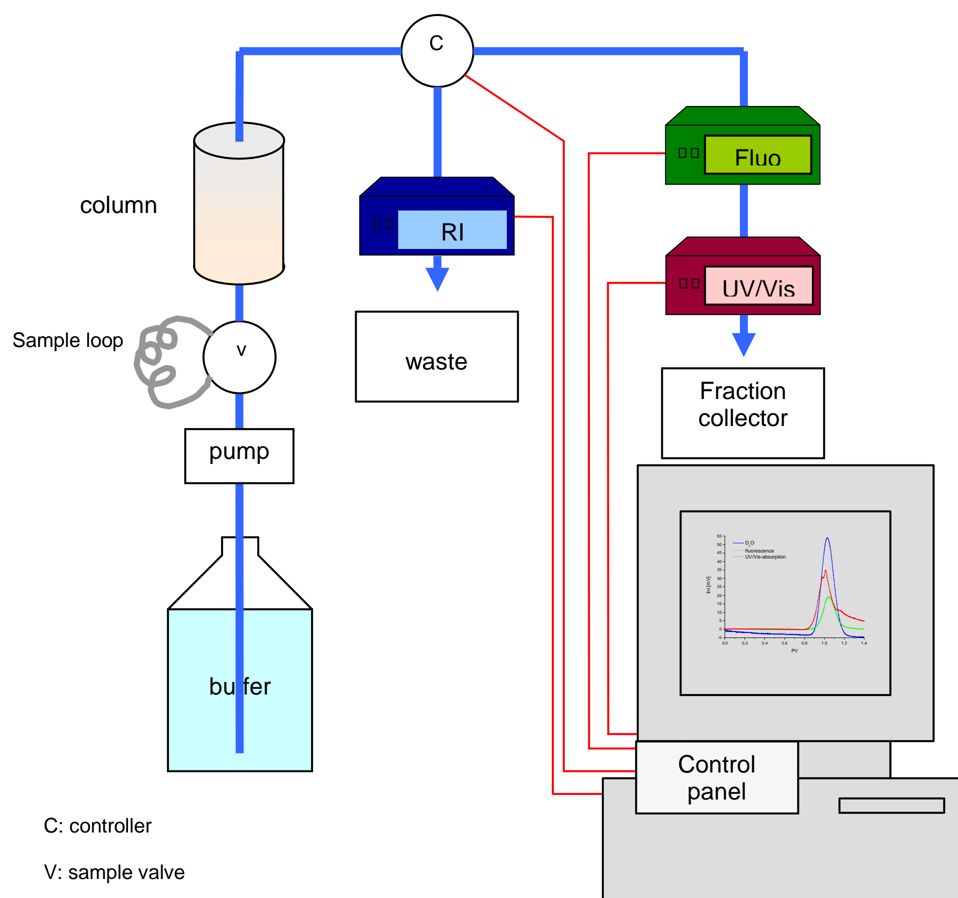

Figure 3-3 Scheme of experimental setup. Column system, detector row and control panel.

The determination of the pore volume was done by calculating the empty column volume minus the volume of the sand (calculated by weight and density of 2.2 cm^3/g). The measurements with conservative tracer were taken with a pulse of 1ml 0.05 M D_2O in M461-medium, the breakthrough was measured by the refractometer and the effluent was directly discarded. For the bacterial breakthrough (pulse) the sample loop was filled with 1 ml of bacterial suspension (cell amount about 10^9 cells), the cell amount was determined before. The breakthrough was monitored by fluorescence-detection (excitation wavelength 360 nm, emission wavelength 520 nm) and by UV/VIS-absorption (660 nm). The effluent was collected in fractions of 4 ml (corresponding to 40 min) in sample tubes containing 400µl 4%

PFA solution (in PBS). The small amount of PFA was used to inactive the leaking cells and prevent further growing during the experimental runtime, but avoiding denaturation of gfp during the long runtime. After 12 h runtime the column was removed from the system and stored at 8°C. Aliquots of fractions (30 µl up to 150 µl, depending on cell amount) were counted for total cell amount (see 3.4.).

For comparison between bacterial and particle breakthrough latter was investigated by pumping a 1 ml-pulse of micropheres (sulforhodamin b encapsulated in melanin, surface-coated with carboxylate groups; Microparticles, Berlin) into a column.

After the breakthrough had finished the column was opened at the bottom (inlet) and 1cm-layers were separated, whereas the layer structure was destroyed. The sand was resuspended in 50 ml of a 1:3-dilution of 4% PFA (in PBS; 12.5 ml) and PBS (37.5 ml) and left on a shaker for 1 h at room temperature. Aliquots of 1 ml supernatant were counted for total cell amount (see 3.4).

3.4 Microscope procedure and data analysis

The total cell amount of the original bacterial suspension as well as aliquots of effluent and supernatant were determined by fluorescence spectroscopy of fluorescence labelled cells. The detection of pure *gfp*-expression under UV-light for counting cells of the samples was not sufficient, because the excitation-light intensity was too high, thus a bleaching of the cells occurred much faster than it took to get the digital images of the samples.

The method for fluorescence labelling of cells used was carried out as described in Weinbauer (1998), modified by Klauth (2004). Therefore the corresponding sample (50-150 µl of fractions, 1 ml of suspended layer, 100 µl of a 1:10 or 1:100-dilution of inoculum, depending on cell-density) was pipetted into a FinStar staining reactor containing 4 ml of PBS buffer (pH 7), as described previously (Poschen 2002). For filtration, an isopore filter (Millipore, 0.2 µm, 25 mm diameter, GTBP, Millipore) was used. The liquid sample was sucked off with 130 mbar underpressure and the retained particles were washed twice with 4 ml PBS buffer (pH 7). The filter was then coated with 1.5 ml of 5 µM Sybr-Green solution in PBS buffer (pH 7) and incubated for 5 min. Two washing steps were performed subsequently.

The filter with the stained cells was mounted into a drop of water on a microscope slide after drying and a drop of water was put on the surface. A cover slide was used and a drop of immersion oil was put on the cover slide. The sample was viewed under blue excitation light (Nikon B-2A, excitation 450-490 nm, dichroic mirror 505 nm, longpass > 520 nm) at 600-fold (in some cases 450- or 1000-fold) magnification. The experimental setup was the same as described at Klauth et al. (2004). In Table 2 the values are given for the regarding lenses in different experiments.

	450-fold	600-fold	1000-fold
Picture size (in PIXEL)	750*545	1232*972	1232*972
1Px=	0.106µm	0.104µm	0.057µm
1µm=	9.43Px	9.7Px	17.5Px
Picture size in $µm^2$	80*5 $=4.6*10^3$	128*101 $=1.29*10^3$	70.2*55.4 $=3.9*10^3$
Filter size in cm^2	4,15	4,15	4,15
Filterfactor =filter size/picture size	$9*10^4$	$3.22*10^4$	$1.07*10^5$

Table 2 Characteristic data and parameters for the different used lenses of the microscope setup.

Each counted cell was classified with 22 parameters, which were designated in the KS400 software (Carl Zeiss, Jena). All raw data (for each counted cell 22 parameters) were stored and left unprocessed in a microsoft access database. By SQL-query morphological portions of each investigated fraction/layer or inoculum could be determined as well as calculations of the biovolume of a regarding sample.

For classification of morphology three limitations were set, deriving from test data (Table 3):

FERETRATIO = ratio of minimal and maximal extent of an object

NCHAINS = number of chain-coded objects in a regarded cell, within the object, three dimensions. Chain coding can begin at any point of the object, so the number is the varying parameter (see Figure 3-4).

NCRACKS = number of chain-coded objects in a regarded cell, along the object, in two dimensions (see Figure 3-4).

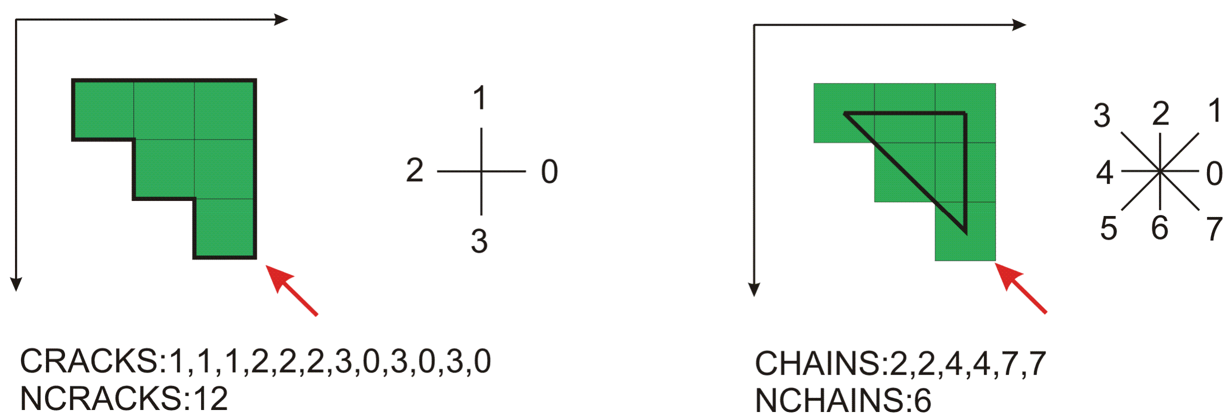

Figure 3-4 Explanation of crack- and chain-code

classification parameter	FERETRATIO			NCRACKS			NCHAINS		
lens	0.45	0.6	1.0	0.45	0.6	1.0	0.45	0.6	1.0
cocci	>= 0.6			≤20	<30	<50	≤11	<20	<30
rods	< 0.6			>20	≥30	≥50	>11	≥20	≥30
ellipsoids	rest of cells, that do not fulfil the criteria above								

Table 3 Classification parameters obtained via digital image analysis

All cells were classified with these limitations. The limitations were combined as “AND”-conditions (all 3 limitations have to be fulfilled, otherwise the corresponding cells was sorted as “ellipsoid”).

This was done outgoing from the 2-dimensional picture of each cell to a 3-dimensional geometric body: A circle-formed cell became a spherical cell, a coccus, whereas a long, straight cell became a rod. Figure 3-5 shows the corresponding figures with the data derived from the digital image analysis.

$V_c = \frac{4}{3}\pi r^3$ Volume of a coccoid cell

$V_R = \frac{4}{3}\pi r^3 + \pi h r^2$ Volume of a rod cell

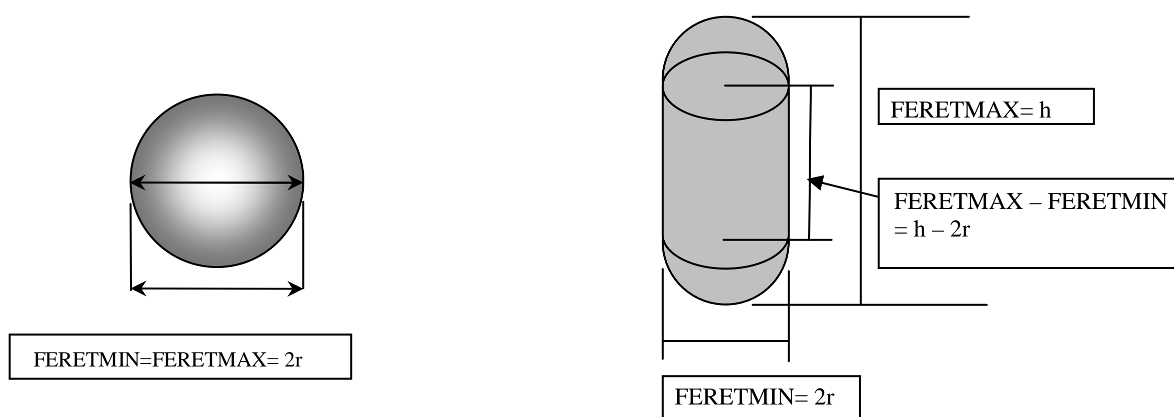

Figure 3-5 Calculation of the biovolume of different types of cells.

Deriving from the parameters FERETRATIO/NCHAINS/NCRACKS another method for balancing the bacterial breakthrough was done by calculating the biovolume: through digital image analysis the volume of each counted cell was determined (see above).

After upscaling for the cell amount in each fraction, layer or in the inoculum the biovolume could be determined. This remains nearly constant for cultures, which derived from older inocula (at least one week old) (Figure 3-6).

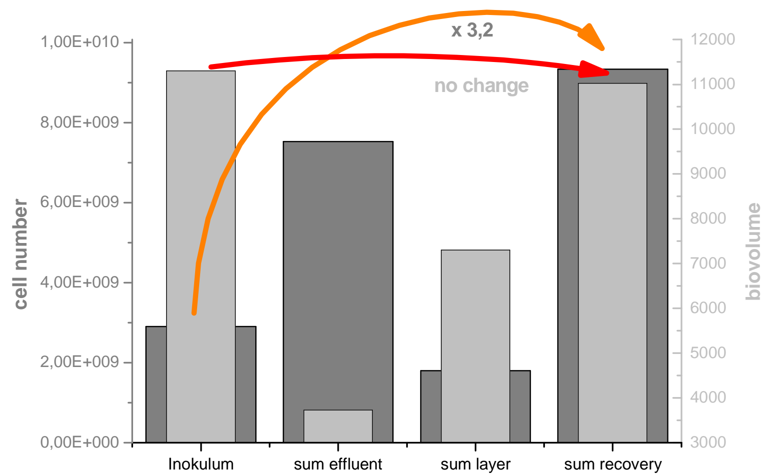

Figure 3-6 Example: Biovolume remains nearly constant. A slight increase is probably due to rest of metabolic useable sources of the culture itself.

For cultures, which derived from fresh inocula, where the metabolic turnover was quite high, the biovolume showed a slightly increasing behaviour. But this increase was very low compared to a biovolume-increase of an actively growing culture with glucose as sole C- and energy source in the same time of 12-18 h runtime.

3.5 Investigation of stress occurring due to lack of oxygen

3.5.1 Investigation of the increase of cells

To find out the reasons for morphological change and cell increase of bacteria during column passage a culture of *P. fluorescens-gfp* was grown according to Tappe et al. (1996) in a chemostat, i.e. without mechanical retardation of bacteria. The dilution rate D was $0.05h^{-1}$ as was the growthrate μ. Two aliquots of this chemostat culture were taken and treated as followed: A control was transferred into a flask and allowed to grow under air access and second a sample in which the solved oxygen was degassed through helium for 1 h and afterwards closed airtight and left growing on the shaker. Both, sample and control were investigated after 3, 6 and 24 hours and the cell amount was determined as described before (see 3.4).

3.5.2 Investigation of the morphological composition of control and stressed culture

For this investigation simple batch experiments were done. Pre-cultures were grown in BC (with 0.5 g/l glucose) over night under air access (shaker). Afterwards the conditions were altered in the means of replacement of oxygen by helium in one of the sample cultures (culture 2), one was left without possible air exchange (culture 3), and one culture was left under standard conditions as control (culture 1). All cultures were grown additionally for 24 hours. Samples from each culture were taken directly before the conditions changed (time 0) and after 24 hours of growing under these changed conditions (time 24hr). The investigation of morphology was executed as before (see 3.4) and was analyzed by database queries, too.

4 Breakthrough of Pseudomonas fluorescens-gfp through quartz sand columns under saturated conditions without substrate

The topic of this work was the investigation of the transport behaviour of *P. fluorescens-gfp* in quartz sand columns under saturated conditions without and with substrate addition. Furthermore the comparison of the obtained breakthrough curves (BTC) and retention profiles with microspheres (MS) has been proved.

The detection of the conservative tracer D_2O, bacterial and microsphere breakthrough occurred after injection of a short pulse (1ml, according to ~0.02 PV). Two different experiment series were run: (1) without substrate addition (only minimal salt medium as growing and transport buffer); (2) continuous substrate addition (0.5 g/l glucose) in minimal salt medium. The variable parameter in each series was the culture age, respective the time of carbon- and energy depletion of *P. fluorescens*-gfp cells. Prior to each experiment, the breakthrough curve of D_2O was recorded, to check if the column was saturated and filled correctly, and as a reference to compare with the bacterial breakthrough curve. Under established standard conditions the breakthrough of microspheres was recorded for comparison of the breakthrough and retardation of particles of bacterial size, shape, and surface characteristics to the one of metabolic active bacteria.

4.1 Results

4.1.1 Comparison between the breakthrough of bacteria and microspheres

The bacterial breakthrough of the genetic modified *P. fluorescence*-gfp strain was detected online by measuring the fluorescence intensity of gfp-expression as a function of cell amount. This method has the advantage that it keeps the cells active and viable prior they enter the column in comparison to works where cells were marked by fluorescence dyes prior to the breakthrough (Becker 2004). The maximum of detected breakthrough of P. *fluorescens-gfp* was found at nearly 1 pore volume (PV) (0.96 and 1.04 PV for fresh and old cells,

respectively; Figure 4-1). Beside the position of the actual maximum the signal intensity was also found to be dependent on the duration of carbon and energy starvation of the injected culture. The signal intensity was high for fresh cells which is indicative for a larger amount of cells broken through while the signal intensity decreased for older cultures.

