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BIOFILM GROWTH KINETICS ON HYDROCARBON IN A POROUS MEDIUM UNDER BIOSTIMULATION CONDITIONS.

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Abstract

The formation of biofilm on hydrocarbon in a porous medium under biostimulation conditions was investigated in order to a better understanding of microbial development during bioremediation of hydrocarbon-contaminated soils. *Pseudomonas alkanolytica* ATCC 21034 was studied as it grows embedded in a biofilm on dodecane surface. First, an experimental protocol for determination of biofilm growth kinetics on hydrocarbon in a porous medium was proposed. Six identical columns packed with sand were run simultaneously. The growth kinetics were obtained by measuring the biofilm dry weight of each column at different times. The reproducibility of the experimental protocol was validated using statistical tests. Then biofilm growth kinetics were established in the porous medium for two specific surfaces of dodecane (large or small droplets). A linear growth was observed due to transport limitation of both dodecane and dissolved oxygen in the biofilm. So the degradation of dodecane was uncompleted. Moreover, difficulty to bring dissolved oxygen in the deeper part of the porous medium was pointed out. All these phenomena could explain some failures encountered during biostimulation processes.

Keywords*: Bioremediation – Pseudomonas alkanolytica – dodecane - packed columns*

List of symbols:

s	standard deviation
m	arithmetic mean
n	sample size
i,j	index indicating the number of the column
$t'_{i,j}$	Student's t-test parameter

INTRODUCTION

Nowadays, pollution of soils with hydrocarbons has become a major environmental problem. Every year, hundreds of contaminated sites are discovered. These pollutants can seriously damage ecosystems. To remedy this situation, several methods for soil decontamination have been proposed (Autry & Ellis, 1992), and the most promising techniques were recently reviewed (Khaitan et al., 2006). Among them, biodegradation has been intensively studied for several years (Atlas, 1981; Bourquin, 1990). It consists of using microorganisms to eliminate hydrocarbons from polluted sites since these compounds are potential carbon sources. One of these techniques, called in-situ biostimulation, consists of saturating the soil with a mineral salt solution in order to improve the growth of indigenous microorganisms. Then the aqueous phase is continuously pumped out of the soil, treated, amended with nutrients and reintroduced into the soil. This process is particularly interesting insofar as it allows a treatment in depth at a lower cost than physical or chemical techniques. During the last two decades, several sites were successfully decontaminated with this method (Bardi et al., 2000; Bourquin, 1990; Iwamoto & Nasu, 2001). However, the process is still at the experimental stage and no systematic procedure has been established yet. Frequent and often unexplained failures were encountered during practical applications of this technique (Delille et al., 2004; Downey & Elliot, 1990; Dua et al., 2002; Heyse et al., 1986; Iwamoto & Nasu, 2001; Margesin & Schinner, 1999; Mohn & Steward, 2000; Seklemova et al., 2001). Sometimes hydrocarbon degradation was incomplete whereas significant growth of microorganisms was observed. A possible explanation could be the formation of a biofilm on hydrocarbon. Indeed, this phenomenon was observed in the laboratory for yeast or bacteria growing on n-alkanes (Blanch & Einsele, 1973; Bouchez-Naïtali et al., 2001; Mallee & Blanch, 1977; Miura et al., 1977). These authors showed that when microorganisms had a strong affinity to oil, they attached themselves on hydrocarbon (direct interfacial uptake) and formed flocs consisting of

a conglomeration of cells, hydrocarbon droplets and air bubbles. The growth kinetics presented a short exponential phase, followed by a linear phase. This last phase was attributed to the mass transfer limitation within flocs. During this linear growth, the contact with hydrocarbon or dissolved oxygen was rate-limiting for cells inside the aggregates (Blanch & Einsele, 1973; Bouchez-Naïtali et al., 2001). When no air bubbles were added to the culture medium, the same phenomenom appeared (Joannis, 1998; Marin et al., 1996) but the term of biofilm, consisting of cells embedded in a polymer around hydrocarbon droplets, was preferred. As direct interfacial uptake appeared to be the most frequent mechanism for soil bacteria degrading long-chain alkane (Bouchez-Naïtali et al., 1999), the formation of biofilm or flocs on hydrocarbon droplets could appear during bioremediation processes. Nevertheless this phenomenon is not taken into account in bioremediation studies.

