

# Characterization of the Diffusive Properties of Biofilms Using Pulsed Field Gradient-Nuclear Magnetic Resonance

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**Abstract:** The mobility of water in intact biofilms was measured with pulsed field gradient nuclear magnetic resonance (PFG-NMR) and used to characterise their diffusive properties. The results obtained with several well-defined systems, viz. pure water, agar, and agar containing inert particles or active bacteria were compared to glucose diffusion coefficients measured with micro-electrodes and those calculated utilising theoretical diffusion models. A good correspondence was observed indicating that PFG-NMR should also enable the measurement of diffusion coefficients in heterogeneous biological systems. Diffusion coefficients of several types of natural biofilms were measured as well and these results were related to the physical biofilm characteristics. The values had a high accuracy and reflected the properties of a sample of ca. 100 biofilms, while non-uniformity or non-geometrical shapes did not negatively influence the results. The monitored PFG-NMR signal contains supplementary information on e.g. cell fraction or spatial organisation but quantitative analysis was not yet possible. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* 60: 283–291, 1998.

**Keywords:** diffusion; biofilms; gels; NMR

## INTRODUCTION

The advantages of immobilized microorganisms compared to suspended cells are widely acknowledged in industrial systems. Immobilized microorganisms can be easily retained in the reactor, which allows high biomass concentrations, short residence times, and facilitates down-stream processing (Karel et al., 1985). Under specific conditions, microorganisms produce exopolymers, which allows them to adhere to surfaces or to each other. Such spontaneously formed biofilms are ubiquitous in nature, and applied in industrial processes like wastewater treatment and off-gas

purification (Characklis and Marshall, 1989; Ottengraf, 1986). Artificial immobilization of microorganisms may be achieved by encapsulation in a polymer matrix, e.g., agar, carrageenan, or alginate (Karel et al., 1985). These artificial biofilms are utilised for the production of fine-chemicals and beverages (Lommi, 1990). In both natural and artificial biofilms, a dense structure is created in which mass transfer is predominantly diffusive. Due to the relatively slow mass transfer rate of substrates and products and the high volumetric reaction rate inside the biofilm, steep concentration profiles develop (De Beer and Van den Heuvel, 1988). This results in less effective, partially penetrated biofilms. Therefore, knowledge of mass transfer properties is considered essential for modeling, design, and scale-up of processes utilizing biofilms.

Biofilm diffusion coefficients are usually obtained from a steady-state flux or an accumulation rate. This requires detailed knowledge of the microbial activity and an appropriate reaction-diffusion model. Measurements are facilitated if the tracer compound is not consumed. To that end, either the biofilm has to be inactivated or a biologically inert tracer has to be used (Libicki et al., 1988; Westrin and Axelsson, 1991). All these methods necessitate biofilms with well-defined shapes, i.e., flat plates, cylinders, or spheres. In practice, however, both natural and artificial biofilms are often irregularly shaped and lack a homogeneous structure (Beefink and Staugaard, 1986; De Beer et al., 1994). This hampers the measurement of effective diffusion coefficients  $D_{\text{eff}}$  in biofilms and, accordingly, reported values vary from 5 to 95% of the diffusion coefficient in pure water (Kitsos et al., 1992; Libicki et al., 1988; Westrin and Axelsson, 1991).

Nuclear magnetic resonance (NMR) has been used to investigate several microbial processes, e.g., in-vivo metabolic activity (Ramos et al., 1994), heavy metal adsorption (Nestle and Kimmich, 1996), membrane permeability (Van

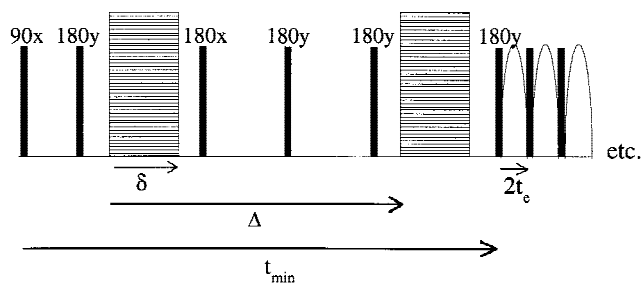
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Zijl et al., 1991), and intracellular diffusion (Tanner, 1983). Because NMR does not require well-defined shapes or homogeneous structures, this technique also seems an ideal method to investigate the diffusional properties of entire microbial biofilms. To this end, pulsed field gradient NMR was used for a systematic study of a series of artificial biofilms with well-defined properties and natural biofilms from several sources. The results were compared to those obtained with microelectrodes to evaluate the accuracy and relevance of the NMR method.

### Diffusion Measurements with Pulsed Gradient Spin-Echo NMR

Some nuclei, *e.g.*,  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{31}\text{P}$ , possess a magnetic dipole moment. The overall magnetic moment of a sample containing these nuclei will orient parallel to an external magnetic field  $B_0$ . When this sample is exposed to a so-called radio frequency  $90^\circ$ -pulse, the overall magnetization will be oriented in the plane perpendicular to  $B_0$ . Subsequently, the spins return to the equilibrium state with their moment aligned to  $B_0$  by means of spin-lattice relaxation, characterized by a relaxation time  $T_1$ . Initially, the precessing spins of all nuclei in the plane perpendicular to  $B_0$  will have an identical phase, but gradually dephasing occurs due to spin-spin relaxation, characterized by a relaxation time  $T_2$ , and inhomogeneities of the magnetic field. Application of a linear magnetic field gradient, instead of using a homogeneous magnetic field, enhances dephasing in a well-defined way. The dephasing rate is monitored by inverting the phase of each spin with a so-called  $180^\circ$ -pulse at a time  $t$  after the  $90^\circ$ -pulse. Provided that the individual spins are subjected to the same magnetic environment, complete refocusing of all phases occurs at time  $2t$ , and a so-called echo is generated. However, displacement of the molecules, *e.g.*, due to Brownian motion, prevents complete phase-refocusing, resulting in an attenuation of the echo amplitude. The attenuation of this echo is quantitatively related to the mean distance traveled by the molecules. More details on theory and practice of diffusion measurements according to this method are described elsewhere (Farrar and Becker, 1971; LeBihan, 1991; Stilbs 1987).