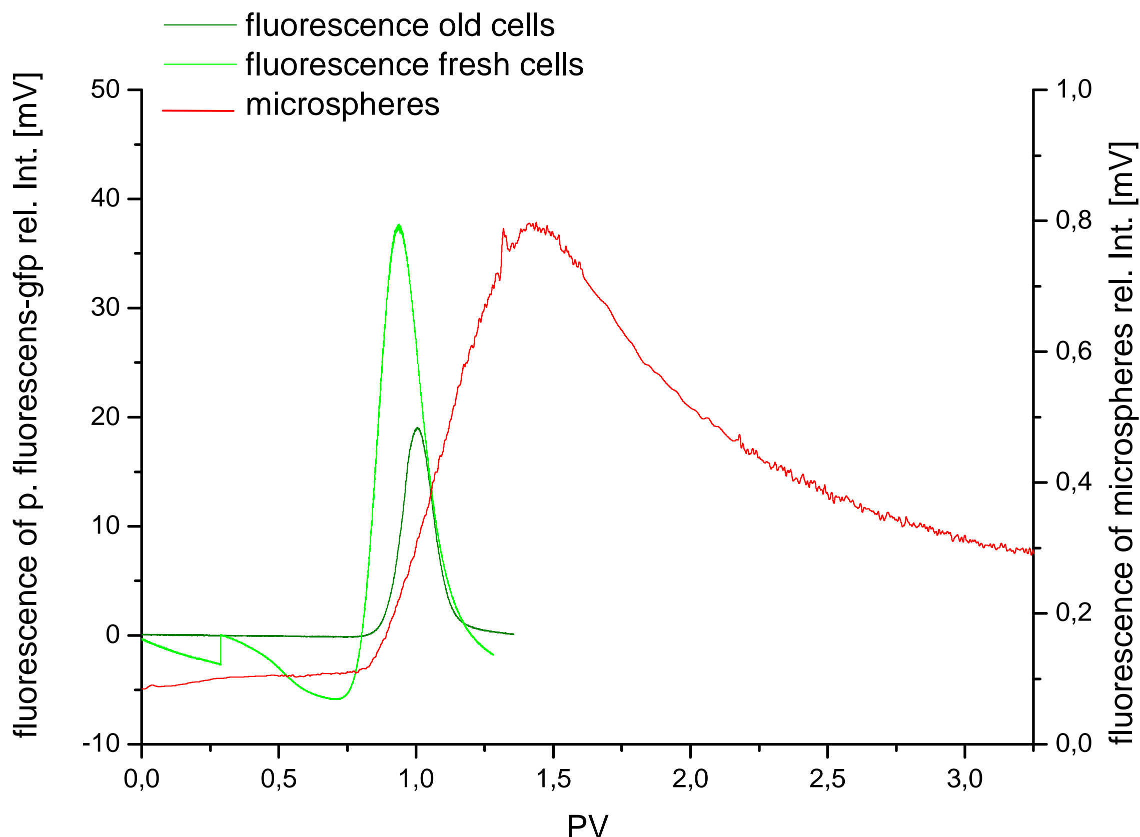

Figure 4-1 Breakthrough curves of D_2O (refractive index), bacteria of different ages and microspheres (each fluorescence) under saturated conditions. Each experiment run with a pulse of 1 ml 50 mM D_2O/Medium 461, cell suspension or microspheres ($1x10^9$ cells, $6x10^9$ MS). Flow rate was 0.1 ml/min

In fact, the intensity signal of this *gfp* expression could not be calibrated as a function of the cell amount because the density of the *gfp* inside the cells was changing: The cell division of especially fresh cells (see 4.1.2) led to a dilution of the *gfp* because the amount of *gfp* proteins did not increase during the division process. On the other hand increasing duration of carbon and energy starvation of a culture led to decreased intensity of fluorescence due to missing protein translation inside the cells. Due to this obtained heterogeneity of each investigated

culture the observed signal of *gfp* fluorescence was taken for qualitative control of bacterial breakthrough as in other works (Dunn 2005). The actual bacterial breakthrough curve was obtained by cell counting of each fraction (as is described in detail later on). The maximum of fluorescence intensity of the injected MS showed a significant later breakthrough (at 1.5 PV, Figure 4-1) for the same experimental conditions (flow rate 0.1 ml/min).

4.1.2 Influence of duration of carbon and energy starvation of *P. fluorescens-gfp* on breakthrough behaviour

For balancing and description of breakthrough and retardation of *P. fluorescens-gfp* the amount of the recovered cells was set as 100 %, because cell multiplication during the breakthrough led to an increase of cell amount (see 4.1.3)

In the effluent as in the column, dependence from duration of carbon and energy starvation of the retained cells was observed (Figure 4-2): Increasing the duration of carbon and energy starvation of culture, the amount of retained cells in the column decreased. For cells of the early log-phase (4h-24h) 57 % to 72 % of the cells (depending on the duration of carbon and energy starvation of the inoculum) were retained in the column, whereas for 336h old cells 95% of the cells were recovered in the effluent. For cells of the early stat-phase (72h) nearly equal distribution of cells in effluent and column was observed.

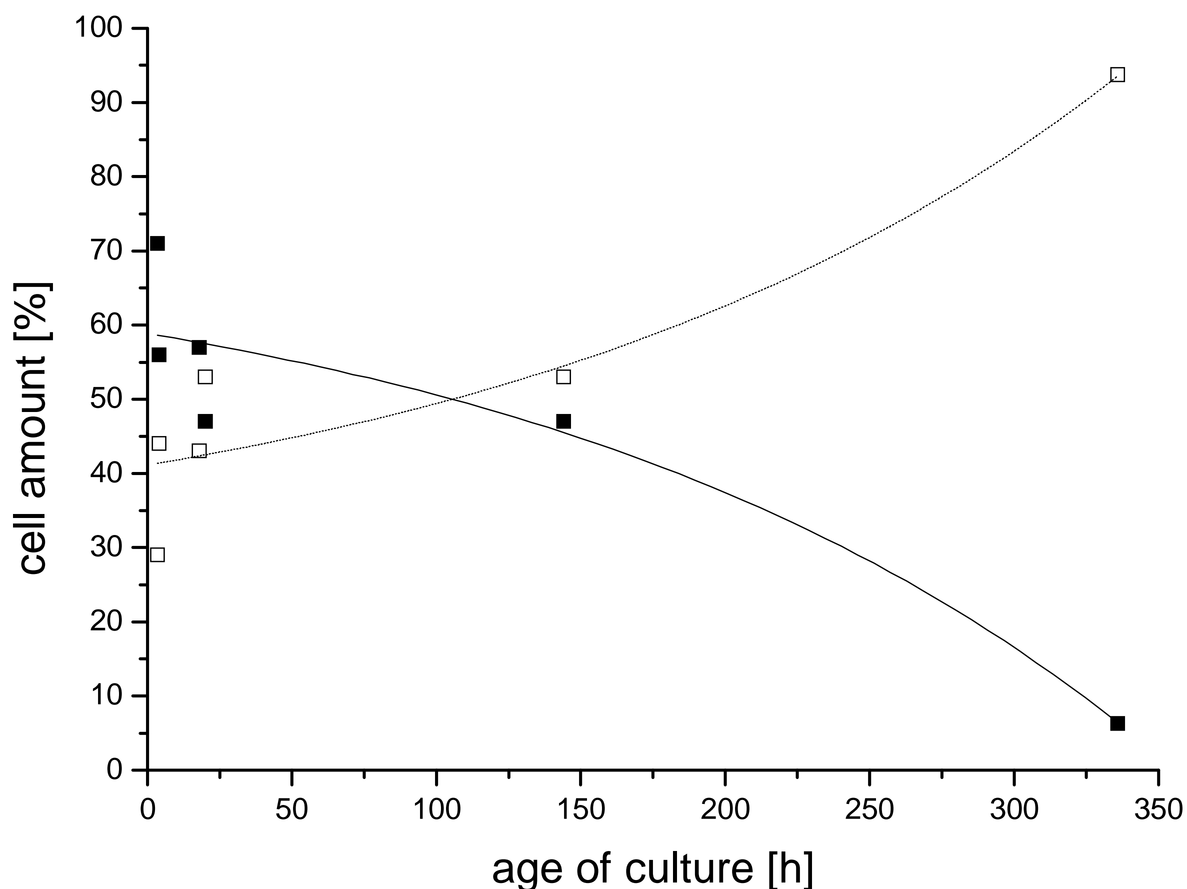

Figure 4-2 Cell amounts [%] of *P. fluorescens-gfp* found in the effluent and retained in the column versus duration of carbon starvation (age of the used culture). Black: column, white: effluent

The actual breakthrough curves were received by cell counting of each fraction of the effluent (Figure 4-3) in the investigated range of pore volume, and were fitted into a Gaussian distribution. In contrast to the MS experiment (where only 0.05 % of particles were recovered in the effluent) all bacteria experiments showed a detectable and countable BTC.

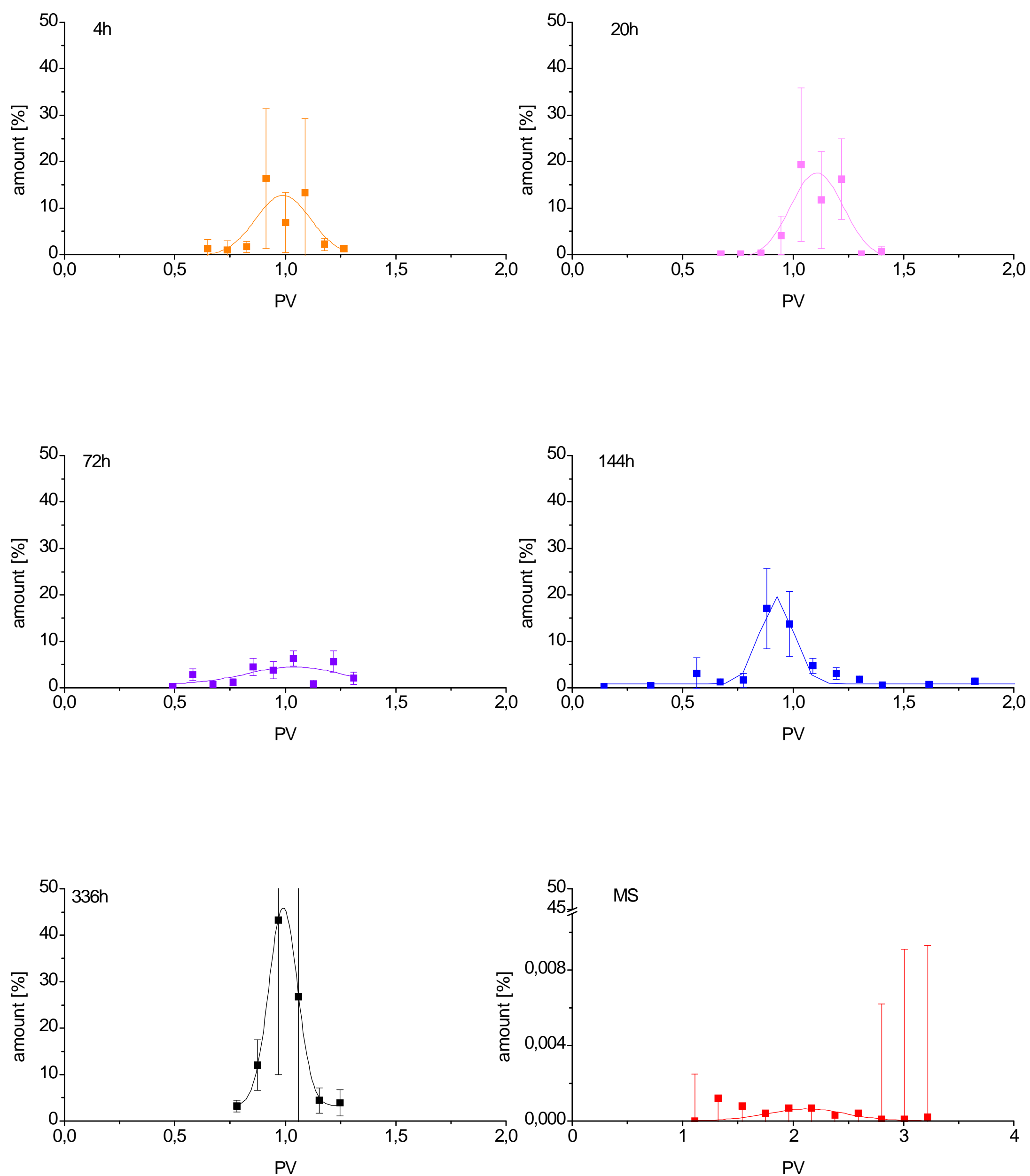

Figure 4-3 Breakthrough curves of *P. fluorescens-gfp* through saturated quartz sand columns at different age of culture compared to the breakthrough curve of microspheres (1 µm of diameter) under the same conditions. The line represents the fitted curve as a Gaussian curve.

The retention profiles are shown in Figure 4-4. Depending on the duration of carbon and energy starvation of the injected culture the distribution of cells within a regarded column changed. Cells of a 4h old culture were retained in two parts of the column. In the first half 41% of all cells were retained, in the second half 15 % of all cells. Cells of 20h were also mainly retained in the first half of a column (40 %) and 7 % in the second half. Increasing the duration of carbon and energy starvation of a culture to 72h led to a distribution where 28 % of all cells were retained in the last third and 32 % in the second third of the column. In the middle third only 13 % were found. In the experiment with the 144h old culture the main part of the cells (38 %) was found in the last third of the column due to the high cell amount in the next to last layer, 9 % of all cells were found nearly completely in the first half of the column. For the 336h experiment only 8 % of all cells were found in the column whereas only 1 % was found in the last half of the column.

In contrast to these bacterial retention profiles the retention profile of microspheres is different, because 97 % of all particles were retained and found nearly completely (87 %) in the first layer of the column.

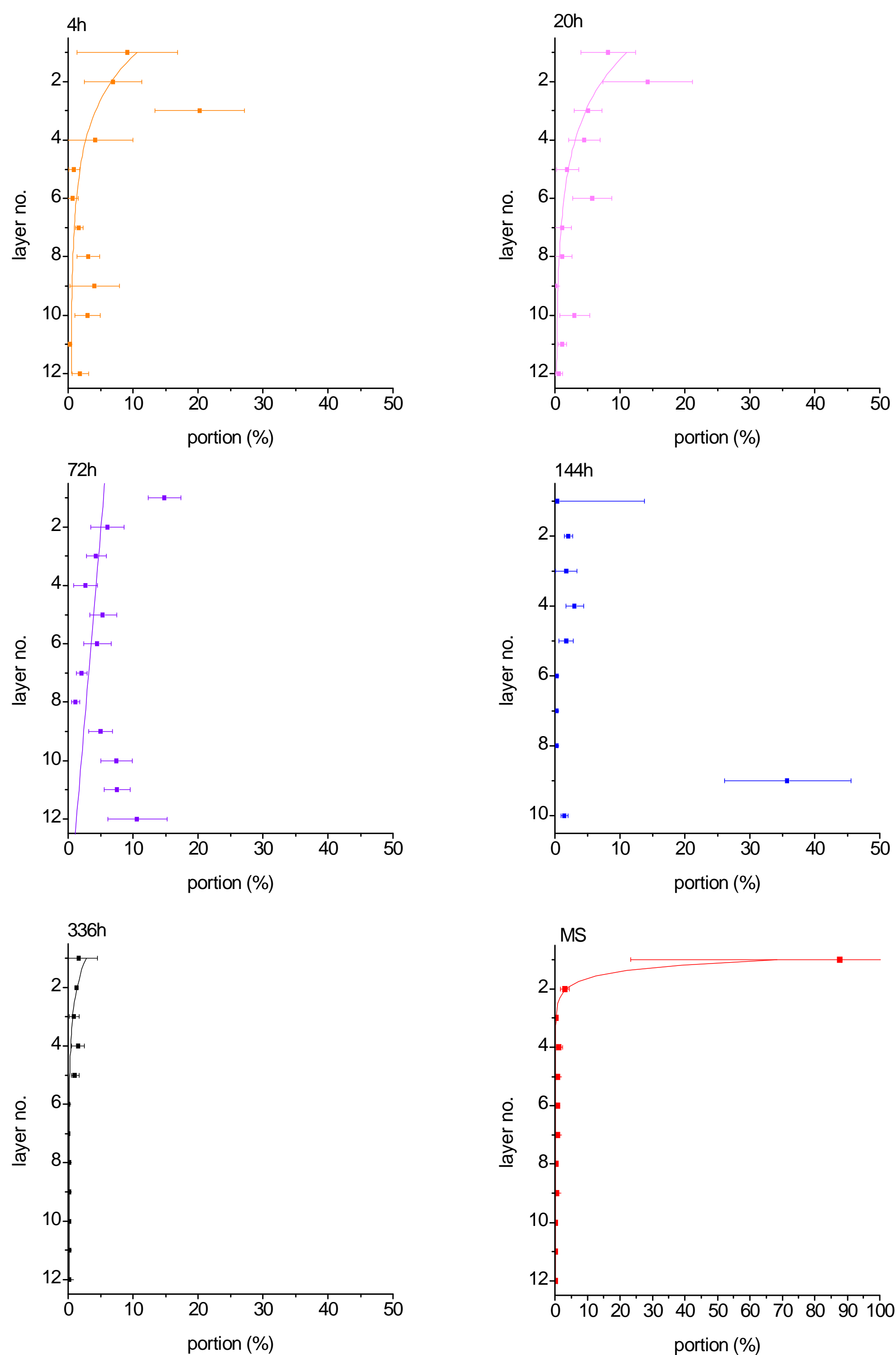

Figure 4-4 Retention profiles of *Pseudomonas fluorescens-gfp* and microspheres. Graphs in descending order: 4h (orange)-20h (pink)-72h (purple)-144h (blue)-336h (black)-ms (red). Data observed from counting each single layer. Layer 1: inlet, layer 10 (or 12 respectively) outlet.