For a better definition of the time interval in which the diffusion is monitored, it is advantageous to use two short magnetic field gradient pulses and superimpose them on the constant and homogeneous field  $B_0$ . There are many variations of the basic pulse sequence, and the one applied in this study is shown in Figure 1 (Van Dusschoten, 1996). This specific PFG-NMR sequence monitors diffusion and  $T_2$  relaxation simultaneously in one set of experiments. In this way, the signal from several fractions of water with a distinct diffusional and/or relaxation behavior, *e.g.*, intracellular water, may be distinguished, and enables a thorough interpretation of the NMR-signal obtained from heterogeneous systems like biofilms. Furthermore, a series of so-called spin-echoes can be generated with the  $180^\circ$ -pulses after the second gradient pulse, and the amplitude of the



**Figure 1.** The applied pulsed gradient echo sequence (Van Dusschoten, 1996). The slim rectangles show the  $90^\circ$  and  $180^\circ$  pulses in the x- or y-plane. The curved lines represent the echoes which are observed after a time  $t_{\min}$  with intervals of  $2t_e$ . In the period  $\delta$  gradient pulses with variable strength are applied.

echo can be monitored in time (Van Dusschoten et al., 1996).

During one set of experiments, different magnitudes of the magnetic field gradient  $G$  are imposed, and after a time interval  $t_{\min}$  successive echoes with an interval of  $2t_e$  are observed. The decay of the monitored signal  $S(t, b)$  is described by the theoretical relation:

$$S(t, b) = \sum_{n=1}^n z_n \cdot S(0, 0) \cdot \exp(-b \cdot D_n) \cdot \exp(-t/T_{2,n}) \quad (1)$$

with

$$b = \gamma^2 \delta^2 G^2 (\Delta - \delta/3)$$

comprising the signals from the magnetic species in  $n$  different physical environments. Each fraction  $z_n$  is characterized by a diffusion coefficient  $D_n$  and a relaxation time  $T_{2,n}$ . The gradient factor  $b$  contains the gyromagnetic ratio  $\gamma$ , the magnetic field gradient  $G$ , and the duration of gradient pulse or labeling period  $\delta$ . The time  $\Delta$  between two gradient pulses can be regarded as the time interval in which the mean square displacement of the nuclei is observed. The additional  $180^\circ$ -pulses between the pair of pulsed field gradients are imposed to diminish the influence of magnetic field distortions and susceptibility artifacts due to the presence of aggregates, for example, on the diffusion measurement (Van Dusschoten et al., 1995a). In comparison with other sequences generally applied, a high signal-to-noise ratio is attained. Water itself was chosen as the tracer molecule because protons have the largest gyromagnetic resonance, and it is the most abundant compound in a biofilm. The diffusional behavior of water is comparable to that of relevant microbiological metabolites, like glucose and oxygen (Tyrell and Harris, 1984).

## EXPERIMENTAL

### Self-Diffusion of Water

The diffusion of water was determined as a function of the temperature to test the experimental set-up and procedure. A 0.5 mM  $\text{MnCl}_2$ -solution was used to reduce the relaxation

time  $T_1$  from 2 s to 50 ms, because a long  $T_1$  hinders a fast scanning procedure. The manganese-ions disturb the local magnetic field and accelerate the dephasing of neighboring protons (Callaghan, 1991).

### Artificial Biofilms

Spherical gels with a diameter of about 4 mm were prepared from agar and alginate, according to Beuling et al. (1995) and Hulst et al. (1989), respectively. The obstruction effect of the polymer matrix was studied in gel beads containing up to 4% w/w agar. The effect of bacteria was simulated with inert and impermeable 0.9  $\mu\text{m}$  polystyrene particles. These particles were dispersed homogeneously in the agar matrix; their volume fraction ranged from 5 to 30% v/v. The influence of real microorganisms was investigated in 1.5% w/w agar containing up to 20% v/v homogeneously dispersed bacteria (*Micrococcus luteus*: ATCC 4698). The volume of these organisms was calculated assuming 250 kg dw/m<sup>3</sup> bacteria (Bakken and Olsen, 1983; Kubitschek et al., 1984).

### Natural Biofilms

Nitrifying biofilms were taken from different heights of a conical fluidized bed reactor described elsewhere (De Beer et al., 1993). Aerobic biofilms, grown on basalt carriers, were obtained from a nitrifying/denitrifying Circox reactor (Freijters et al., 1996). Mesophilic methanogenic granules were obtained from three different full-scale wastewater treatment plants: a papermill (Industriewater BV, Eerbeek, The Netherlands), a potato processing plant (CAB, Wezep, The Netherlands), and a citric acid production plant (ADM, Ringaskiddy, Ireland).

### Biofilm Characterization

Biofilm density, dry weight, and ash contents were analyzed as described by Hulshoff Pol et al. (1986).

### Diffusion Experiments

An NMR spectrometer (Smis, Guildford, UK) equipped with a 0.5 T electromagnet (Bruker, Karlsruhe, Germany) was used for the diffusion experiments. A probe with an internal diameter of 0.03 m, and actively shielded gradients (Doty Scientific, Columbia, USA) was fitted in the 0.14 m gap of the magnet. Magnetic field gradients were applied up to 375 mT/m (Van Dusschoten et al., 1995b).

A glass tube containing 10 mL of sample was placed in the probe, and kept at  $30 \pm 1^\circ\text{C}$ , unless specified otherwise. The gelbeads and biofilms were submerged in a buffer solution containing a small amount of dextran (MW ca. 150 kg/mole), chelated to  $\text{Fe}_2\text{O}_3$  as described by Hnatowich et al. (1983) to reduce the  $T_2$  of the external water phase, and it was not monitored with PFG-NMR. Diffusion measurements were performed by recording 2000 echoes generated

after two field gradient pulses using,  $\delta = 5.0$  ms,  $\Delta = 12.1$  ms, and  $2t_e = 0.6$  ms. The measurements were repeated 11 times with an increasing magnitude of the gradient pulses; the repetition time was set at 6 s. In this way, a two-dimensional data set was obtained as a function of time and  $G$ . The signals were fitted with Eq. (1) assuming that the sample contained only one fraction, unless mentioned otherwise. The two-dimensional fitting routine was based on the Marquardt-Levenberg algorithm (Press et al., 1989). The signal obtained at  $G = 0$  was not used.

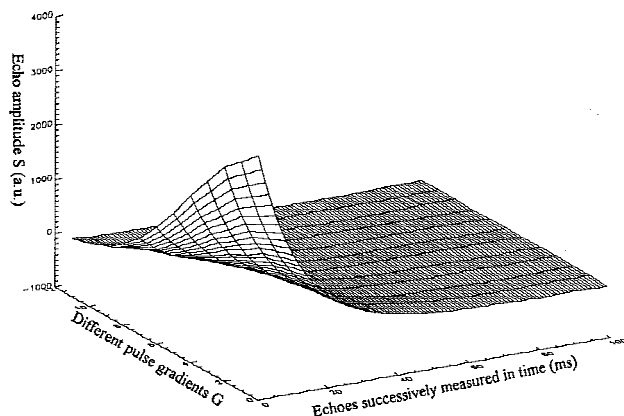
Microelectrode measurements were performed at  $30 \pm 0.1^\circ\text{C}$  as described by Cronenberg and Van den Heuvel (1991). A glucose microelectrode with a tip diameter of 8  $\mu\text{m}$  was positioned in the center of a gelbead or aggregate that was submerged in a 50 mM  $\text{K}_2\text{HPO}_4$  buffer. The response was monitored on a stepwise concentration change in the well mixed bulk liquid from 0 to 2 mM glucose. This curve was fitted with the theoretical relation given by Crank (1975). Prior to these measurements, the immobilized microorganisms were inactivated by incubation in a 0.2% w/w  $\text{HgCl}_2$  solution during 15 h.

The variance of the results was calculated using Student's t-test, and expressed as a 95% confidence interval.

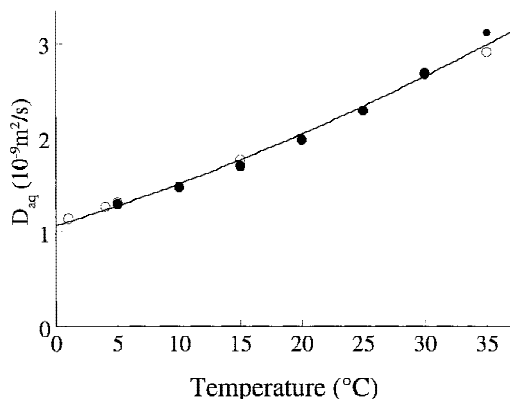
## RESULTS

### Self-Diffusion of Water

Typical decay curves of the echo amplitude of water is given in Figure 2. The average values for diffusion coefficients thus obtained at various temperatures are given in Figure 3; the variance amounted to 2%. The theoretical temperature dependency was calculated according to the Stokes-Einstein relation (Cussler, 1976), using a molecular radius of 1.0 nm and a viscosity of water as given by Weast and Astle (1980). The experimental data reported by Tyrrell and Harris (1984) are also inserted in this figure for com-



**Figure 2.** A typical example of an experimental two-dimensional data set obtained from water at  $30^\circ\text{C}$ . One experiment consists of a series of echo sequences with an increasing magnitude of the magnetic gradient  $G$ . After each pulse sequence, the amplitude  $S$  of the successive echoes is monitored.



**Figure 3.** The influence of the temperature on the self-diffusion of water determined with PFG-NMR (●), compared to the Stokes-Einstein relation (solid line). The experimental values (○) reported by Tyrrell and Harris (1984) are inserted as well.

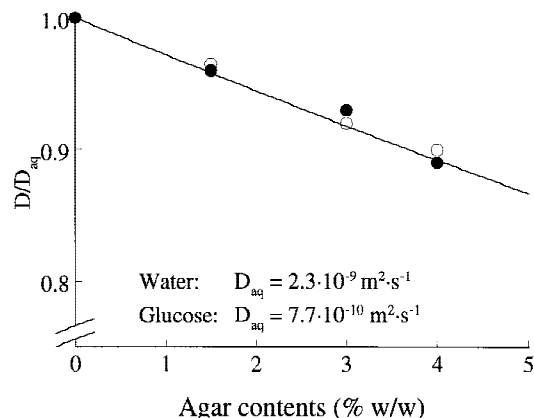
parison. Obviously, our results are in good accordance with these other sources.

### Diffusion in Artificial Biofilms

The influence of a polymer matrix on the diffusion coefficient of water was determined in gels containing up to 4% w/w agar. Figure 4 shows that the diffusion coefficient decreased with the polymer contents. The results can be described satisfactorily by the model of Mackie and Meares (Muhr and Blanshard, 1982), which is generally applied to describe diffusion in gels. Furthermore, the relaxation time  $T_2$  was found to decrease from 100 to 38 ms when the agar concentration was increased from 1.5 to 4% w/w (data not shown). The results of all measurements with glucose microelectrodes will be treated separately at the end of this section.

The results obtained in a 1.5% w/w agar matrix containing up to 30% v/v polystyrene particles are given in Figure 5. The diffusion coefficient of water decreased with an increasing polystyrene fraction and the normalized values are predicted satisfactorily by the relation of Fricke (1924), which describes diffusion through dispersions of impermeable spheres. The relaxation time  $T_2$  of the water inside the gelbeads was not affected by the dispersed polystyrene and amounted to 100 ms. Variation of the time interval  $\Delta$  from 12.1 to 200 ms had no effect on the diffusion coefficients obtained.