4.1.3 Balance of breakthrough based on the total amount of cells. Morphological change of *Pseudomonas fluorescens-gfp* during the breakthrough through saturated sand columns

Comparing the cell amount of injected inocula and the amount of recovered cells of each experiment it was observed that

● the cell amount increased during the passage of cells through a column and

● a morphological change occurred

Both, increase of cell amount and morphological change were dependent on the duration of carbon and energy starvation of the used culture (Table 4 and Figure 4-5). The increase was due to cell-division in means of stress-response (see 4.1.3). The small increase of the 4h-old culture was caused by the delayed beginning of growing of a 3 weeks old inoculum.

duration of carbon and energy starvation of culture [hr]	Enhancement (ratio recovered to inoculated amount of cells) x-times	Phase of cell cycle
4	7.5	early lag
19	123.5	logarithmic
144	13.5	stationary
336	4.4	late stationary/decay

Table 4 Increase of cell amount depending on duration of carbon and energy starvation of culture. Increase x-times: cell amount of recovered cells compared to injected cells (inoculum).

Morphological investigations of the inocula (Figure 4-5) as well as of samples from effluent and layers showed that fresh cells looked mostly rod-shaped whereas old cells seemed to be of a coccoid form. The third morphological species, the ellipsoid shaped cells, was mentioned only in this investigation in detail, further investigations focussed on the rod-shaped and coccoid cells.

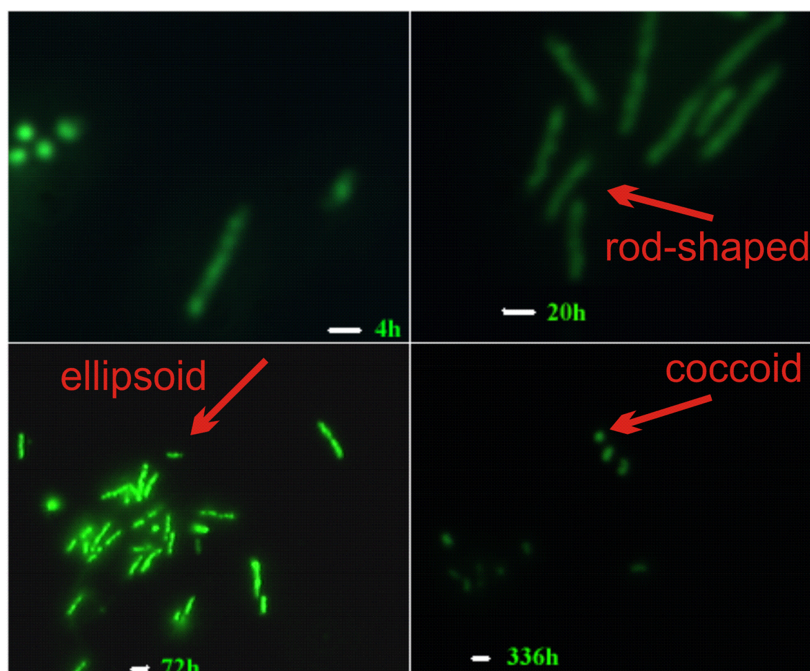

Figure 4-5 Different observed morphological species of *P. fluorescens-gfp* inocula. Pictures obtained from fluorescence microscopy of different inocula. green: age of culture in h, white line: scaling for 1 µm

The cell shape was different comparing fresh and old inocula: cells from the late exponential phase (20h) were long rod-shaped (up to 5 µm in longitudinal extension), cells from the resting phase (336h) were of mainly coccoid shape (about 0.5 mm in diameter). Cells which passed the exponential and were about to enter the resting phase (72h) showed still longitudinal alignment (ellipsoid shape) but one could often find the small arising gap which were the division seam, which is finally disrupted when the small coccoid cells emerge. Cells which were in the early log-phase (4h) showed either coccoid- or rod-shaped profile, depending on the duration of carbon and energy starvation of the used preculture. It took a longer time for older cells to enter the exponential phase – that means to start growth - than for fresh cells. This effect was described before (Klein 1996), where the recovery time (time that cells needed to enter the early log phase to start the exponential growing) was increasing with increasing duration of carbon and energy starvation of the cells.

Figure 4-6 shows the dependence of the amount of rod-shaped and coccoid cells in a determined inoculum from the age of the used culture.

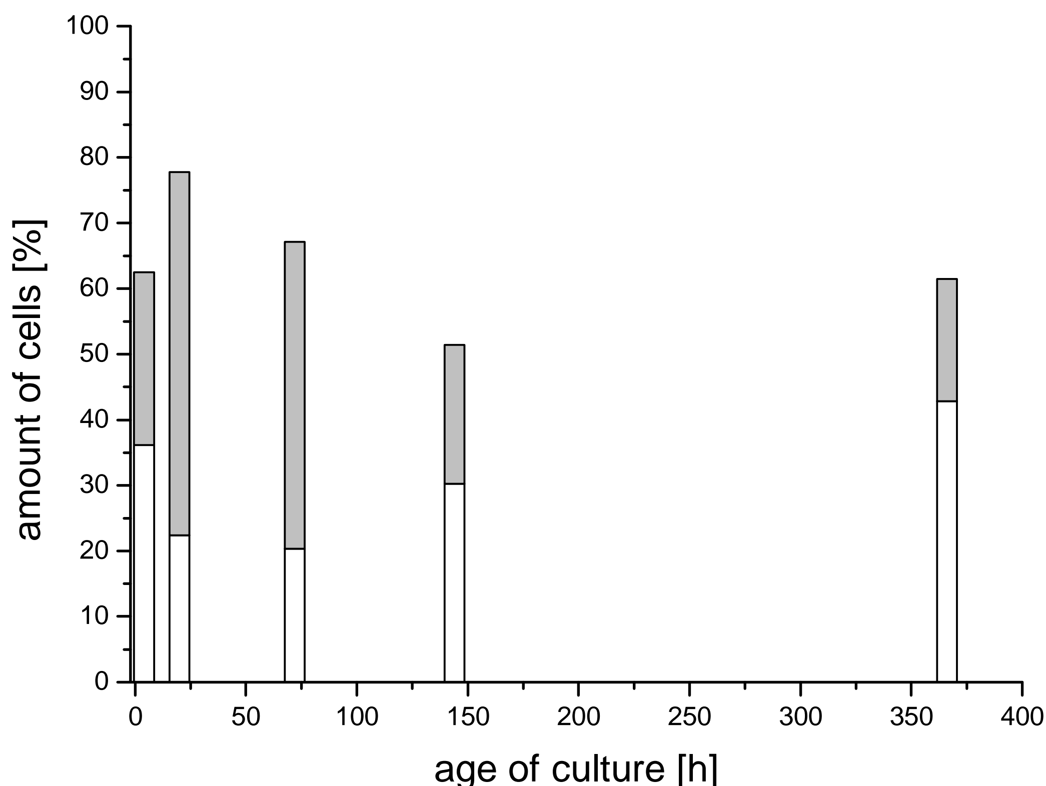

Figure 4-6 Change of the morphological composition of *P. fluorescens-gfp* inocula as a function of the age [h]. White: coccoid, grey: rod-shaped cells.

The highest amount of long rod-shaped cells (55 %) was observed for log-phase (20h old) cultures, whereas this amount decreased with increasing age (cultures of the stationary phase): 47 % for 72h, 21 % for 144h and only 19 % for 366h. The small amount for the 4h old culture was due to the fact that at this time the culture was starting the growth. The amount of coccoid cells was increasing from 23% to 43% with increasing duration of carbon and energy starvation from 20h to 144h and older.

The duration of carbon and energy starvation of a culture was the main factor affecting the distribution of coccoid and rod-shaped cells either in effluent or in a column. In Figure 4-7 amounts of coccoid and rod-shaped cells in effluent and in columns were plotted. The dotted trend lines indicated that with increasing age the amount of coccoid and rod-shaped cells increased in effluent and decreased inside the column. The reason for the different slope of

the trend was the presence of the third morphological species, ellipsoid shaped cells (see Figure 4-5), which built the in-between of coccoid and rod-shaped cells.

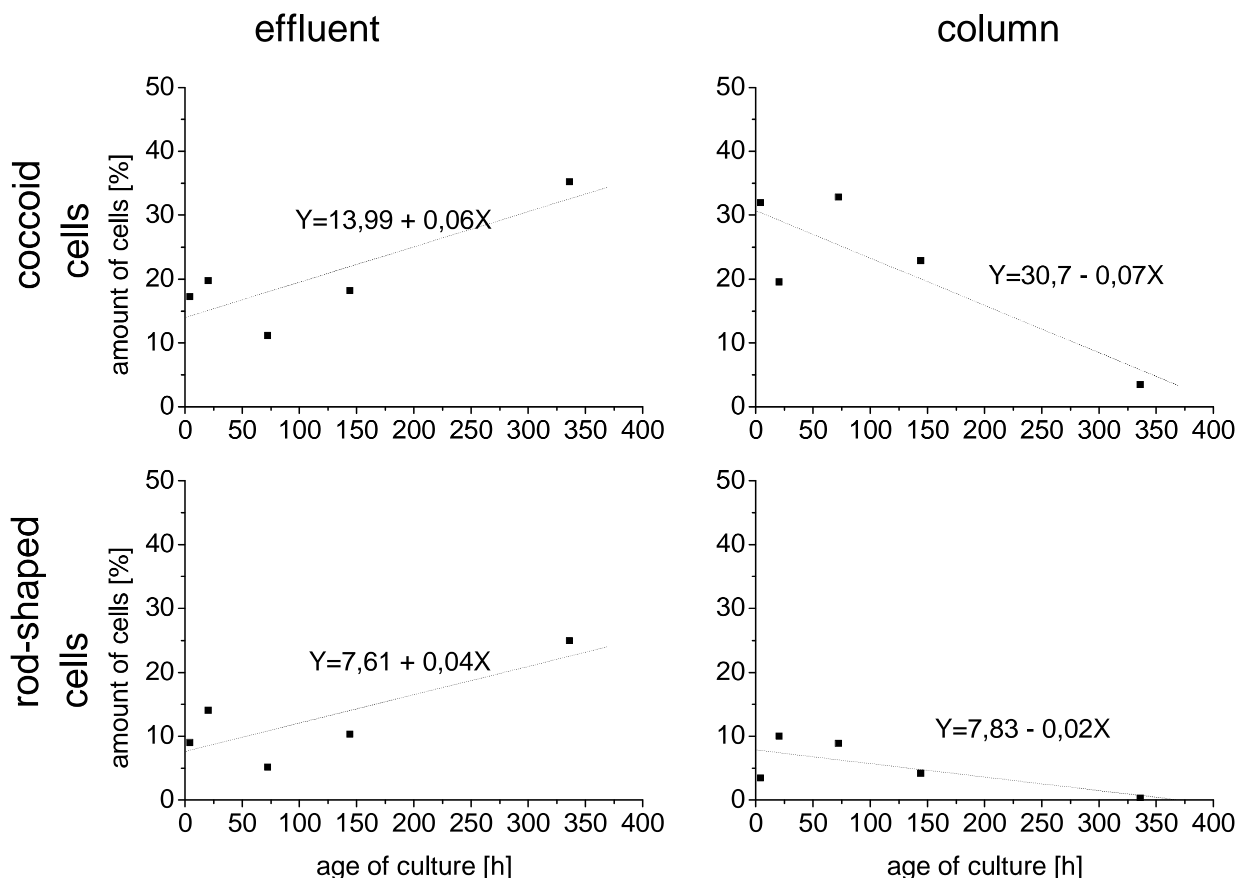

Figure 4-7 Composition of effluent (left band) and columns (right band) with coccoid (upper band) and rod-shaped (lower band) cells versus the age of a culture.

Detailed observation of the depth profiles (Figure 4-8) suggests that the amount of rod-shaped cells was slightly decreasing to the outlet whereas the amount of coccoid cells was not changing very much within the single layers of a column. The amount of rod shaped cells changed for fresh cells (20h) from 20 % (average) in the first four layers to 13 % (average) in the last three layers. For old cells (144h) the amount decreased from 12 % (average) in the first four layers to about 7 % in the last three layers. The portion of coccoid cells showed a broader variability in the first layers than in the last: In the first four layers the range was from

35 % to about 50 % for 20h-old cells to 55 % to 60 % for 336h-old cells. The amount of coccoid cells in the last three layers slightly increased for the 20h-old culture from 45 % to 50% (20h) and remained constant at 55 % for the 336h-old culture.

Comparing the effect of the age, a trend was observed: By increasing the age the amount of rod-shaped cells in the first three layers decreased (from mean 23 % for logarithmic phase cells to mean 16 % for late stationary phase cells) whereas at the same time the portion of coccoid cells was increasing (from mean 39 % for logarithmic phase cells to mean 50 % for late stationary phase cells).

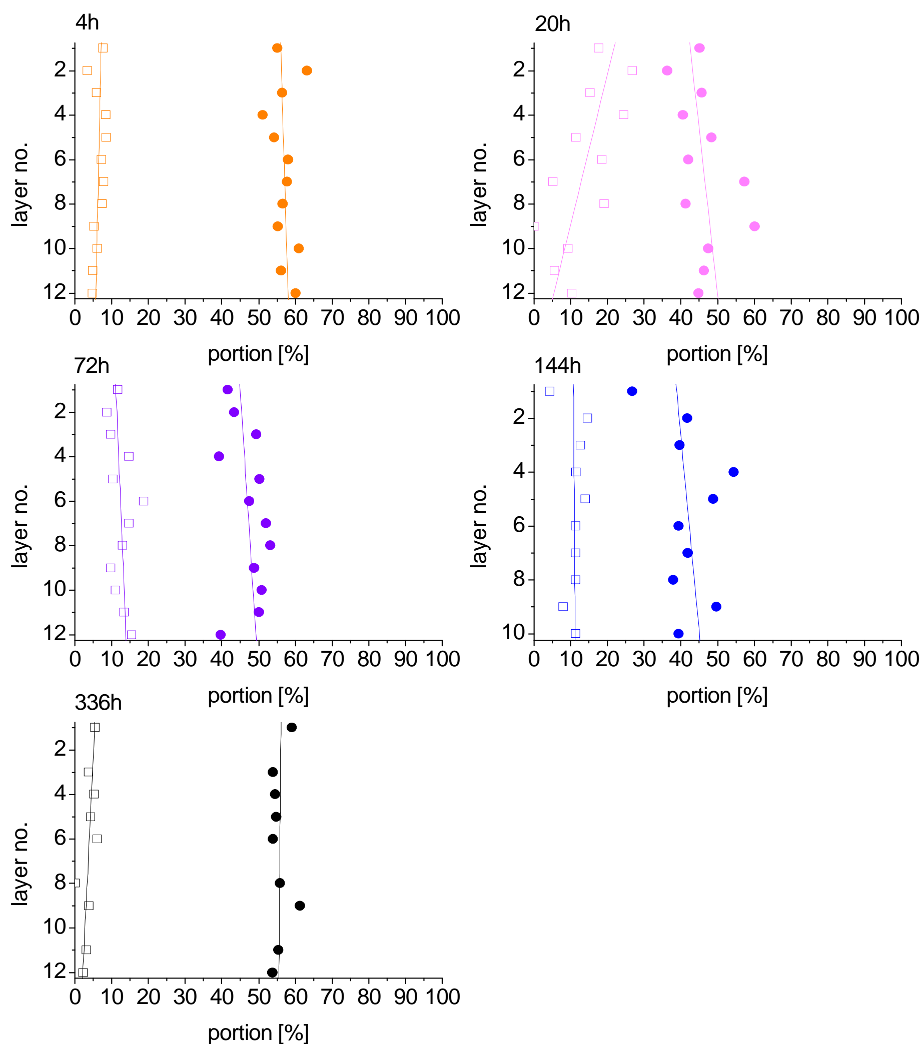

Figure 4-8 Distribution of coccoid and rod-shaped cells in layers. % related to the cell amount in the regarding layer (100%). Points: coccoid cells, squares: rod-shaped cells.

Investigations of the morphology of the cells found in the effluent (Figure 4-9) showed that the amount of rod-shaped cells is highest in fractions in proximity of the maximum breakthrough. At the same time the amount of coccoid cells is decreasing in the proximity of the maximum breakthrough.