The influence of immobilized bacteria on the water mobility was investigated in 1.5% w/w agar containing up to 20% v/v bacteria. From the obtained water diffusion coefficients presented in Figure 6, it is clear that the effect of bacteria is comparable to that of polystyrene particles. The average relaxation time  $T_2$  equaled 80 ms, and was not affected by the magnitude of the bacterial fraction within the range measured. Further analysis of the data sets revealed the existence of another water fraction with a significantly lower  $T_2$  of about 30 ms. This fraction amounted

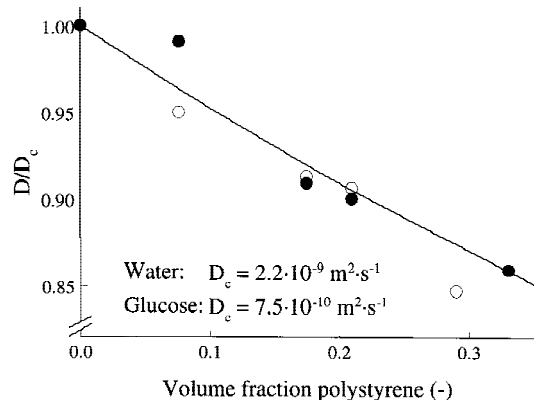


**Figure 4.** The influence of the agar concentration on the diffusion coefficients obtained with NMR (●) at 25°C, the results of the microelectrode experiments with glucose are inserted as well (○). Solid line: model of Mackie and Meares (Muhr & Blanshard, 1982). The measured values are normalized with the diffusion coefficients of pure water  $D_{aq}$ .

to ca. 15% of the detected water, and its diffusion coefficient varied from  $1.36 \cdot 10^{-9}$  to  $1.94 \cdot 10^{-9}$  m<sup>2</sup>/s. There was no quantitative relation between the magnitude of the fraction with the shorter  $T_2$  value and the concentration of the bacteria. Diffusion coefficients and relaxation times of the other fraction equaled the values obtained with a plain monoexponential analysis, although the experimental variance of the calculated diffusion coefficient amounted to 8%.

### Diffusion in Natural Biofilms

The diffusional properties of several types of natural biofilms were determined from the experimental decay curves using monoexponential analysis. The average values calculated from at least three such experiments are presented in Table I, together with the experimental dry weight and ash contents. The diffusion coefficients were normalized on  $D_{aq}$



**Figure 5.** Influence of dispersed polystyrene particles on the diffusion coefficient of water in 1.5% w/w agar obtained with PFG-NMR (●) at 25°C. The diffusion coefficients of glucose obtained with microelectrodes are inserted as well (○). Solid line: model of Fricke (1924). The measured values are normalized with the diffusion coefficients in a proper agar gel  $D_c$ .

of pure water to obtain a temperature independent value; the variance was less than 2%. Inactivation of the biofilms with  $\text{HgCl}_2$  did not influence the results of the diffusion experiments.

Biexponential analysis of these decay curves again revealed the existence of two phases. In methanogenic granules, the dominating diffusion coefficient amounted to  $1.1 \cdot 10^{-9} \text{ m}^2/\text{s}$ , while a small fraction of 10% was obtained with a much higher value of  $1.9 \cdot 10^{-9} \text{ m}^2/\text{s}$ . The concomitant relaxation times  $T_2$  amounted to 15 and 70 ms, respectively. About 60% of the detected water in aerobic biofilms exhibited a diffusion coefficient of  $2.2 \cdot 10^{-9} \text{ m}^2/\text{s}$  and a  $T_2$  of 200 ms. A slightly lower value of  $1.7 \cdot 10^{-9} \text{ m}^2/\text{s}$  and a  $T_2$  of 40 ms were obtained for the remaining part.

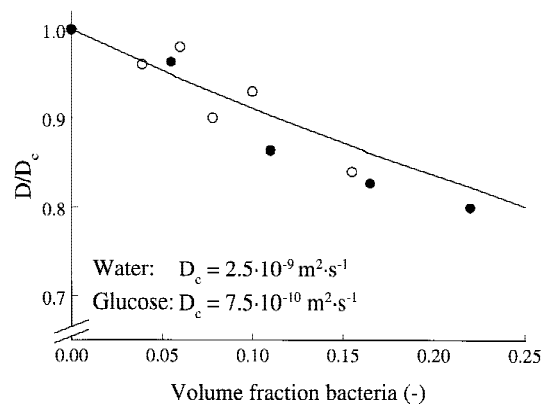
### Diffusion of Glucose Measured with Microelectrodes

The glucose diffusion coefficients obtained from the microelectrode measurements in proper gels, polystyrene loaded gels, and gels containing immobilized bacteria, are inserted in Figures 4, 5, and 6, respectively. A good correspondence with the PFG-NMR results is apparent. Only the natural biofilms from the potato processing plant were sufficiently spherical to allow supplementary microelectrode experiments; the diameter of these granules averaged 2.5 mm. A glucose diffusion coefficient of  $(4.8 \pm 0.3) \cdot 10^{-10} \text{ m}^2/\text{s}$  was obtained at  $30^\circ\text{C}$ , *i.e.*, 63% of the diffusion coefficient in pure water (Longworth, 1953).

## DISCUSSION

The diffusion coefficients of water, obtained at various temperatures, were proportional to the ratio of the temperature  $T$  and the viscosity  $\eta$ , and could be described well with the Stokes-Einstein relation (Cussler, 1976). A similar temperature dependence is observed for diffusion coefficients of small molecules dissolved in water, while the absolute values agree well with the diffusion coefficients of water reported in the literature (Tyrrell and Harris, 1984). Thus, the self-diffusion of water is accurately measured with PFG-NMR, and its temperature dependence shows a regular behavior.