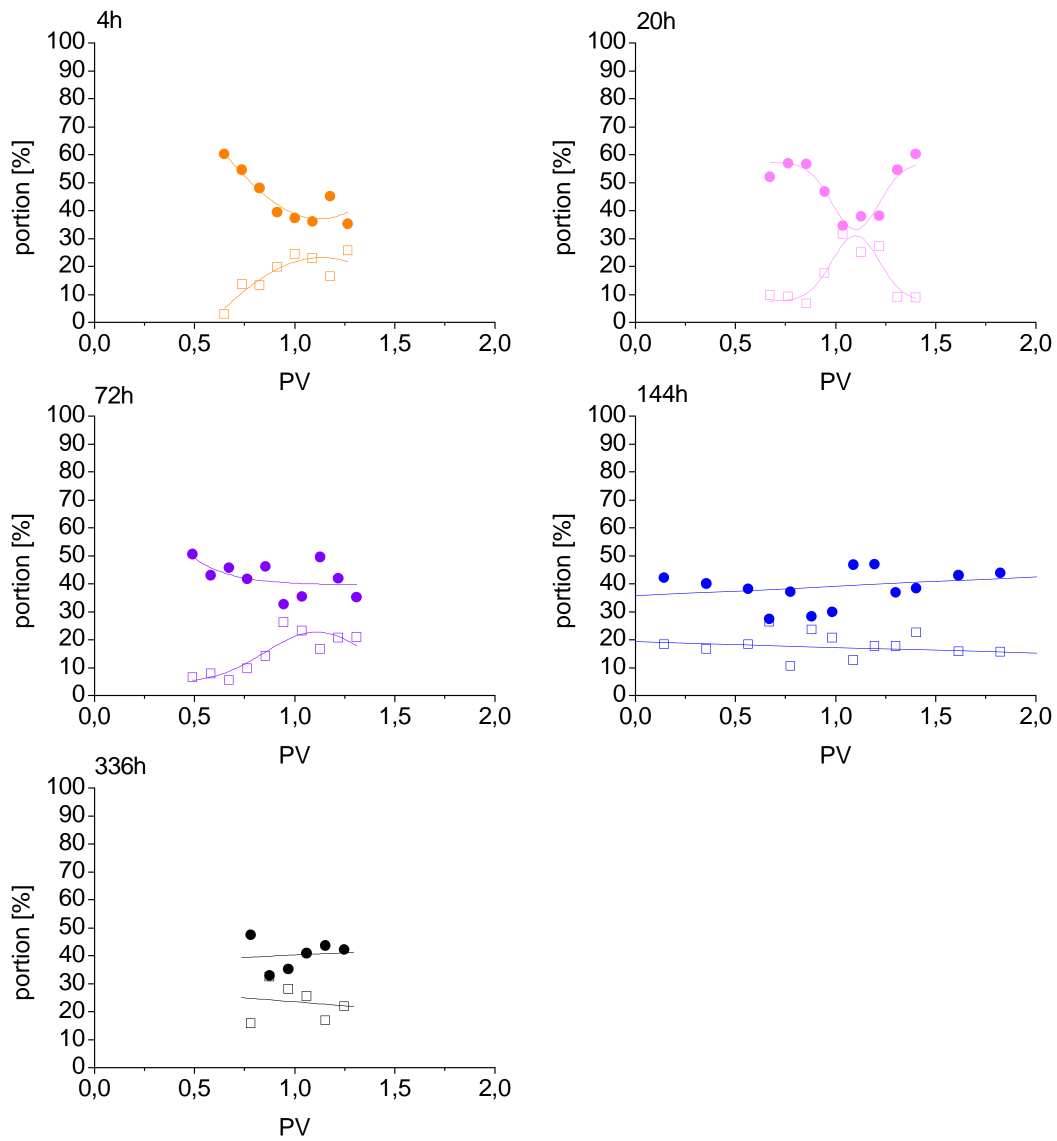

Figure 4-9 Distribution of coccoid and rod-shaped cells in the effluent. % related to the cell amount in the regarding layer (100%). Points: coccoid cells, squares: rod-shaped cells

A general conclusion is that an increase of duration of carbon and energy starvation leads to a greater breakthrough of cells. The morphology of the cells in the effluent is tending to smaller cells whereas the cell retarded inside a column are more rod-shaped.

4.1.4 The morphological change of *P. fluorescens-gfp* under static conditions

The cell increase during the column passage and the change of morphology was examined in batch experiments. It was assumed that during the passage of bacterial cells through a column (>10 hours), the oxygen deficiency was a stress factor. Response to this stress could be the multiplication of cells leading to morphological changes from rod-shaped cells into coccoid cells. The biomass stayed constant.

The experiments were carried out as described before (see chapter 3.5.2). The increase of the amount of cells was determined as the quotient of cell amount after growing without oxygen for 24h and cell amount of a control culture growing with oxygen.

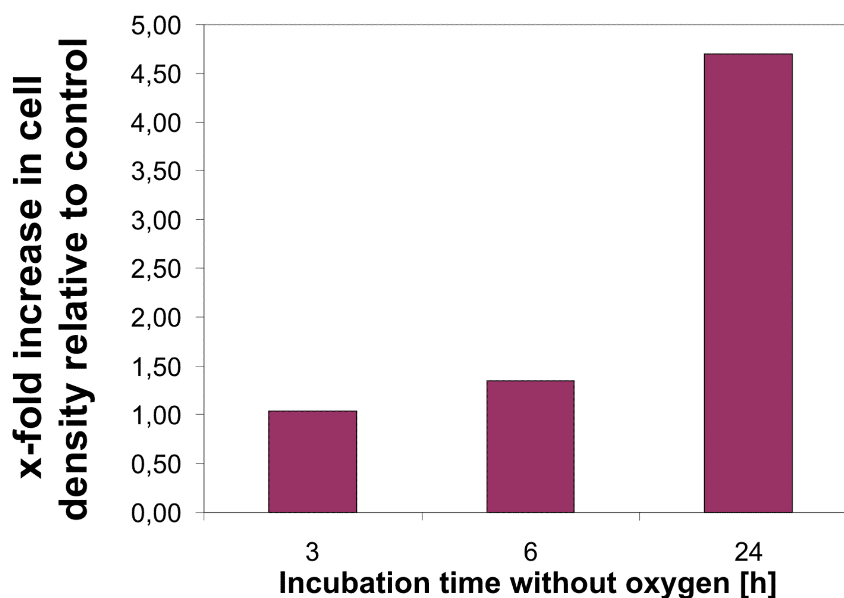

Figure 4-10 Culture of *P. fluorescens-gfp* (18h old, log-phase) were degassed with helium for 1 h and afterwards incubated without oxygen. The increase of cell amount (y-axis) is plotted against the incubation time (x-axis) after degassing.

In Figure 4-10 the increase of cell amount is plotted against the incubation time of a culture after degassing the oxygen for 1 h with helium. At the start the cell amount of degassed and control culture do not differ, but after additional 6 h the degassed sample shows a cell increase of approximately 1.5 time more than the control under normal oxygen conditions. The effect is greatest after 24h when the degassed sample consists of more than 4.5 time more cells than the control culture does. After growing under degassed conditions samples were taken to investigate the growing potential of the stressed cells. To control the recover potential of stressed cells in comparison to cells grown under standard conditions the cfu quota was determined (Figure 4-11). The degassed sample (30 min) delivered a value of 0.6, the control one of 0.55, thus, the stressed cells can recover to a standard level and are able to build more colony forming units.

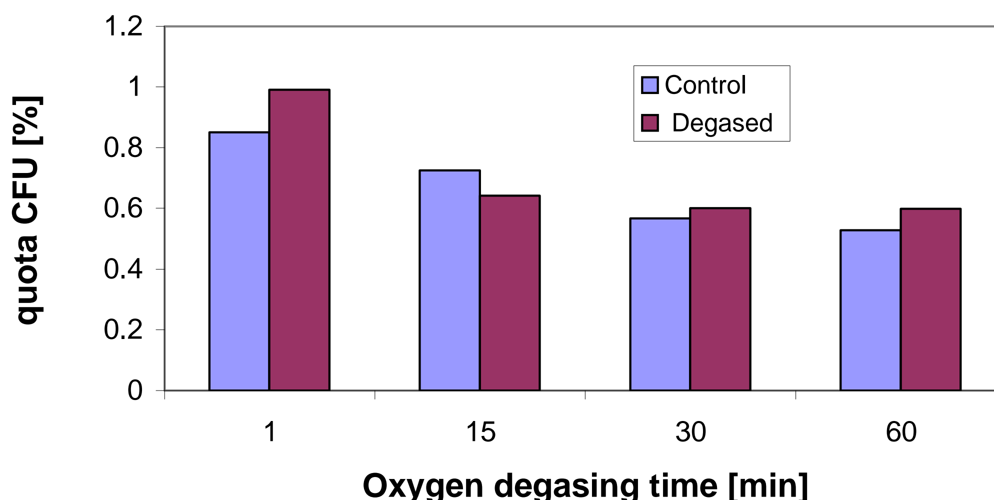

Figure 4-11 Quota of viable cells after different oxygen degasing times. The viability is measured by colony forming units of treated cells in comparison to a control. Quota cfu [%]: amount of cells that are able to recover.

The morphological change which occurs after 24h in a degassed culture (Figure 4-12) was significant compared to the control, in which air access was possible. In a control the amount of rod-shaped cells is higher (36 %) than the amount of coccoid cells (26 %), whereas the ratio in the degassed culture is vice versa (35 % coccoid cells, 25% rod-shaped cells). For a culture where no fresh air has access (contains a rest of oxygen in its atmosphere, sample 3) the ratio is similar but not so large (34 % coccoid cells and 28 % rod-shaped cells).

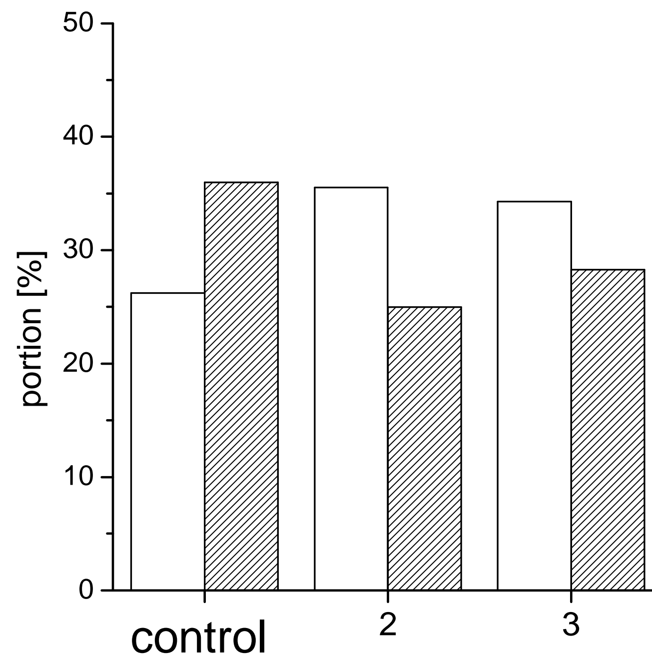

Figure 4-12 Morphological change of degassed cultures after 24 h incubation without oxygen. **control**: shaker, air access possible, **2:** shaker, degassed with He, no air exchange, **3:** shaker, airtight (no fresh air, rest of oxygen). White: coccoid cells, grey: rod-shaped cells.

Therefore, lack of oxygen is a stress factor which leads to an increase of cells, which are mostly coccoid. Although these cells are stressed, they are able to grow again when transferred to normal growing conditions, even able to form cfu. This also implicates, that for the breakthrough of bacteria the conditions inside a column are characterized by insufficient air-exchange and therefore magnification occurs without culture growing.

4.1.5 Influence of experimental runtime on bacterial breakthrough

In this work pulse technique was used instead of continuous addition of bacteria. Only few results are presented obtained by pulse technique (Chen J.Y. 2001; Hall J.A. 2005). The influence of runtime on the bacterial breakthrough had to be mentioned because an increase of runtime was assumed to cause elution of cells not in the means of actual breakthrough but in the means of continuous release. This effect was investigated for old cells (late stationary phase) and fresh cells (early logarithmic phase). In literature there are normally investigations

attempted with continuous addition of particle solution (Bradford 2002, 2003, 2006) which led of course to an extension of runtime.

The amount of cells found in the effluent and in the layers was dependent from the runtime of the experiment: Increase of runtime led to continuous release of cells. This effect was significant in experiments with cells of the stationary phase (Figure 4-13). Elongation of runtime from 1.4 PV to 4.8 PV led to a decrease of amount of cells in especially the first half of the column from 38 % to 10 %, the amount of cells in the last section increased from 34 % to 37 %. In sum the amount of cells which was retained in a column decreased from about 73 % to 47 % when the runtime was increased.

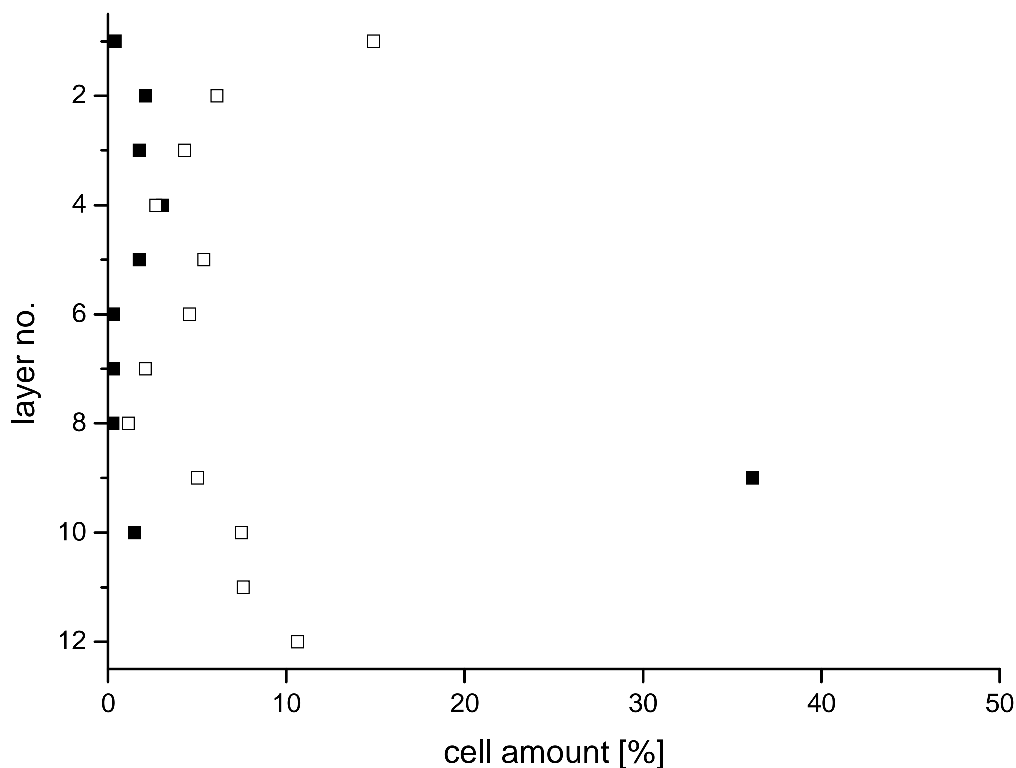

Figure 4-13 Influence of experimental runtime on the distribution of stationary phase cells in a column after breakthrough. White: 1.4 PV, black: 4.8 PV

The effect of runtime on the distribution of cells of the early log phase (Figure 4-14) inside a column was as expected from the experiments with old cells. Experiments were conducted for the duration of 1.3 PV and of 2 PV. The total amount of cells which was retarded inside the

column decreased from 84 % to 19 %. In detail, 28 % of the recovered cells are found in the first half of a column after a short runtime and 57 % in the second half. At increase of the runtime to 2 PV the amount of cells in the first half decreases to 14 % and in the second half to 5 %.

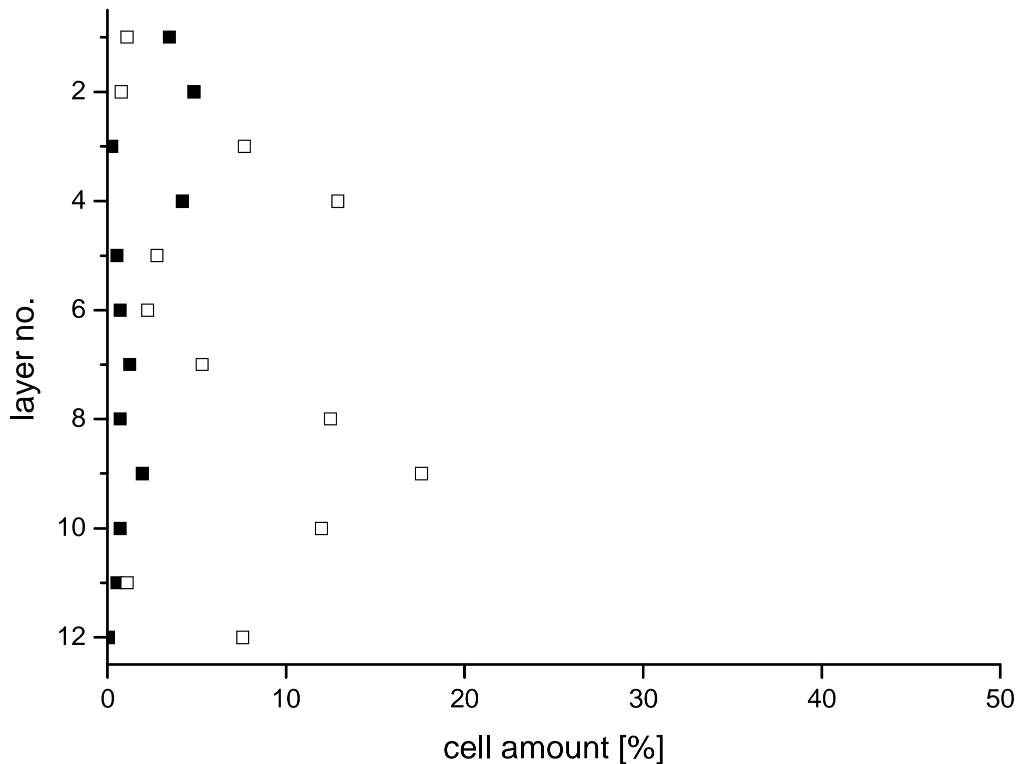

Figure 4-14 Influence of runtime on the distribution of log phase cells in a column after breakthrough under saturated conditions. White: 1.3 PV, black: 2 PV

An increase of the experimental runtime appeared in an increased breakthrough of cells, as well for older as for fresh cells. This indicates that for continuous flow rate a larger amount of cells was washed out which may be an effect of straining.