The decay of the NMR signal amplitude is not only influenced by translational diffusion of the water molecules, but also by the relaxation time  $T_2$ , which is mainly determined by the molecular environment (Callaghan, 1991). In case the signal of an ensemble of protons extinguishes within the observation time, *i.e.*,  $T_2 < \Delta$ , it will not contribute to the observed amplitude. This enables masking of the liquid phase surrounding the gelbeads or biofilms by addition of ironoxide, which reduces the relaxation time  $T_2$  to less than 1 ms. For this purpose, ironoxide was chelated to dextran macromolecules with such a low diffusivity that it could not penetrate gelbeads and biofilms (Lebrun and Junter, 1993). This was confirmed by the constant signal obtained during more than 2 h. Because this signal is di-



**Figure 6.** The influence of bacteria, *i.e.*, *Micrococcus luteus*, immobilized in 1.5% w/w agar on the diffusion coefficient. Results obtained with PFG-NMR (●) at  $30^\circ\text{C}$ , and the results measured with a glucose microelectrode (○). Solid line: model of Fricke (1924). The measured values are normalized with the diffusion coefficients in a proper agar gel  $D_c$ .

rectly related to the detectable amount of water, it can be concluded that the chelated ironoxide was effectively excluded from the gelbeads and the biofilms. It should be noted that the possibility to mask external water is restricted to dense biofilms. Biofilms with an open structure like fungal pellets contain large pores and displayed a decreasing signal amplitude during a series of experiments (data not shown).

The  $T_2$  of the water inside the agar matrix depended on the polymer concentration, but was not affected by the presence of polystyrene. Apparently, gel fibers have a much larger impact on the relaxation behavior of the labeled protons. This can be ascribed to the chemical exchange of the protons between water and the sugar monomers of the agar matrix (Hills et al., 1989). In comparison with agar, the contact surface between water and the nonporous particles is only small. Moreover, polystyrene does not possess such exchangeable protons and, hence, its presence does not affect the  $T_2$ .

The NMR technique monitors the average displacement of water molecules within a certain time interval  $\Delta$ . The concomitant mean square displacement  $\langle x^2 \rangle$  of an ensemble molecules in one direction of an infinite medium is related to the diffusion coefficient  $D$  according to (Tyrrell and Harris, 1984):

$$\langle x^2 \rangle = 2 \cdot D \cdot \Delta \quad (2)$$

The mobility of dissolved molecules in a continuous liquid phase is hindered by the presence of dispersed solid obstructions. If the observation time  $\Delta$  is sufficiently short, such that the average displacement of the molecules is comparable to the length-scale of the obstructions and the distance between them, the value obtained for the diffusion coefficient will depend on the time interval applied. In this way, it is possible to gain information regarding the length-scale of obstructions and their average distance (Cotts, 1991). It may even be possible to get information about the

**Table I.** Diffusion coefficients of water in several biofilms obtained from monoexponential analysis of the PFG-NMR data. Physical characteristics of the biofilms are also included.

Biofilm type	D [m <sup>2</sup> /s]	T <sub>2</sub> [ms]	Temp [°C]	D/D <sub>aq</sub> [-]	Density [kg/m <sup>3</sup> ]	Dry weight [kg/m <sup>3</sup> ]	Ash [%dw]
Nitrifiers							
Height 1	1.88 · 10 <sup>-9</sup>	nd	26	0.85	1015	42.5	nd
Height 2	1.76 · 10 <sup>-9</sup>	nd	26	0.80	1018	71.8	nd
Aerobes	2.10 · 10 <sup>-9</sup>	192	28	0.89	1016*	45.6*	5%
Methanogenes							
Paper	1.38 · 10 <sup>-9</sup>	20	28	0.59	1043	107.1	16%
Potato	1.42 · 10 <sup>-9</sup>	38	25	0.64	1046	130.6	30%
Citric acid	1.71 · 10 <sup>-9</sup>	62	28	0.73	1034	90.4	20%

\*Experimental values were corrected for presence of the basalt carriers assuming an ash content of 5% w/w in the attached biofilm (Characklis and Marshall, 1989).

spatial distribution of particles, ranging from monodisperse to different types of clustered distributions. However, no effect of the time interval  $\Delta$  was found down to 12.1 ms, and the corresponding displacement of 7.7  $\mu\text{m}$  for a diffusion coefficient of  $2 \cdot 10^{-9} \text{ m}^2 \cdot \text{s}^{-1}$  obviously exceeded the typical distance between the polystyrene particles. Microscopical observations revealed that the particles were distributed individually and homogeneously through the agar matrix. Thus, the average distance between the particles for the 8% v/v loading amounted to ca. 12  $\mu\text{m}$ , and a small increase of the diffusion coefficient was to be expected at a  $\Delta$  of 12.1 ms compared to the values obtained at higher  $\Delta$ . Apparently,  $\Delta$  should be reduced more drastically to have an appreciable effect. It should be noted that the time interval required to observe an average displacement of 1  $\mu\text{m}$  in pure water amounts to only 0.25 ms. Unfortunately, this option was not yet available on the equipment utilized.

The presence of a fraction  $\phi$  of dispersed impermeable particles affects the effective diffusivity in two ways, *viz.* (1) reduction of the volume available for diffusion, *i.e.*, exclusion, and (2) increase of the diffusive pathlength by steric hindrance, usually referred to as the tortuosity  $\tau$  of the material. The relation between the diffusion coefficient in the continuous phase  $D_c$  and the effective diffusion coefficient  $D_{eff}$  is given by (Epstein, 1989):

$$D_{eff} = \frac{(1 - \phi)}{\tau^2} \cdot D_c \quad (3)$$

The square of the tortuosity is required, because dispersed particles both increase the pathlength of diffusing molecules, and decrease the steepness of the concentration gradient experienced. Effective diffusion coefficients are obtained directly from steady-state fluxes monitored in a classical diffusion cell, for example. This is not so, if a measurement is performed under transient circumstances, *e.g.*, all step-response methods. Then, the diffusion coefficient is obtained from the pace at which the new equilibrium is reached, and a so-called transient diffusion coefficient  $D_{eff}/(1-\phi)$  is obtained (Axelsson and Persson, 1988; Beuling et al., 1995; Libicki et al., 1988). PFG-NMR yields this diffusion coefficient as well, because it determines the av-

erage displacement of ensemble molecules while the magnitude of this ensemble is not monitored. Consequently, determination of the effective diffusion coefficient from NMR-data requires further information on the porosity ( $1-\phi$ ) of the dispersed system. For that purpose, it should be worthwhile to calibrate the obtained NMR signal in such a way that the porosity of the material could be deduced directly from the signal amplitude. This has already been done in the well-logging field (Kleinberg et al., 1992) and would enable measurement of the transient diffusion coefficient and the porosity with the same experimental technique. It should be noted, however, that during the echo sequence part of the signal may be lost between the two pulsed gradients. As a consequence, the measured amplitude is only an indication for the amount of water.