It can be assumed that for endless application of aqueous solution in case for stationary phase cells all cells are found in the effluent. Considering the viability of cells (even after occurred breakthrough and even for stationary phase cells) and transferred these results to natural environment one can assume that for potentially pathogenic cells the passage from the aquifer to the groundwater is possible and therefore a contamination can take place. In cases of

biofilm production this effect may cause a continuous leaching of cells into the surrounding aqueous system and therefore may act as a (pathogenic) reservoir of cells.

4.2 Discussion

For all experiments the pulse technique was used to add the bacterial suspension onto the column. This was performed to limit the added cells to a level that could be counted after the breakthrough. A continuous addition of a bacterial solution – as described in literature elsewhere - would have made cell counting after breakthrough impossible. In this work the added cell amount remained in the range of 10^8 and 10^9 cells per experiment, continuous addition meant the addition of 100-times of this amount.

4.2.1 Bacterial breakthrough compared to breakthrough of microspheres and D_2O

Microspheres in similar shape and dimension as used tracer bacteria were applied in transport experiments as a kind of standard. Shape shifting and particle increase effects could be normalized to this standard.

The possible reasons for the simultaneous breakthrough of *P. fluorescens-gfp* and D_2O at ~ 1 PV and the significant earlier breakthrough of *P. fluorescens-gfp* compared to the microspheres assumed to be (1) different sizes of bacteria and microspheres, (2) different surface-characteristics, (3) movement by flagella and (4) oxygen-stress and favourite routes along the matrix-column body interface.

(1) For the old small bacteria (1-4 µm length, ~ 1 µm diameter) on the one hand and the small particles (1 µm in diameter) on the other hand straining is the main reason for the delayed breakthrough and high retardation (Bradford div.; Murphy 2000). This effect elucidates the phenomenon that small particles are trapped in micropores and throats of the matrix which disables further transport. The older cells reach a maximum breakthrough of pore volumes short past 1 whereas the larger fresh cells exhibit a breakthrough before 1 PV. For the inactive microspheres this is significantly shifted to pore volumes of ~1.5. A concentration dependent

straining can be excluded, because both experiments were conducted with a total of ~ 10^9 cells/particles. The same results are obtained by Becker et al. (Becker 2004), where compared to bacterial breakthrough the breakthrough of negatively charged microspheres took place later. At low flow rates bacterial attachment increases to increase the ability to grow. But for motile bacteria like P. fluorescens the flagella are not only the part for preferred attachment to matrix surfaces but also the part which enable the bacterium to free itself, to detach (McCaulou 1995, McClaine 2002). Thus, at inconvenient conditions this detachment behaviour of especially fresh cells would explain the earlier breakthrough. The other way round, inorganic particles are only attached by surface-interactions and cannot free themselves actively.

(2) The more hydrophobic cell-surface of bacteria prevents interactions with the silica matrix, whereas the microspheres are involved in stronger H-bonds between their surface and the matrix. The repulsion forces of bacterial cell surface and the matrix are responsible for the bacteria resting in a resulting fast water front. This leads to an early breakthrough of < 1 PV (LIT).

(3) Fresh cells of *Pseudomonas fluorescens-gfp* exhibit flagella which allow forward movement additionally to the movement caused by water flux. Increasing age of bacteria leads to decreasing content of flagella, which is completely missing for resting cells.

(4) The lack of oxygen inside the column causes a directed movement of especially fresh cells of about 40 µm/sec (Schlegel 1992; Sen 2005) on the one hand towards the column outlet and on the other hand along favoured routes at the column-matrix interface that may exist. These routes are accessible for both, bacteria and microspheres, and could be visualized in a system of quartz sand under saturated conditions and microspheres by the method of macrophotography (Klauth P. submitted). It could be demonstrated that a high amount of particles were attached to the regions adjacent to the column body, assuming favourable breakthrough of particles. However, parallel straining and absent active motility prevented the small particles from breaking through the column. In contrast to this hypothesis, a gradient of oxygen and chemotaxis could lead to movement towards the inlet, where oxygen is supplied by fresh medium.

4.2.2 Morphological change of *Pseudomonas fluorescens-gfp* as a cause for breakthrough behaviour and retardation. The impact of oxygen deficiency as observed under static conditions.

Beside the flagella movement mentioned above two main aspects are assumed to have a high impact on the transport behaviour of *P. fluorescens-gfp* through quartz sand columns and the succeeding distribution inside the columns: (1) different sizes and shapes as well as (2) changing hydrophobicity. Varying age of the used cultures and the oxygen deficiency inside the column were the reasons for these two effects explained in the following.

(1) Wilhelm et al. (1998) showed that the size of resting cells of *P. fluorescens* from the stationary phase was only a third of the size of growing cells (log phase) as it was observed by Smets et al. (1999), too. In the experiments of this thesis the size shrinkage was strongly dependent on the age - and therefore the duration of carbon starvation - of the used culture. The metabolic active cells of about 20h showed a higher potential for the cell division of the rod-shaped cells into several coccoid cells than the older cells did. Only the size shrinkage with cell enhancement in parallel would result in a later breakthrough of older cells compared to the breakthrough of fresh cells because the old, smaller cells would have been trapped in micropores and the fresh, larger cells go through a process like in size exclusion chromatography.

(2) The surface composition of cells changes significantly during the cell cycle. Chen and Strevett (Chen 2001; Strevett 2003) showed that although the zeta-potential was not changing during the lifetime of a culture, the composition of the cell membrane did. Results from Sanin et al. (Sanin 2003) obtained by MATH-test showed changes in hydrophobicity with increasing age of the cells. Older cells showed a higher hydrophobicity than fresh cells did. Assigned to the system used in this work the effect of higher hydrophobicity of old cells would lead to an earlier breakthrough of stationary phase cells of P. *fluorescens-gfp* caused by lower interactions of cell and matrix surface. If only the hydrophobicity would be considered for bacterial breakthrough, and retention respectively, logarithmic phase cells would break through later due to stronger interactions of the regarding surfaces. Moreover this would lead to a retardation of cells. This phenomenon is described in literature dealing with breakthrough experiments under unsaturated conditions, where the breakthrough behaviour of different bacteria with different cell-surface characteristics were conducted as a function of the degree of saturation (Schaefer 1998).

Only theoretical investigations about total free energy were made by Chen and Strevett (2001). They came to the conclusion that cells of the logarithmic phase showed less hydrophobicity (higher hydrophilicity) than stationary phase cells. At the same time these stationary phase cells were calculated to have a higher trend to attachment which resulted in higher deposition on the matrix. In sum, they proposed, that if only the total free energy would be mentioned, logarithmic cells would break through in a higher amount, stationary phase cells would be retained. The results of this work are not consistent with the described results of Chen and Strevett. The observed different breakthrough curves and retardation profiles can not be explained by only one physico-chemical value. Also the chemical factors like surface-composition and physical dimensions like size have an impact on the distribution between effluent and column of cells of a certain age

In Figure 4-15 a sketch is shown to summarize the obtained features of breakthrough and retardation of *P. fluorescens-gfp*. The resulting effect is dependent from the age of the cells. Weighting of a single effect is changing with varying age of the culture.

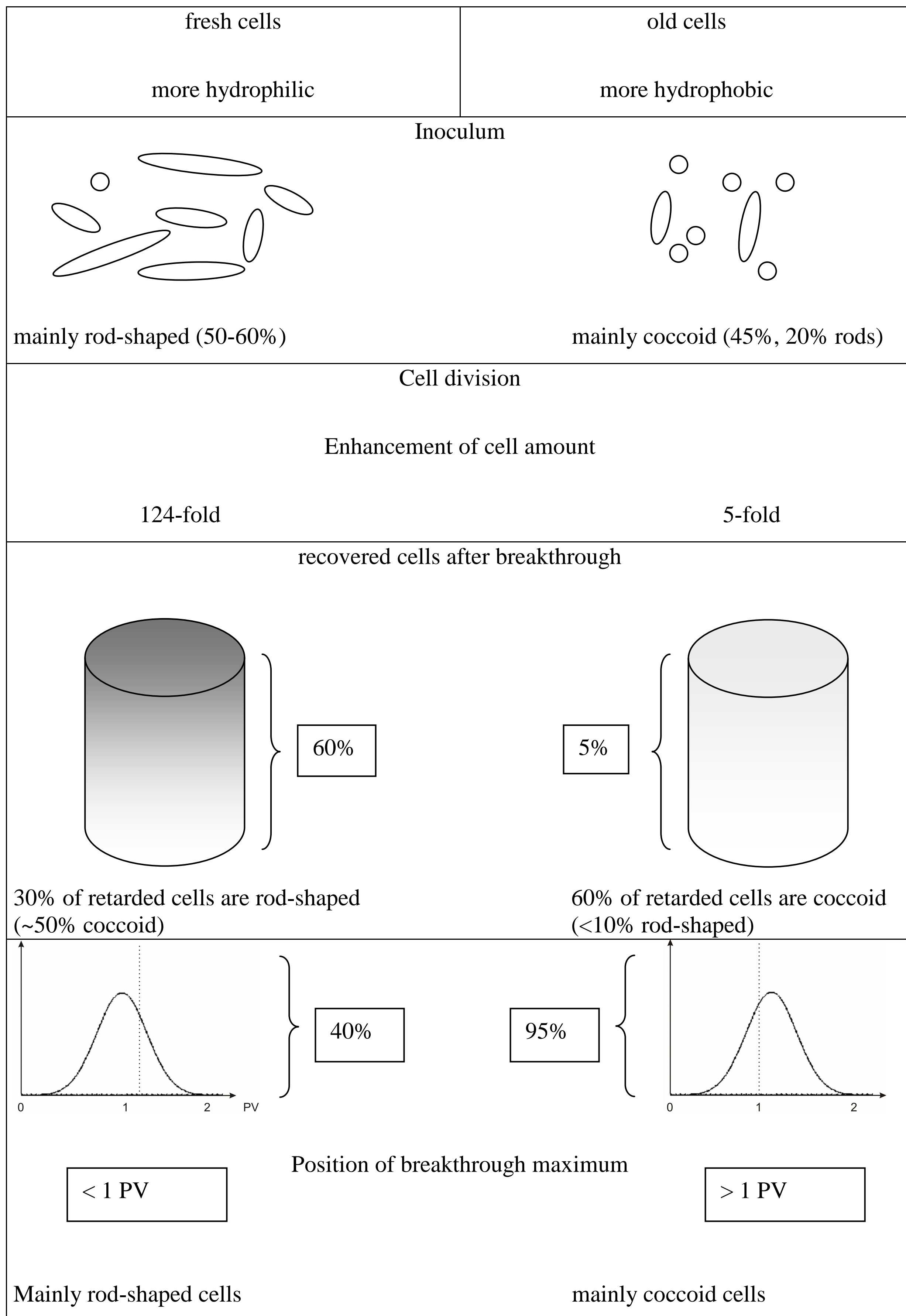

Figure 4-15 Summary of results obtained by breakthrough as a function of age of culture.

Conferred to the obtained data in this work the quantity and relevance of a single effect (trapping in micropores, size-exclusion and hydrophobicity) could not be determined because in some cases they went in opposite directions:

● Fresh, hydrophilic cells broke through earlier than old cells: The large cells which missed cell division broke through at first due to size exclusion. The left coccoid cells were trapped in pores inside the matrix as occurred in the experiments with old cells. For a great part of the large cells inside a column cell-division led to an increase of cell amount, that is, the emerging small cells were trapped in micropores inside the column. .Additionally the cells were attached to the matrix due to H-bonds of the more hydrophilic cell-membrane of the fresh cells. These effects resulted in a higher deposition/retardation and lower breakthrough of the greatest part of fresh cells.

● Although older cells were assumed to be trapped in micropores (which is the explanation for the later breakthrough maximum), they were nearly found quantitatively in the effluent due to the higher hydrophobicity, which prevented them from being attached to the matrix. This detachment seemed to be the main factor influencing the breakthrough of older cells, because there was hardly any deposition found.

The obtained data of the breakthrough curves and retention profiles indicated that the effect of hydrophobicity and therefore the interaction between the surfaces of cells and matrix is smaller than the effect of size exclusion of large and trapping of small cells. Therefore, the changing shape from rod-shaped to coccoid and the possibility of fresh cells to move on their own are the main factors influencing the actual breakthrough and the distribution inside a column.

4.2.3 Investigations of the morphological change of *P. fluorescens-gfp* under static conditions in chemostat and batch experiments

Sanin et al. (2003) found size shrinkage of *Pseudomonas* from rod shaped to spherical shape with increasing age after starvation for carbon (or nitrogen). The reason for this behaviour was that the cells tend to increase their surface area to volume ratio as a response to starvation. Thereby the ability to transport necessary nutrients into the cells with minimum energy consumption should be adhered.

The effect of oxygen deficiency can be described according to these results, because cell-size-decrease may be the reason for the response to the oxygen-deficiency which occurred during the passage of cells inside the column although the time range of the oxygen-starvation (hours) was significantly smaller than the one for chemical starvation (days or weeks). The strength the response occurred was dependent from the age of the used culture. For other gram-negative bacteria it is known that older cells are more stress-resistant than fresh cells are and therefore the response does not have to be as large as for the fresh cells (Smeulders 1999). This is consistent with the results of this work where the increase of cell amount due to stress-response for old cells is a fractional part (4-fold increase compared to the inoculated amount of cells) of the one of fresh cells (125-fold increase compared to the inoculated amount of cells).

To get an idea how the oxygen deficiency has an influence on the bacterial behaviour, the experiments were performed under static conditions. These chemostat and batch experiments showed that oxygen deficiency causes fast cell response (in hours) similar to chemical starvation for carbon of nitrogen (days and weeks). It could be proved that oxygen deficiency caused cell size shrinkage, and therefore morphological change occurred. The size shrinkage was attended by increase of cell amount due to cell division. This cell division was basically possible for young cells, so that the increase of cell amount was observed especially in fresh cultures.

The resulting size shrinkage as a result of oxygen deficiency is a fast occurring effect in the range of several hours in contrast to real nutrient starvation and it results not only on size/shape but also on surface composition after days or weeks. Therefore, the oxygen deficiency should be considered for experiments under saturated conditions as a main cause for cell enhancement when working with logarithmic phase cells.

In sum, the factors which are responsible not only for the breakthrough but also on the spatial distribution inside a column are found at the side of the used cultures as well as at the side of the used system. For the cultures the age is the main factor affecting the size and shape as well as the composition of the outer cell membrane. For the system the saturated conditions without substrate lead to limited oxygen availability and therefore a cell response occurs via cell division.

5 Breakthrough behaviour of *Pseudomonas fluorescens-gfp* under continuous addition of substrate

5.1 Results

5.1.1 Distribution of cells in effluent and column depending on the age of the used cultures

The experiments under saturated conditions were performed under continuous substrate addition at a concentration of 0.5 g/l glucose in minimal medium (M 461), the experimental runtime was extended due to the start of growing inside the column and therefore not only the pure first breakthrough could be detected by fluorescence signal but also the breakthrough behaviour afterwards in dependence from the age of the inoculated culture (Figure 5-1). The enhancement of cell amount (ratio recovered to inoculated cells) is given in Table 5. As mentioned before 4.1.1 the fluorescence signal was observed for qualitative causes. The BTC themselves were plotted as cell amount versus PV (Figure 5-1). The acquired cell amount again was the sum of the recovered cells in effluent and column, a detailed partition in cell growing and cell-division as stress response was therefore not possible.

duration of carbon and energy starvation of inoculated culture [h]	Enhancement (ratio recovered to inoculated amount of cells), x-times
5	8.7
24	81.7
96	3.1
500	12

Table 5 Recovered amount of cells compared to inoculated cells. Enhancement in dependence of duration of carbon and energy starvation of the inoculated culture [h].