Water diffusion coefficients were obtained in model systems containing various amounts of agar and dispersed polystyrene particles. The obstruction effect of the agar matrix was described satisfactorily by the model of Mackie and Meares (see Fig. 4), which approaches diffusion as a statistical movement of small molecules through a lattice of polymers. The influence of the dispersed polystyrene particles was described well by the model of Fricke (see Fig. 5), which idealizes diffusion in heterogeneous systems as a well-defined macroscopical process. The presence of polymer chains affects the diffusion coefficient more than the same fraction of dispersed particles, illustrating that the scale of the obstructions compared to the diffusing molecules plays an important role, and that this difference is adequately monitored with NMR.

Glucose diffusion coefficients obtained independently with microelectrodes were affected similarly by the polymer matrix and the polystyrene particles, while water diffusion coefficients in alginate matrices measured with NMR (data not shown) corresponded well with normalized diffusion coefficients of oxygen and lactose reported in the literature (Axelsson and Persson, 1988; Hulst et al., 1989). Apparently, the mobility of water is affected just as the mobility of these other small molecules dissolved in the continuous liquid phase. Thus, it can be concluded that water serves as a suitable analyte for the characterization of mass transfer

properties of heterogeneous media like these biofilm simulating model systems.

Bacteria contain ca. 75% water, and are enclosed by a hydrophobic semipermeable membrane (Brock and Madigan, 1991). Thus, matrices that contain immobilized bacteria constitute an extra aqueous phase, separated from the water phase in the polymer matrix. Biexponential analysis of the NMR-data obtained from agar containing immobilized bacteria, indeed gave two water fractions. The  $T_2$  of the largest fraction (80 ms) was comparable to the value for the 1.5% w/w agar gel itself, while the other fraction exhibited a much smaller  $T_2$  (30 ms). Because such a fraction was not encountered in agar containing polystyrene, it has to be ascribed to the presence of the bacteria, and indeed, Monte Carlo simulations performed to test the reliability of the fitting procedure (Van Dusschoten et al., 1996) have shown that the signals of such different water fractions can be separated using this specific pulse sequence. However, the amplitude of this fraction did not correlate with the cell loading, while both  $T_2$  and the concomitant diffusion coefficients showed a large variation. This may be explained by the permeability of the bacterial cell envelope for water (Brock and Madigan, 1991), allowing to some extent that water moves in and out of the cells during the measurement. Due to this exchange, the observed fractions and relaxation times may partly mix up and, in general, these values no longer can be assigned to a specific fraction directly (Van Dusschoten, 1996). Unfortunately, it is not yet possible to obtain quantitative information on this exchange of water from these NMR-data (Van Dusschoten et al., 1996). It may explain, however, the observation that the magnitude of the diffusion coefficient belonging to this small fraction corresponded to an average displacement of at least 6  $\mu\text{m}$ , which grossly exceeds the length-scale of the microorganism applied, *i.e.*, 1  $\mu\text{m}$ .

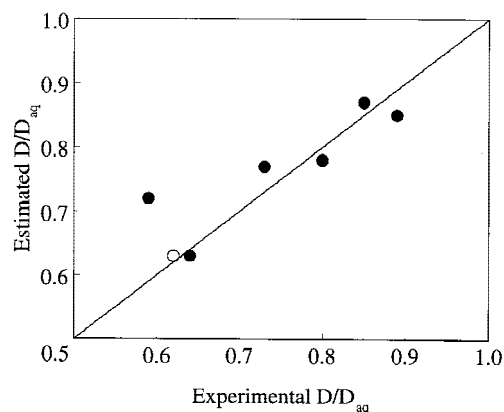
Diffusion coefficients obtained from monoexponential analysis of these NMR-data corresponded well with the diffusion coefficients of the largest fraction after biexponential analysis. The fact that the diffusion coefficient obtained with monoexponential analysis is hardly affected by the bacterial fraction may be explained by the short time scale of about 15 ms needed for the diffusion labeling. The exchange of water between the bacteria and their surroundings during this period may be assumed negligible. Furthermore, the slow and restricted diffusion of water present in the bacteria (Franks and Mathias, 1982), slightly attenuates the signal of this fraction.

The overall diffusion coefficient obtained with monoexponential analysis decreased with increasing fraction of bacteria, just like the diffusion coefficients of glucose measured independently with microelectrodes (Beuling et al., 1995). Hence, in these well-defined, artificial biofilms the overall water mobility as obtained from a monoexponential analysis of the NMR data displays a similar behavior as the diffusivity of a dissolved compound in the continuous liquid phase of the biofilm. From this finding, it can be inferred that monoexponential analysis enables adequate character-

ization of diffusion coefficients in these particularly active bacterial biofilms.

The diffusion coefficients obtained with PFG-NMR in natural biofilms were evaluated by comparing them to estimates based on physical biofilm characteristics. For that purpose, dry weight and ash contents were used to calculate the volume fractions of both bacteria and polymers. Consequently, the effect of both constituents on the biofilm diffusion coefficient is calculated with the models of Fricke, and Mackie and Meares, respectively (Beuling, 1998). A parity plot of the diffusion coefficients thus calculated, and those obtained with NMR is given in Figure 7, and it can be concluded that monoexponential analysis enables characterization of these natural biofilms as well. Naturally formed biofilms are highly inhomogeneous and multifractional analysis of the data revealed, indeed, the existence of compartments with different properties. Unfortunately, it is not yet possible to relate the obtained information to the structure of the biofilm, for example, and further investigations on this subject will be performed. The overall  $T_2$  decreased with the dry weight content.

From all these arguments; it can be stated that the PFG-NMR technique used for this study is a powerful tool for the characterization of diffusional properties of natural biofilms and has some distinct advantages. Shape, uniformity, or even solid particles have no negative effect on the results. Furthermore, the natural variation is averaged, because a sample of ca. 100 biofilms is measured within a single experiment. The most prominent advantage, however, is the possibility to measure in living biofilms without any knowledge of the microbial activity. Finally, NMR is relatively fast and accurate: One experiment takes only 5 min and exhibits a variance of 2%. Typical values for other techniques amount to 30 min and 5%, respectively (Westrin, 1991).



**Figure 7.** Parity plot of the normalized diffusion coefficients obtained with NMR (●) compared to estimates calculated with biofilm composition and theoretical models. The value obtained with microelectrodes is inserted as well. (○). The diffusion coefficients are normalized with its value in water  $D_{aq}$ .

## CONCLUSION

PFM-NMR is a fast and elegant technique, which enables reliable measurement of the water mobility inside complex heterogeneous systems like natural and active biofilms. Diffusion coefficients measured in both well-defined biofilms and spontaneously grown aggregates corresponded well to glucose diffusion coefficients determined in the same matrices. The results could be fitted with appropriate diffusion models of Mackie and Meares, and Fricke for the polymer matrix and obstructing microorganisms, respectively. Diffusion coefficients of the natural biofilms could be related to their physical characteristics. Quantitative analysis of the supplementary information contained in the PFM-NMR signal on cell fraction and spatial organization was not yet possible.

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## NOMENCLATURE

$b$	gradient factor [ $s \cdot m^{-2}$ ]
$D$	diffusion coefficient [ $m^2 \cdot s^{-1}$ ]
$G$	magnetic field gradient [ $T \cdot m^{-1}$ ]
$S$	amplitude [–]
$t$	time [s]
$t_e$	time between the 180°-pulses [s]
$t_{min}$	time interval after which the first echo is observed [s]
$T$	temperature [K]
$T_1$	spin-lattice relaxation time [s]
$T_2$	spin-spin relaxation time [s]
$\langle x^2 \rangle$	mean square displacement [ $m^2$ ]
$z$	fraction [–]
$\Delta$	time between two gradient pulses [s]
$\delta$	duration of a gradient pulse [s]
$\phi$	volume fraction of the dispersed phase [–]
$\gamma$	gyromagnetic ratio: for protons $4.2576 \cdot 10^7$ [ $s^{-1} \cdot T^{-1}$ ]
$\tau$	tortuosity [–]
$\eta$	viscosity [ $N \cdot s \cdot m^{-2}$ ]

### Subscripts

$aq$	water
$c$	continuous phase

## References

Axelsson, A., Persson, B. 1988. Determination of effective diffusion coefficients in calcium alginate gel plates with varying yeast cell content. *Appl. Biochem. Biotechnol.* **18**: 231–250.

Bakken, L. R., Olsen, R. A. 1983. Buoyant densities and dry-matter content of microorganisms: Conversion of a measured biovolume into biomass. *Appl. Environm. Microbiol.* **45**: 1188–1195.

Beefink, H. H., Staugaard, P. 1986. Structure and dynamics of anaerobic bacterial aggregates in a gas-lift reactor. *Appl. Environm. Microbiol.* **52**: 1139–1146.

Beuling, E. E., Van den Heuvel, J. C., Ottengraf, S. P. P. 1995. Determination of biofilm diffusion coefficients using micro-electrodes. In:

R. H. Wijffels, R. M. Buitelaar, C. Bucke, and J. Tramper (eds.), *Immobilized cells: Basics and applications*. Elsevier, Amsterdam.

Beuling, E. E. 1998. Mass transfer properties of biofilms. Ph.D. thesis, University of Amsterdam, The Netherlands.

Brock, D. B., Madigan, M. T. 1991. *Biology of microorganisms*. 6th edition. Prentice-Hall, Englewood Cliffs, NJ.

Callaghan, P. T. 1991. *Principles of nuclear magnetic resonance microscopy*. Clarendon Press, Oxford.

Characklis, W. G., Marshall, K. C. 1989 *Biofilms*. Wiley, New York.

Cotts, R. M. 1991. Diffusion and diffraction. *Nature* **315**: 443–444.

Crank, J. 1975. *The mathematics of diffusion*. 2nd edition. Oxford Science Publications, Oxford.

Cronenberg, C. C. H., Van den Heuvel, J. C. 1991. Determination of glucose diffusion coefficients in biofilms with micro-electrodes. *Biosens. Bioelectron.* **6**: 255–262.

Cussler, E. L. 1976. *Diffusion: Mass transfer in fluid systems*. Cambridge University Press, Cambridge.

De Beer, D., Van den Heuvel, J. C. 1988. Gradients in immobilized biological systems. *Anal. Chim. Acta* **213**: 259–265.

De Beer, D., Van den Heuvel, J. C., Ottengraf, S. P. P. 1993. Microelectrode measurements of the activity distribution in nitrifying bacterial aggregates. *Appl. Env. Microbiol.* **59**: 573–579.

De Beer, D., Stoodley, P., Roe, F., Lewandowski, Z. 1994. Effects of biofilm structures on oxygen distribution and mass transport. *Biotechnol. Bioeng.* **43**: 1131–1138.

Epstein, N. 1989. On tortuosity and the tortuosity factor in flow and diffusion through porous media. *Chem. Eng. Sci.* **44**: 777–779.

Farrar, T. C., Becker, E. D. 1971. *Pulse and fourier transform NMR*. Academic Press, New York.

Franks, F., Mathias, S. F. 1982. *Biophysics of water*. Wiley, Chichester.

Fricke, H. 1924. A mathematical treatment of the electric conductivity and capacity of disperse systems. *Phys. Rev.* **24**: 575–587.

Freijters, C. T. M. J., Eikelboom, D. H., Mulder, A., Mulder, R. 1996. Treatment of municipal wastewater in a circos airlift reactor with integrated denitrification. In: P. Harremoës (ed), *Proceedings of the 3rd IAWQ Conference on Biofilm Systems, Vol. 2 (C)*. Int. Assoc. on Water Quality, London.