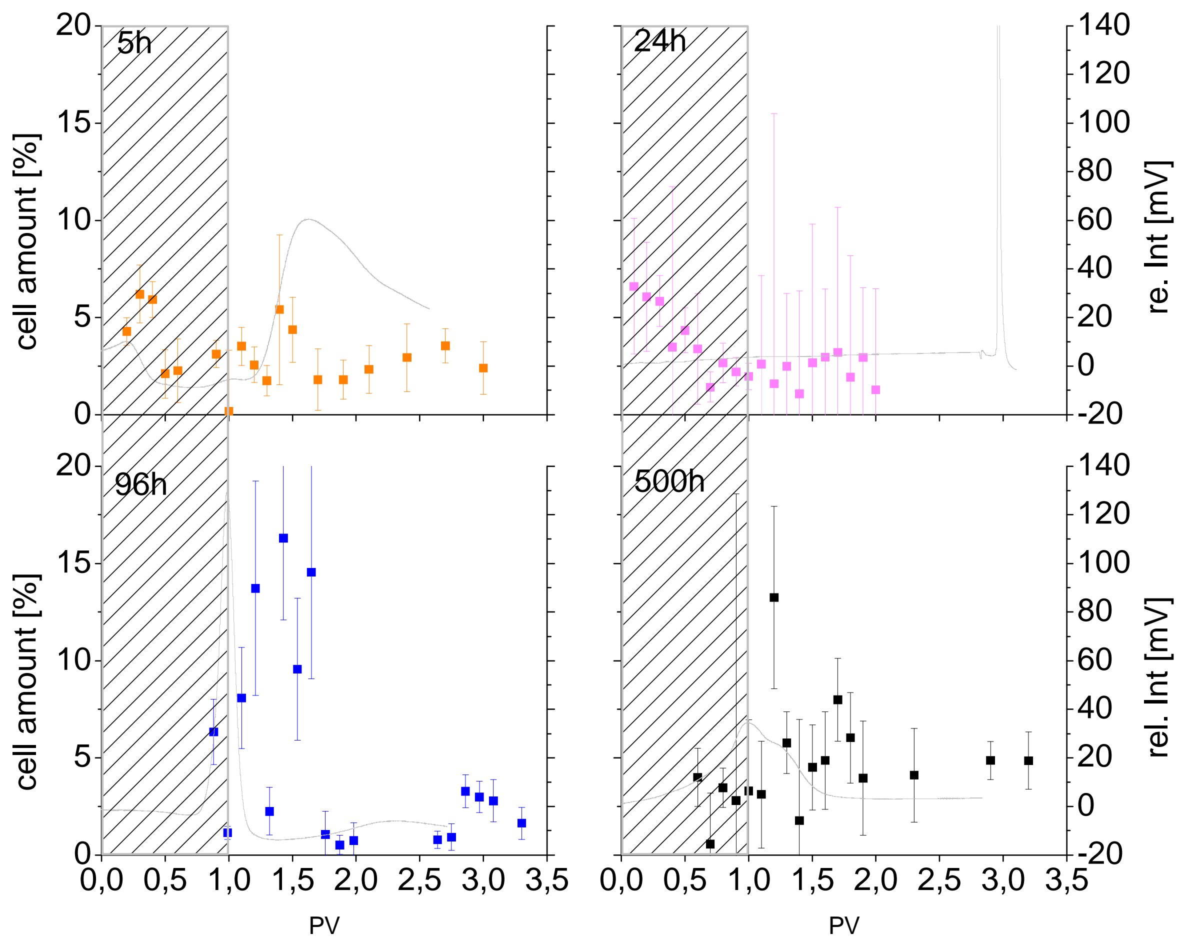

Figure 5-1 Breakthrough curves of *P. fluorescens-gfp* (different ages) through quartz sand columns under saturated conditions and continuous addition of substrate (glucose (0.5 g/l)). A fitting was not possible due to the complex shape of the obtained BTCs. Grey line: fluorescence signal, grey box: indicates the part of the BTC < 1.0 PV

For further investigations of cell amount the part of the BTC before 1.0 PV and after 1.0 PV were examined in detail. The obtained amounts of cells are listed in Table 6.

Age of culture [hr]	Amount of cells found in the effluent before 1.0 PV [%]	Amount of cells found in the effluent after 1.0 PV [%]	Amount of cells in effluent [%]
5	24.1	32.4	56.5
24	38.0	22.8	60.8
96	7.5	79.2	86.7
500	14.1	65.1	79.1

Table 6 Amount of recovered cells in the effluent before and after 1.0 PV in dependence from the age [% of total recovered amount of cells in the experiment]

For a culture of 5h of growing, where the change in morphology, physiological state and surface characteristics are mostly different. The first breakthrough observed by fluorescence detection took place nearly directly after injection, which contains 24.1 % of all cells. A small shoulder before 1 PV and the main breakthrough after 1.6 PV contained 32.4 % of all cells. As well as for the experiment without substrate in the regarded substrate experiments it was important to note, that the recovery time (the time the culture needed to start growing and to enter the logarithmic phase) of the inoculum of the culture for the 5h experiments had an influence on the original composition of the inoculum of the column (see 4.1.2).

A fresh growing culture of 24h showed no unique breakthrough, the fluorescence signals showed only a slight increase during the runtime, the high peak at the end was a technical artefact. To keep the division of the BTC as in the other experiments the amount of cells before and after 1.0 PV were determined: 38 % of all cells broke through before and ~23 % after 1.0 PV.

The best detectable breakthrough curve via fluorescence signal was obtained for 96h old cultures with a main peak and a shoulder of breakthrough later at about 2.3 PV. The main breakthrough obtained by cells counting was shifted to higher pore volumes. Thus, before 1.0 PV only 7.5 % of all cells were recovered and the largest amount of about 79 % afterwards.

Old cultures of about 500h showed a broader peak of the fluorescence signal suggesting two occurring breakthrough curves which could not be examined separately. The defined border of examination of 1.0 PV delivered 14 % of all cells before and 65 % of all cells after this border 1.0 PV.

The age of culture had also an important influence not only on the shape of breakthrough curve itself but also on the cell distribution between effluent and column (Figure 5-2).

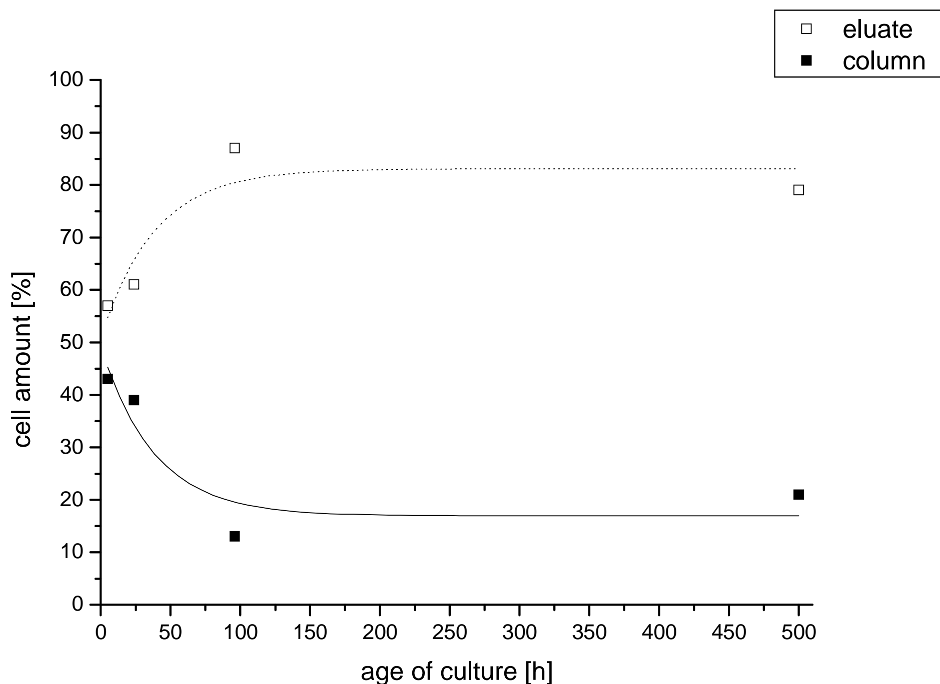

Figure 5-2 Distribution of cells in effluent and column depending on the age of a culture.

For log-phase cells, 5 and 24 h old, 44 % and 40 % respectively of these cells remained in the column, even after a long runtime of more than 2.5 PV. This is in contradiction to the results of experiments without substrate, in which 20 % of the used fresh cells were retained in the column after a runtime of 2 PV (see Figure 4-14). For older cells the pattern looked corresponding: despite the long runtime (of more than 2 PV) 15 % to 20 % of cells remained in the column. Without substrate cells of these old cultures broke through nearly to an amount of 95 %.

The retention profiles of the breakthrough experiments are shown in Figure 5-3. There was no distinct pattern in either experiment. Within a regarded column the distribution of cells was nearly unique, only a slightly higher amount of cells at the inlet and the outlet was observed. Among several layers the difference of retained cells was about 1.1% to 7 % (for the 5h old culture), 1 % to 4.1 % (for the 24h old culture), 0.1 % to 1.5 % (for the 96h old culture, 6 % of cells in the first layer should be the exception) and 0 % to 5.8 % (for the 500h old culture).

For *Pseudomonas fluorescens-gfp* the addition of substrate led to a greater breakthrough than without substrate. The retention profiles showed no definite shape for none of the used cultures, that means the distribution of cells inside the column with substrate seems to have no preferential paths or deposition sites.

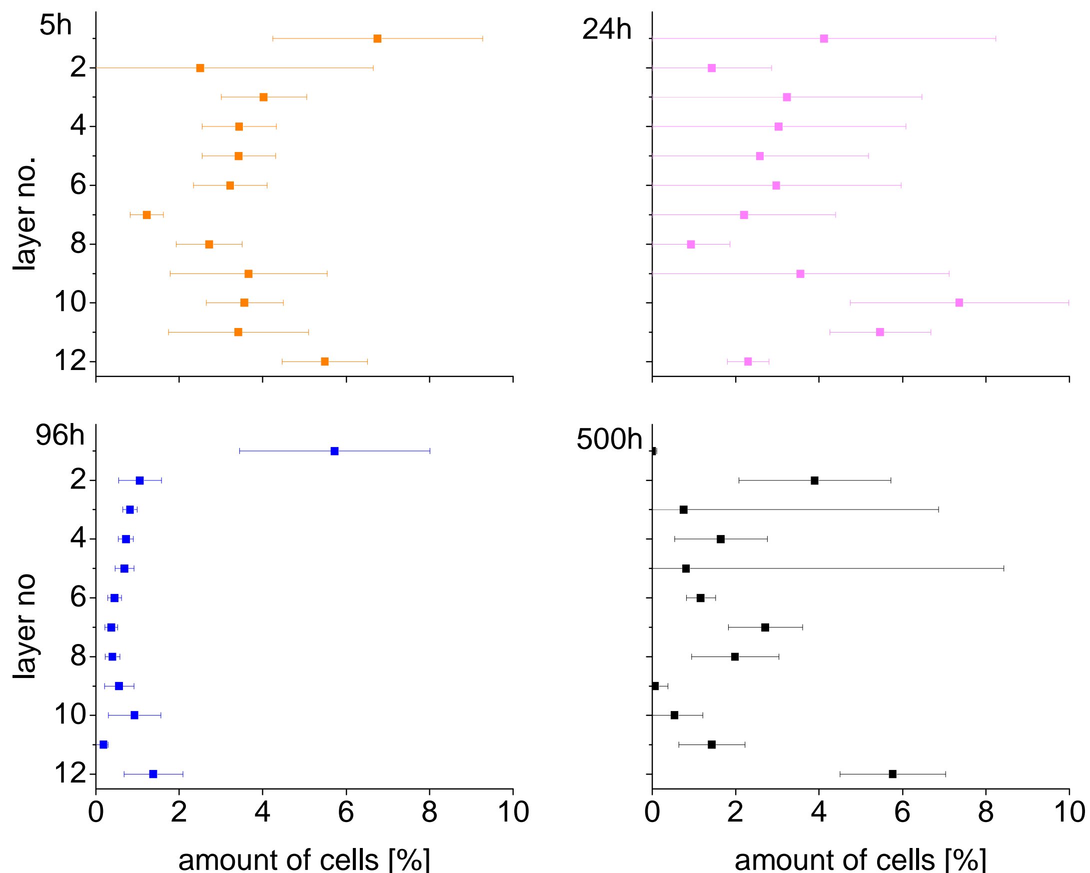

Figure 5-3 Retention profiles of columns after breakthrough of *Pseudomonas fluorescens-gfp* of different ages under continuous substrate addition. Layer numbers (from inlet to outlet) versus retained cell amount.

5.1.2 Morphological aspects

The morphological composition of effluent and column is given in Figure 5-4. In these investigations only coccoid and rod-shaped cells were considered. The morphological species of ellipsoid cells was ignored as mentioned before.

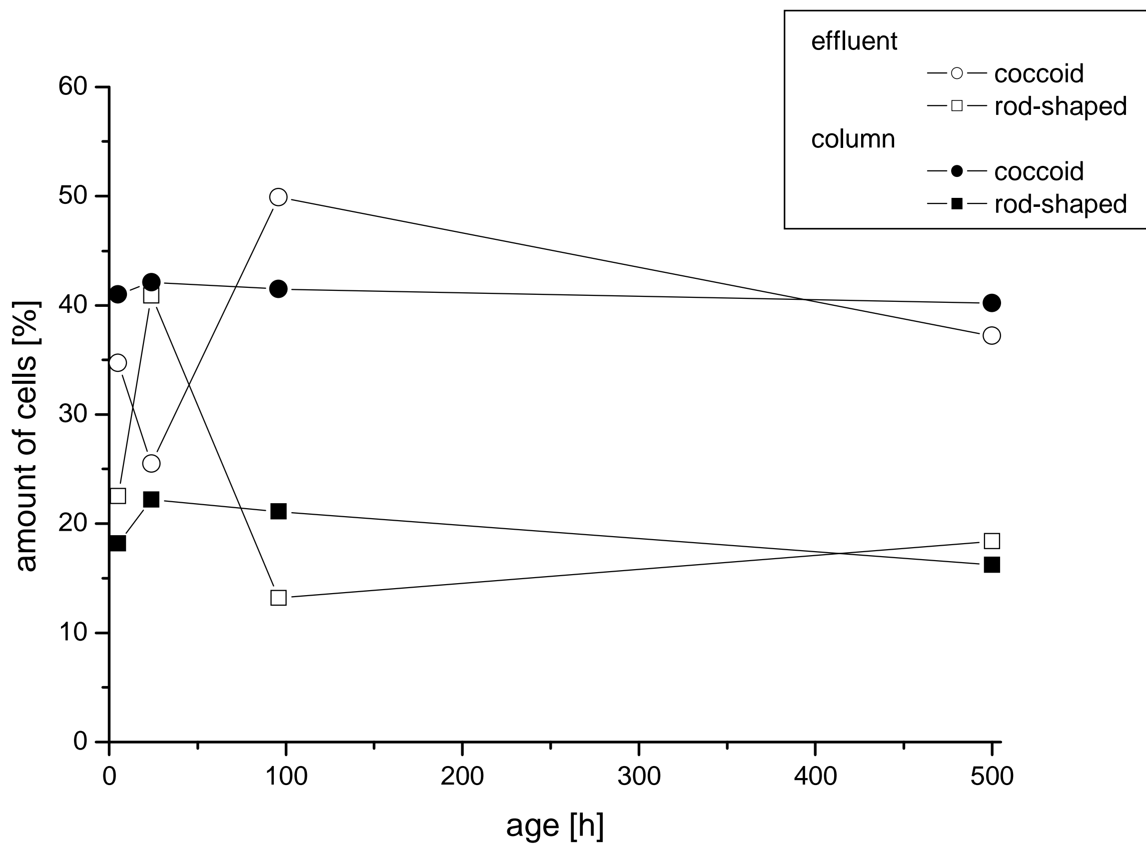

Figure 5-4 Distribution of coccoid and rod-shaped cells in effluent and column after the breakthrough under substrate-addition as a function of duration of carbon and energy starvation of the used culture [h].

The amount of coccoid and rod-shaped cells in the column appeared to be independent from the age of a culture; the amount remained constant about 20 % and 40 % respectively with a slight decrease. The portions in the effluent were more influenced by the age of cultures: the amount of coccoid cells was decreasing with increasing age from 5h to 24h whereas the amount of rod-shaped cells was increasing. Further increase of age to 96h and 500h the

amount of coccoid cells increased again to a level of 50 % and finally reached an amount of 39 %, whereas at the same time the amount of rod-shaped cells decreased to a level of 13 % and ended at 19 %.

For the old culture of 500h the differences in composition of effluent and column were evened: The amount of each morphological species in effluent and column was nearly on the same level: about 20 % rod-shaped cells and 40 % coccoid cells.

Figure 5-5 shows the different compositions of the effluent after detailed examination. The amount of the morphological species for the 5h old and the 500h old culture are nearly the same. The greatest difference in composition is found for the 96h old culture.