Hills B. P., Wright K. M., Bilton P. S. 1989. NMR studies of water proton relaxation in Sephadex bead suspensions. *Mol. Phys.* **76**: 193–208.

Hulst, A. C., Hens, H. J.H., Buitelaar, R. M., Tramper, J. 1989. Determination of the effective diffusion coefficient of oxygen in gel materials in relation to gel concentration. *Biotechnol. Techn.* **3**: 199–204.

Hulshoff Pol, L. W., De Zeeuw, W. J., Velzeboer, C. T. M., Lettinga, G. 1986. Granulation in UASB reactors. *Wat. Sci. Technol.* **15**: 291–304.

Hnatowich, D. J., Layne, W. W., Childs, R. L., Lanteigne, D., Davis, M. A., Griffin, T. W., Doherty, P. W. 1983. Radioactive labeling of antibody: A simple and efficient method. *Science* **20**: 613–615.

Karel, S. F., Libicki, S. B., Robertson, C. R. 1985. The immobilization of whole cells: Engineering principles. *Chem. Eng. Sci.* **40**: 1321–1354.

Kitsos, H. M., Robert, R. S., Jones, W. J., Tornabene, T. G. 1992. An experimental study of mass diffusion and reaction rate in an anaerobic biofilm. *Biotechnol. Bioeng.* **39**: 1141–1146.

Kleinberg, R. L., Sezinger, A., Griffin, D. D., Fukuhara, J. 1992. Novel NMR apparatus for investigating an external sample. *J. Magn. Reson.* **97**: 466–485.

Kubitschek, H. E., Baldwin, W. W., Schröder, S. J., Graetzer, R. 1984. Independence of buoyant density and growth rate in *Escherichia coli*. *J. Bacteriol.* **158**: 296–299.

Lebrun, L., Junter, G.-A. 1993. Diffusion of sucrose and dextran through agar gel membranes. *Enzyme Microb. Technol.* **15**: 1057–1062.

Libicki, S. B., Salmon, P. M., Robertson, C. R. 1988. The effective diffusive permeability of a nonreacting solute in microbial cell aggregates. *Biotechnol. Bioeng.* **32**: 68–85.

LeBihan, D. 1991. Molecular diffusion nuclear magnetic resonance imaging. *Magn. Reson. Quart.* **17**: 1–30.

Lommi, H. O. 1990. Immobilized yeast for maturation and alcohol-free beer. *Brew. Distil. Internat.* **21**: 22–23.

Longworth, L. G. 1953. Diffusion measurements, at 25°C, of aqueous

- solutions of amino acids, peptides and sugars. *J. Am. Chem. Soc.* **75**: 5705–5709.
- Muhr, A. H., Blanshard, J. M. V. 1982. Diffusion in gels. *Polymer* **23**: 1012–1026.
- Nestle, N. F. E. I., Kimmich, R. 1996. NMR imaging of heavy metal absorption in alginate, immobilized cells, and kombu algal biosorbents. *Biotechnol. Bioeng.* **52**: 538–543.
- Ottengraf, S. S. P. 1986. Exhaust gas purification of waste gases. In: H. J. Rehm, G. Reed, (eds.), *Biotechnology* **8**. VCH verlagsgesellschaft, Weinheim.
- Press, W. H., Flannery, B. P., Teukolsky, S. A., Vetterling, W. T. 1989. *Numerical recipes in pascal: The art of scientific computing*. Cambridge University Press, Cambridge.
- Ramos, A., Jordan K. N., Cogan, T. H., Santos, H. 1994. <sup>13</sup>C Nuclear magnetic resonance studies of citrate and glucose cometabolism by *Lactococcus lactis*. *Appl. Environ. Microbiol.* **60**: 1739–1748.
- Stilbs, P. 1987. Fourier transform pulsed-gradient spin-echo studies of molecular diffusion. *Progr. N.M.R. Spectr.* **19**: 1–45.
- Tanner, J. E. 1983. Intracellular diffusion of water. *Arch. Biochem. Biophys.* **224**: 416–428.
- Tyrrell, H. J. V., Harris, K. R. 1984. *Diffusion in liquids: A theoretical and experimental study*. Butterworths, London & Boston.
- Van Dusschoten, D., De Jager, P. A., Van As, H. 1995a. PFG-NMR desensitized for susceptibility artefacts, using the PFG multiple-spin-echo sequence. *J. Magn. Reson. A*, **112**: 237–240.
- Van Dusschoten, D., De Jager, P. A., Van As, H. 1995b. Extracting diffusion constants from echo-time-dependent PFG NMR data using relaxation-time information. *J. Magn. Reson. A*, **116**: 22–28.
- Van Dusschoten, D., 1996. *Probing water motion in heterogeneous systems*. Ph.D. thesis, Wageningen Agricultural University, The Netherlands.
- Van Dusschoten, D., Moonen C. T. W., De Jager, P. A., Van As, H. 1996. Unraveling diffusion constants in biological tissue by combining Carr-Purcell-Meiboom-Gill imaging and pulsed field gradient NMR. *Magn. Reson. Med.* **36**: 907–912.
- Van Zijl, P. C. M., Moonen, C. T. W., Faustino, P., Pekar, J., Kaplan, O., Cohen, J. S. 1991. Complete separation of intracellular and extracellular information in NMR spectra of perfused cells by diffusion-weighted spectroscopy. *Proc. Natl. Acad. Sci. USA* **88**: 3228–3232.
- Weast R. C., Astle, M. J. 1980. *CRC Handbook of chemistry and physics*. CRC Press, Inc., Boca Raton, FL.
- Westrin, B. A. 1991. *Diffusion measurements in gels: A methodological study*. Ph.D. thesis, Lund University, Sweden.
- Westrin, B. A., Axelsson, A. 1991. Diffusion in gels containing immobilized cells: A critical review. *Biotechnol. Bioeng.* **38**: 439–446.