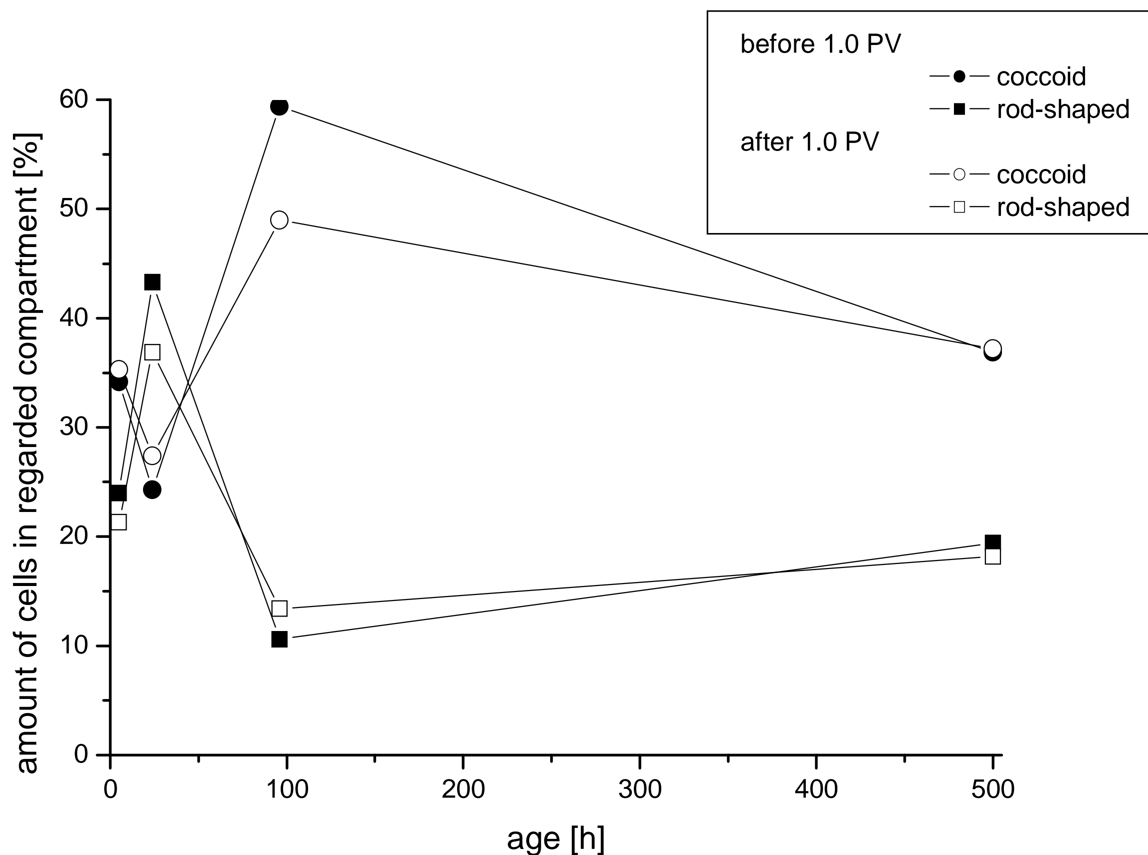

Figure 5-5 Morphological composition of different regions of effluent and column after breakthrough versus duration of carbon and energy starvation (age of culture [h]). left: coccoid cells, right: rod-shaped cells

The investigation of the morphological composition of cells inside the column layers after bacterial breakthrough is shown in Figure 5-6. For the 5h experiment the portion of the coccoid cells as well as the one of rod-shaped cells was not varying significantly within the column layers, 37 % to 45 % for coccoid cells, and 13 % to 23 % for rod-shaped cells. For the 24h experiment a general tendency was observed: the amount of rod-shaped cells was increasing within the column from 10 % at the inlet to 33 % at the outlet, whereas the amount of coccoid cells was decreasing in the same direction from 50 % to 34 %. That means, for the logarithmic phase cells the last layers of a column consisted of nearly the same amount of coccoid and rod-shaped cells.

For the 96h old (stationary phase) cells the amount of coccoid (about 43 %) and rod-shaped cells (about 20 %) within the column remained nearly constant with a slight increase of each species towards the outlet.

For the late stationary/decay phase cells (500h) for coccoid and rod-shaped cells no unique pattern was observed. The fitted line indicated an increase of coccoid and rod-shaped cells from inlet to outlet. The quota of rod-shaped cells with values from 12 % to 20 % was not as much differing as the quota of the coccoid cells with values of 30 % to 50 %.

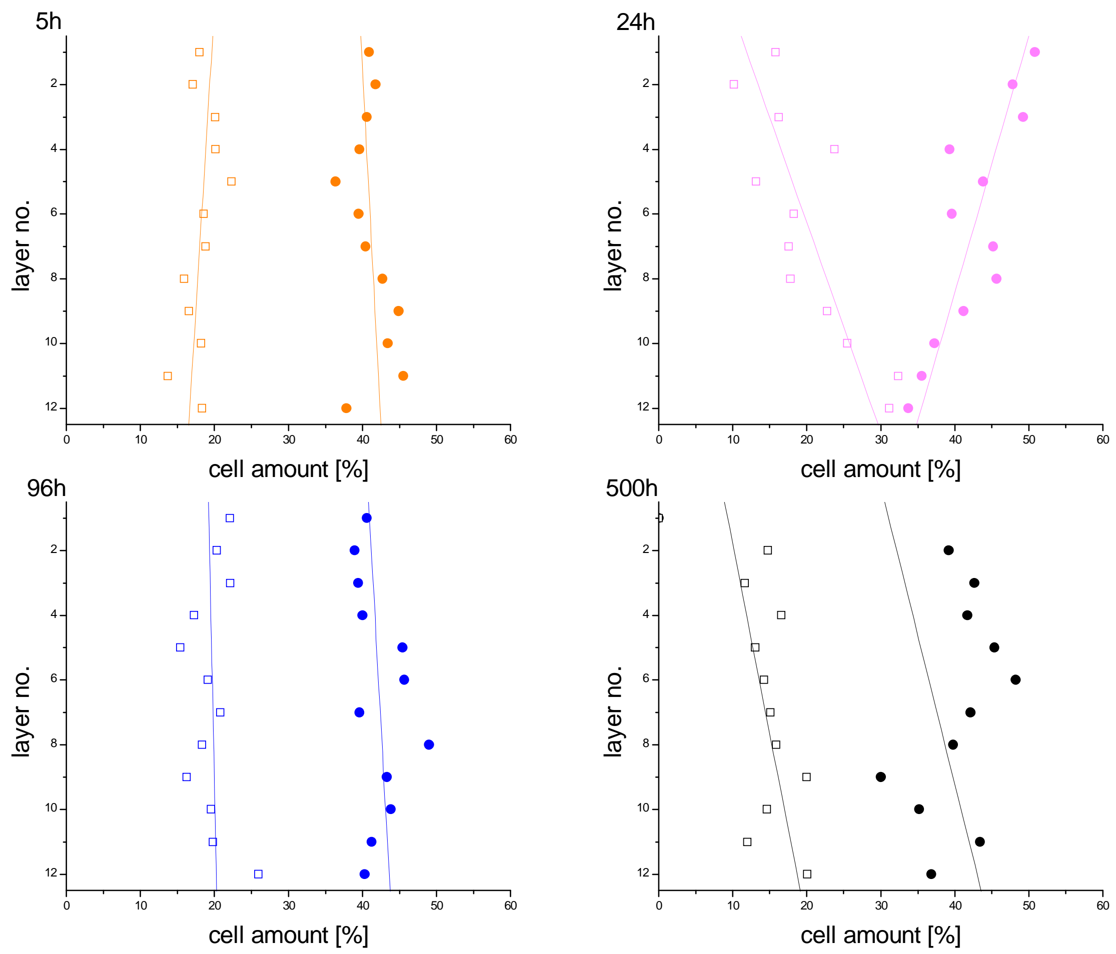

Figure 5-6 Morphological composition of single layers in columns after breakthrough. Points: coccoid, squares: rod-shaped cells

5.2 Discussion

5.2.1 General aspects

Up to now there are only a few paper in which results of bacterial transport under saturated conditions and simultaneously substrate addition (Murphy 1997, 2000; Jordan 2004) were obtained. To simulate the aquifer saturated with the water of the groundwater beneath, the cell amount should not exceed a level of 10^5 to 10^6 per ml. Pure groundwater generally exhibits a sum cell concentration of 10^4 per ml (Uhlmann 1982), therefore the adjacent soil should have a cell amount of 10^4 or one to two magnitudes higher. Hence the disadvantage of the above mentioned works is the high cell amount of cells due to equilibration of saturated columns with cell suspensions of 10^7 cells per ml throughout the hole columns. The resulting changes and observations can only be seen as results of biodegradation essays, where the limited amount of substrate (salicylate) is degraded by an excess of biomass and therefore no culture growing is observable. Conditions in natural systems are different.

In contrast to the setup used in literature, the cell amount used in this work was limited by inoculating only approximately 10^8 to 10^9 cells (for a filling of 100 g sand) to ensure that any effect of substrate-addition could be acquired. Assuming real growing of culture the amount of the emerging cells should not exceed a value compared to the amount of cells in natural systems. As observed from results of experiments without substrate the limiting factor inside a column was the oxygen availability. This oxygen could act as the final electron acceptor of the degradation of excess of glucose by a definite amount of biomass. Moreover, influence of growth on the bacterial breakthrough and spatial distribution inside the column could be detected.

As described for the experiments without substrate the breakthrough behaviour of cells was unexpected. Especially the breakthrough of the young cultures of 5h and 24h had to be investigated carefully. On the base of the fluorescence signal breakthrough appeared directly after injection. The corresponding cell amount of the regarding fractions of the effluent, measured as microscopic counts, showed also a breakthrough of cells. Furthermore, comparison with the regarding breakthrough curve of D_2O indicated that the package of the column was filled correctly. Compared to results in literature the breakthrough of cells before 0.8 PV is described the first time. This early breakthrough can be explained by the oxygen

deficiency inside the column, as mentioned above (see 4.2.3). This aerotactic effect could be seen as a special chemotactic effect as described by Murphy (Murphy 2000) and Sen et al. (2005) in their modelling considerations. Other possible reasons like preferential flow paths or boundary effects could be responsible, too, but have been present also in the experiments without substrate (see above). These boundary effects were excluded by conducting D_2O-breakthrough before bacteria were added.

Chemotactic effect in the regarding system is not based on the substrate availability but on the limitation of oxygen inside a column. As results without substrate indicate the oxygen deficiency leads to cell increase accompanied with a change in the morphological ratio. Under substrate addition the increase of cells is enhanced additionally. This phenomenon was described as mother-daughter or shedding cells (Murphy 2000). It is assumed that the mother cell is attached to the matrix and the emerging daughter cell is released to the aqueous phase. This behaviour would explain the higher amount of cells in the effluent, but not the faster breakthrough of cells in the effluent in presence of substrate than in the effluent without substrate. An explanation could be the oxygen deficiency of not only the saturated system but also the substrate availability resulting in a faster use of oxygen. This forces the cells to a faster breakthrough. This faster breakthrough can be established by the own movement by flagella motion. The assumed velocity of a bacterium that is able to move via flagellae is about 20 to 60 µm/sec (Schlegel 1992; Sen 2005). Assuming a mean velocity of 40 µm per sec, the covered distance of a cell would be 2.4 mm per minute or 14.4 cm per hour. Transferred to the experimental setup of this work (12 cm length of column), the cells could reach the column outlet in 1 hour. In 1 hour a column (PV of ~42 ml) is washed for a seventh part (flow rate 0.1 ml/min). If this could happen, the first cells could reach the outlet after 1/7=0.16 PV. In this work breakthrough experiments were performed with fresh cells under simultaneous substrate addition were the first cells were recovered after 0.2 PV.

A model of Sen et al. (2005) predicts no influence of substrate to bacterial breakthrough for experiments lasting less than 10 pore volumes at all. Their model is only valid for more than 10 up to 60 pore volumes therefore it cannot be transferred to the system of this work. The general statement that for low pore volumes the influence of substrate addition is negligible is disproved in this work. In the same paper estimations about the influence and the magnitude of chemotaxis are described.

Under substrate-missing conditions the cells do not show this behaviour, although they are active (they divide) and they pass the column significantly earlier than inactive particles do. Hence it is assumed that substrate addition leads to a higher chemotactic effect, because the limiting factor – oxygen-availability – is under growing conditions worse than just for conditions in which the cells tend to keep their survival.

Under this aspect the breakthrough of a higher amount of cells under substrate addition becomes clear because inside the column the conditions for effective use of the glucose are worse than in the effluent where oxygen is available again. This effect results in a cell amount of maximum of 8 % cells in each layer. The distribution of fresh cells inside a regarded column was not significantly changing compared to the one of old cells. In either of the experiments cells were found throughout the column without preferences. The morphological species inside single layers were changing only for logarithmic cells.

The morphological change of cells in the presence of substrate was more complex than in the experiments without substrate. Not only the stress of oxygen led to an increase of cell amount also real culture growing occurred. This was not as much as under standard conditions in liquid culture due to a limitation of oxygen. The culture growth had an effect on the breakthrough shape of the fluorescence intensity signal. For logarithmic phase cells the signal showed a broad range with no distinct peaks. These peaks were observed only for old stationary phase cells. The spreading of morphological different species (coccoid and rod shaped cells) between effluent and column in dependence of the age of the culture showed that for young cells (logarithmic and early stationary phase) the amount of rod shaped cells in the effluent decreased whereas the amount of coccoid cells increased. The amount of cells which is retarded inside a column is decreasing until an age of culture of 24h, at the same time the amount of cells breaking through was increasing. From 24h on the amount in both compartments stayed constant. For cells of the stationary phase the amount of every species in each investigated compartment stayed constant. This is observed for the species distribution inside a column, too. That means no change from coccoid to ellipsoid shape or from rod to ellipsoid shape occurred.

5.2.2 Comparison of bacterial breakthrough without and with substrate-addition

Compared to the work of Jordan et al. (2004) the fairly simple system used in this work (quartz sand under saturated conditions and addition of substrate continuously) has the advantage that results of the experiments and conclusions from experiments without substrate could be transferred to the substrate experiments. The results of Jordan et al. omits the fact that transport of bacteria through any saturated sand or soil column leads to stress response of the cells. As shown in this work this response may lead to cell enhancement that has to be distinguished from with real culture growth. This enhancement occurred due to cell division of fresh rod-shaped cells into several smaller coccoid cells.

The most striking difference of substrate-missing and substrate-addition experiments was the amount of cells in effluent and column as a function age of the used culture. In experiments without substrate the increase of cells in effluent and simultaneously the decrease of cells in the retention profile of the column showed exponential decrease in depth. This resulted in a point of intersection at the age of ~ 100h. From this age onwards the gap of cell amount in effluent and column was increasing with increasing age. Before this point of intersection the different amounts were not so significant. This point of intersection may be assumed as a point where a change in cell cycle occurred, the change from stationary to decay phase, accompanied by surface composition and size shrinkage. This change was also seen in investigations of Chen et al. (2003) where different surface compositions were observable in IR-measurements.

The amount of cells in effluent and column as a function of age under substrate addition showed exponential decrease of biomass in depth, too. The resulting amount of cells in effluent was always higher than the amount inside the column. With increasing age the amount of cells in effluent increased and in column decreased (see Figure 5-2). The distribution between effluent and column did not change with increasing age, i.e. there was no point of intersection where the distribution changed. This indicated that due to substrate addition the differences of cells of different ages where negligible that means that all cells started growing. The only difference was the fact that older cells needed more time to recover to enter the logarithmic growth phase.

The amount of coccoid cells in a column decreases linearly with increasing age when there is no substrate available. The amount of rod-shaped cells was also decreasing linearly, too, but

with a smaller slope of the fitted straight line. The amount of coccoid and rod-shaped cells in the effluent increased linearly with similar slopes. The change of total cell amount in the regarding compartment is due to the increase the third morphological species, the ellipsoid cells. These cells were not taken into account in all the investigations because their quota was calculated as the difference to 100 % beside coccoid and rod-shaped cells.

The results from the experiments with substrate addition showed that with increasing age decrease of retarded cells occurred uniformly and affected both, coccoid and rod-shaped cells in the same level: the amount stayed constant. In contrast, the increase of cells in the effluent accompanied by changing composition of coccoid and rod-shaped cells occurred until it reached a constant level at the age of 100h.

Results lead to the conclusion, that without substrate and for culture ages of less than 100h the stationary phase characteristics are valid. Thus the breakthrough takes place for about 50 % of the cells. At the same time the increase of cell amount compared to the amount of the inoculum is reaching its maximum. For breakthrough experiments under substrate addition the spreading of cells between effluent and column, as well as spreading between coccoid and rod-shaped cells, occurs for culture ages of less than 100h.

6 Summary, conclusion and Outlook

The transport-behaviour of active cells differed from the one of inactive particle like microspheres. The breakthrough of active P. fluorescens-gfp took place in the same range than the one of a conservative tracer like D_2O. The gfp-modification of *Pseudomonas fluorescens* is suitable for online monitoring bacterial breakthrough in column-experiments. The used pulse-technique for application of the cell-suspension onto the column had the advantage of limited and countable amounts of cells. The continuous application of cell suspension as described in literature makes it impossible to monitor single differences or even to detect effects concerning single cells. The used saturated conditions of a quartz sand column gave the basic results for balancing bacterial breakthrough in order to examine the effect of substrate availability. The breakthrough of *Pseudomonas fluorescens*-gfp was extremely depending on the age of the used bacterial culture, i.e. duration of carbon and energy starvation prior to the breakthrough experiment, in both cases, without and with substrate-addition. Bacterial breakthrough without available substrate occurred in the same time range than conservative tracers like D_20 do. Compared to this, breakthrough of inactive particles like microspheres occurred far later. One possible explanation could be the presence of flagella in *P. fluorescens*. They enable the cell not only for preferred attachment but also for preferred detachment under inconvenient conditions. So this effect had a higher influence for fresh than for old cells. A second explanation would be trapping of microspheres in micropores, which prevented them to be flushed out. Old cells were found nearly completely in the effluent after the breakthrough. The retardation of fresh cells was higher than for old cells, whereas the part of the fresh cells that broke through showed a faster breakthrough than old cells do. The fresh cells were found in the regions near the column inlet. With increasing age of the used culture the distribution of the cells extended throughout the whole column. The morphological differences of the used inocula were determined and the morphological change during the breakthrough experiment was examined. During the breakthrough an increase of amount of cells occurred, whereas this increase was higher for fresh than for old cells. Along with this increase of cell amount came a morphological change from long rod-shaped cells to small coccoid cells. This change was a result of the limited oxygen availability inside the column. This effect was also examined and described for batch-experiments, where oxygen-limitation leaded to a cell amount increase inside liquid-culture flasks. The resulting small cells were viable at a level of nearly 100%. The resulting breakthrough of the different

morphological species in the same culture can be detected as a distinct distribution of cells in column and effluent.

Continuous substrate addition with a pulse of bacteria at the same time led to a detected breakthrough of cells far earlier than under substrate-free conditions. The distribution of cells in column and effluent differed not so much than for substrate-free conditions. The increase of culture age showed no significant change of effects for cells older than 70 hours. All distributions (cells in column/effluent) or quota of coccoid/rod-shaped cells reached constant levels and showed parallels whereas under substrate-free conditions compared values diverged.

In general for further breakthrough experiments the metabolic state of used active cells has to be monitored. For balancing the transport and prediction of bacterial breakthrough factors like stress during the experiment or growing of culture during transport processes have to be evaluated before, the assumption of bacteria behave like inorganic, immobile particles is obsolete. In experiments especially with substrate alls factors influencing bacterial breakthrough have to be determined before under substrate-free conditions, the effect of oxygen-limitation has to be controlled, especially when xenobiotic should serve as substrates. The morphological change of *P. fluorescens* is probably also valid for other bacterial strains even when cells of different ages show normal morphological differences (like *E. coli*). For other bacterial strains the basic experiments of the given setup (quartz sand, saturated conditions, no substrate) should be conducted to examine whether the morphological change occurs or other effects occur. A general prediction of bacterial transport behaviour seems to be difficult. The transport of bacteria through larger soil columns or lysimeters may only be summarized by counting cells in the effluent but prediction of bacterial behaviour in soil, where substrate is available but not evenly distributed, or even prediction of bacterial location seems impossible. For this purpose, the effect of soil heterogeneity in composition and particle size, the presences of macropores (e.g. worm-wholes) and local available substrate (“hot spots”) should be determined first.

7 References

Agranovski V., Ristovski Z., Hargreaves M., Blackall P. J., Morawska L. (2003). "Performance evaluation of the UVAPS: influence of physiological age of airborne bacteria and bacterial stress." Aerosol science **34**: 1711-1727.

Becker M. W., Collins S. A., Metge D. W., Harvey R. W., Shapiro A. M. (2004). "Effect of cell physicochemical characteristics and motility on bacterial transport in groundwater." Journal of Contaminant Hydrology **69**: 195-213.

Bergey`s manual of systematic bacteriology, 1st. edition, 1986

Bradford S. A., Simunek J., Bettahar M., van Genuchten M. T. Yates S. R. (2003). "Modelling Colloid Attachment, Straining, and Exclusion in Saturated Porous Media." Environmental Science and Technology **37**: 2242-2250.

Bradford S. A., Yates S. R., Bettahar M.,Simunek J. (2002). "Physical factors affecting the transport and fate of colloids in saturated porous media." Water resources research **38**(12,1327): 1-12.

Bradford S. A., Bettahar M. (2006). "Concentration dependent transport of colloids in saturated porous media." Journal of Contaminant Hydrology **82**: 99-117.

Brunninger B.M., Mano D. M. S., Scheunert I., Langenbach T. (1999). "Mobility of the Organochlorine Compound Dicofol in Soil Promoted by *Pseudomonas fluorescens*." Ecotoxicology and Environmental Safety, Environmental Research, Section B **44**: 154-159.

Bundt M., Widmer F., Pesaro M., Zyer J., Blaser P. (2001). "Preferential flow paths: biological "hot spots" in soils." Soil Biology & Biochemistry **33**: 729-738.

Camper A. K., Hayes J. T., Sturman P. J., Jones W. L., Cunningham A. B. (1993)."Effects of motility and adsorption rate coefficient on transport of bacteria through saturated porous media" Applied and Environmental Microbiology **59** (10): 3455-3462

Chen J.Y., Ko C.-H., Bhattacharjee S., Elimelech M. (2001). "Role of spatial distribution of porous medium surface charge heterogeneity in colloid transport." Colloids and Surfaces A: Physicochemical and engeneering aspects **191**: 3-15.

Chen G., Strevett K. A. (2001). "Impact of surface thermodynamics on bacterial transport." Environmental Microbiology **3**(4): 237-245.

Chen G., Zu H. (2004). "Bacterial deposition in porous medium as impacted by solution chemistry." Research in Microbiology **155**: 467-474.

Corapcioglu M. Y., Haridas A. (1984). "Transport and fate of microorganims in porous media: a theoretical investigation." Journal of Hydrology **72**: 149-169.

Dong H. (2002). "Significance of electrophoretic mobility distribution to bacterial transport in granular porous media." Journal of Microbiological Methods **51**: 83-93.

Dunn A. M., Silliman S. E., Dhamwichukorn S., Kulpa C. F. (2005). "Demonstration of microbial transport into the capillary fringe via advection from below the water table." Journal of Hydrology **306**: 50-58.

Ellwood D. C., Keevil C. W., Marsh P.D., Brown C. M., Wardell J. N. (1982). "Surface-associated growth." Philos. Trans. R. Soc. London Ser. B **297**: 517-532

Foppen J.W.A., Mporokoso A., Schijven J.F. (2005). "Determining straining of *Escherichia coli* from breakthrough curves." Journal of Contaminant Hydrology **76**: 191– 210.

Fouchard S., Abdellaoui-Maane Z., Boulanger A., Llopiz P., Neunlist S. (2005). "Influence of growth conditions on *Pseudomonas fluorescens* strains: a link between metabolite production and the PLFA profile." FEMS Microbiology Letters **251**: 211-218.

Gannon J. T., Manilal V. B., Alexander M. (1991) "Relationship between Cell Surface Properties and Transport of Bacteria through Soil" Applied and Environmental Microbiology **57** (1): 190-193

Ginn T. R., Wood B. D., Nelson K. E., Scheibe T. D., Murphy E. M., Prabhakar Clement T. (2002). "Processes of microbial transport in the natural subsurface." Advances in Water research **25**: 1017-1042.

Givskov M., Eberl L., Møller S., Poulsen L.K., Molin S. (1994). "Responses to nutrient starvation in Pseudomonas putida KT2442: analysis of general cross-protection, cell shape, and macromolecular content." Journal of Bacteriology **176**(1): 7-14.

Hall J.A., Mailloux B. J., Onstott T.C., Scheibe T.D., Fuller M.E., Dong H., DeFlaun M.F. (2005). "Physical versus chemical effects on bacterial and bromide transport as determined from on site sediment column pulse experiments." Journal of Contaminant Hydrology **76**: 295– 314.

Hase C., Moënne-Loccoz Y., Défago G. (2001)."Survival and cell culturability of biocontrol *Pseudomonas fluorescens* CHA0 in lysimeter effluent water and utilization of a deleterious genetic modification to study the impact of the strain on numbers of resident culturable bacteria". FEMS Microbiology Ecology **37**(3): 239-249

Jewett D.G., Logan B. E., Arnold R. G., Bales R. C. (1999). "Transport of *Pseudomonas fluorescens* strain P17 through sand columns as a function of water content." Journal of Contaminant Hydrology **36**: 73-89.

Jordan F. L., Sandrin S. K., Frye R. J., Brusseau M. L., Maier R. M. (2004). "The influence of system complexity on bacterial transport in saturated porous media." Journal of Contaminant Hydrology **74**: 19-38.

Klauth P., Wilhelm R., Klumpp E., Poschen L., Groeneweg J. (2004). "Enumeration of soil bacteria with the green fluorescent nucleic acid dye Sytox green in the presence of soil particles." Journal of Microbiological Methods **59**: 189-198.

Klauth P, BauerR., Ralfs C., Ustohal P., Vanderborght J., Vereecken H., Klumpp E. (submitted)."Fluorescence macrophotography as a tool to visualise and quantify spatial distribution of deposited colloid tracers in porous media" Colloids and Surfaces A

Klein, I. (1996). Reaktivierung von Pseudomonas fluorescens aus Hungerkulturen. Fachbereich Chemie und Biotechnik. Fachhochschule Aachen.

Leeson A., Kelly M. E., Rifai H. S., Magar V. S., Ed. (2001). International In Situ and On-Site Bioremediation Symposium, San Diego, California, June4-7, 2001. Columbus, Ohio, Battelle Press.

de Lorenzo V., Herrero M., Jakubzik U., Timmis K. N. (1990). "Mini-Tn5 Transposon Derivatives for Insertion Mutagenesis, Promoter Probing, and Chromosomal Insertion of Cloned DNA in Gram-Negative Eubacteria." Journal of Bacteriology **172**(11): 6568-6572.

Lovins K. W., Angle J. S., Wiebers J. L. and Hill R. L (1993). "Leaching of *Pseudomonas aeruginosa*, and transconjugants containing pR68.45 through unsaturated, intact soil columns" FEMS Microbiology Ecology **13** (2): 105-111

Mallen G., Maloszewski P., Flynn R., Rossi P., Engel M., Seiler K.-P. (2005). "Determination of bacterial and viral transport parameters in a gravel aquifer assuming linear kinetic sorption and desorption." Journal of Hydrology **306**: 21–36.

McCaulou, D.R., McCaulou, R.C., McCaulou, B., Arnold, R.G. (1995)."Effect of temperature controlled motility on transport of bacteria and microspheres through saturated sediment." Water Resources Research **31** (2): 271–280.

McClaine, J.W., Ford, R.M. (2002)."Characterizing the adhesion of motile and nonmotile *Escherichia coli* to a glass surface using a parallel-plate flow chamber." Biotechnology and Bioengineering **78** (2): 179– 189.

McGechan M. B., Lewis D. R. (2002)."Transport of Particulate and Colloid-sorbed Contaminants through Soil, Part 1: General Principles" Biosystems Engineering **83** (3): 255–273

Murphy E. M., Ginn T. R., Chilakapati A., Resch C. T., Phillips J. L., Wietsam T. W., Spadoni C. M. (1997). "The influence of physical heterogeneity on microbial degradation and distribution in porous media." Water resources research **33**(5): 1087-1103.

Murphy E. M., Ginn T. R. (2000). "Modelling microbial processes in porous media." Hydrogeology Journal **8**: 142-158.

Oates P. M., Castenson C., Harvey C. F., Polz M., Culligan P. (2005). "Illuminating reactive microbial transport in saturated porous media: demonstration of a visualization method and conceptual transport model." Journal of Contaminant Hydrology **77**: 233-245.

Ojha A. K., Mukherjee T. K., Chatterji D. (2000). "High intracellular level of guanosine tetraphosphate in *Mycobacterium smegmatis* changes its morphology of the bacterium." Infection and Immunity **68**(7): 4084-4091.

Pang L., Close M., Noonan M. (1998). "*Rhodamin* WT and *Bacillus subtilis* transport through an alluvial gravel aquifer." Ground Water **36**(1): 112-122.

Pitkäjärvi J., Räsänen L. A., Langenskiöld J., Wallenius K., Niemi M., Lindström K. (2003). " Persistence, population dynamics and competitiveness for nodulation of marker gene-tagged *Rhizobium galegae* strains in field lysimeters in the boreal climatic zone." FEMS Microbiology Ecology **46** (1): 91-104

Poschen L., Klauth P., Groeneweg J., Wilhelm R. (2002). "A filtration, incubation and staining reactor including a new protocol for FISH." Journal of Microbiological Methods **50**: 97-100.

Rijnaarts H. H. M., Norde W., Lyklema J., Zehnder A. J.B. (1995). "The isoelectric point of bacteria as an indicator for the presence of cell surface polymers that inhibit adhesion." Colloids and Surfaces B: biointerfaces **4**: 191-197.

Ripp S., Nivens D. E., Werner C., Sayler G.S. (2001). "Vertical transport of a field-released genetically engineered microorganism through soil." Soil Biology & Biochemistry **33**: 1873-1877.

Rockhold M.L., Yarwood R. R., Niemet M.R., Bottomley P.J., Selker J.S. (2002). "Consideration for modelling bacterial-induced changes in hydraulic properties of variably saturated porous media." Advances in Water research **25**: 477-495.

Sambanis, A. (1985). Experimental and modelling studies on the dynamics of culture of the ciliate *Tetrahymena pyriformis* grown on several bacterial species. Minneapolis, University of Minnesota.

Sanin S. L., Sanin F. D., Bryers J. D. (2003). "Effect of starvation on the adhesive properties of xenobiotic degrading bacteria." Process Biochemistry **38**: 909-914.

Schaefer A., Ustohal P., Harms H., Stauffer F., Dracos T., Zehnder A. J. B. (1998). "Transport of bacteria in unsaturated porous media." Journal of Contaminant Hydrology **33**: 149-169.

Schlegel, H. G. (1992). Allgemeine Mikrobiologie. Stuttgart - New York, Georg Thieme Verlag.

Scott G. A., Sloman K. A. (2004). "The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity." Aquatic toxicology **68**: 369-392.

Smets B.F., Grasso D., Engwall M.A., Machinist B.J. (1999). "Surface physicochemical properties of *Pseudomonas fluorescens* and impact on adhesion and transport through porous media." Colloids and Surfaces B: Biointerfaces **14**: 121-139.

Smeulders M., Keer J., Speight R., Williams H. D. (1999). "Adaptation of *Mycobacterium smegmatis* to stationary phase." Journal of Bacteriology **181**(1): 270-283.

Stevik T. K., Aa K., Ausland G., Hanssen J. F. (2004). "Retention and removal of pathogenic bacteria in wastewater percolating through porous media: a review." Water research **38**: 1355-1367.

Tan Y., Gannon J. T., Baveye P., Alexander M. (1994). "Transport of bacteria in an aquifer sand: experiments and model simulations." Water resources research **30**(12): 3243-3252.

Tappe W., Tomaschewski C., Rittershaus S., Groeneweg J. (1996). "Cultivation of nitrifying bacteria in the retentostat, a simple fermenter with internal biomass retention." FEMS Microbiology Ecology **19**: 47-52.

Taylor R., Cronin A., Pedley S., Barker J., Atkinson T. (2004). "The implications of groundwater velocity variations on microbial transport and wellhead protection - review of field evidence." FEMS Microbiology Ecology **49**: 17-26.

Timms-Wilson T. M., Bailey M. J. (2001). "Reliable use of green fluorescent protein in fluorescent pseudomonads." Journal of Microbiological Methods **46**: 77-80.

Tufenkji N., Redman J. A., Elimelech M. (2003). "Interpreting deposition patterns of microbial particles in laboratory-scale column experiments." Environmental Science and Technology **37**: 616-623.

Unc A., Gross M. J. (2004). "Transport of bacteria from manure and protection of water resources." Applied soil ecology **25**: 1-18.

van Loosdrecht M. C. .M., Lyklema J., Norde W., Zehnder A. J. B. "Influence of Interfaces on Microbial Activity." Microbiological Reviews **54** (1): 75-87.

van der Mei H. C., van de Belt-Gritter B., Busscher H. J. (1995). "Implications of microbial adhesion to hydrocarbons for evaluating cell surface hydrophobicity. 2. Adhesion." Colloids and Surfaces B: Biointerfaces **5**: 117-126.

Weinbauer M.G., Beckmann C., Höfle M.G. (1998). "Utility of green fluorescent nucleic acid dyes and aluminium oxide membrane filters for rapid epifluorescence enumeration of soil and sediment bacteria." Applied and Environmental Microbiology **64**(12): 5000-5003.

Wilhelm R., Heller O., Bohland M., Tomaschewski C., Klein I., Klauth P., Tappe W., Groeneweg J., Soeder C. J., Jansen P., Meyer W. (1998). "Biometric analysis of physiologically structured pure bacterial cultures recovering from starvation." Canadian Journal of Microbiology **44**: 399-404.

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“Fluorescence macrophotography as a tool to visualise and quantify spatial distribution of deposited colloid tracers in porous media”. Peter Klauth, Reimar Bauer, Carla Ralfs, PetrUstohal, JanVanderborght, Harry Vereecken, Erwin Klumpp. "Colloids and Surfaces A" submitted