This paper proposes an experimental device and a quantification method to estimate biofilm growth on hydrocarbon in a porous medium under biostimulation conditions. Contrary to usual bioremediation studies at the laboratory scale which used a single packed column (Antizar-Ladislao & Galil, 2003; Balfanz & Rehm, 1991; Boopathy, 2004; Song et al. 1990; Weissenfels et al., 1990; Zhou & Crawford, 1995), we proposed the use of 6 packed columns run simultaneously in order to avoid disturbance due to sampling. Each column was periodically removed from the system to estimate biofilm dry weight content to obtain biofilm growth kinetics. First, the proposed device was validated with statistical tests. Then biofilm growth kinetics on hydrocarbon in a porous medium were obtained and discussed.

MATERIAL AND METHODS

Microorganisms and culture conditions

Bacteria

Pseudomonas alkanolytica ATCC 21034 was used for all the experiments. The strain was maintained at 4°C on an agar Petri dish. The storage medium composition was: digested soy

peptone: 5 g, meat extract:1 g, yeast extract:2 g, NaCl:5 g, agar:15 g in 1 liter of deionised water. The pH was adjusted to 7.2 with 1M NaOH.

Culture medium

The hydrocarbon was n-dodecane (Fluka) and the mineral salt medium composition was: $(NH_4)_2SO_4$:2 g, Na_2HPO_4:3.61 g, KH_2PO_4:1.75 g, $MgSO_4,7H_2O$:0.2 g, $CaCl_2$:50 mg, $FeSO_4,7H_2O$:1 mg, $CuSO_4,5H_2O$:50 mg, H_3BO_3:10 mg, $MnSO_4,5H_2O$:10 mg, $ZnSO_4,7H_2O$:70 mg, $(NH_4)_6Mo_7O_{24},4H_2O$:10 mg in 1 liter of deionised water. The pH was adjusted to 7 using 1 M NaOH and the medium was autoclaved for 20 min at 121°C.

Starter preparation

The biofilm introduced in the porous medium came from a two-phase culture: mineral salt solution and dodecane in order to adapt the microorganisms to the hydrocarbon. The culture was carried out in a shake flask (200 ml of mineral salt solution and 4 ml of dodecane) inoculated from storage medium. After a 6 day incubation (250 rpm, 30°C), the culture was centrifuged for 10 minutes at 20,000 rpm. The pellet was washed three times with deionised water in order to remove residual dodecane and then suspended in about 300 ml of mineral salt solution to obtain an optical density of 0.4 which is equivalent to a biofilm dry weight concentration of 0.5 $g.l^{-1}$. This suspension was used to inoculate the porous medium.

Experimental device

Six columns made of glass (Pharmacia Biotech, 40-cm long and 2.4 cm inlet diameter) were used (Figure 1). The columns were packed with 150 g of sterile sand (400-µm diameter, bed porosity 0.34, permeability 8.2 10^{-12} m^2). Sand was chosen as dodecane and *Pseudomonas alkanolytica* do not adhere on it (Joannis, 1998, Omar & Rehm, 1988). So dodecane was capillary trapped in the porous medium.

Dodecane was first introduced to improve the immobilisation of *Pseudomonas alkanolytica*. The columns were saturated either with pure dodecane or with a water-dodecane emulsion (2

% v/v of dodecane). The hydrocarbon excess was removed by washing the columns with deionised water until no dodecane came out. This excess was weighed after being separated from the water by settling. The total dodecane trapped into the packed bed was estimated by the difference between the quantities introduced and recovered. Microbial inoculation was then carried out by recirculating the starter suspension. This solution was percolated through each column and recycled for a period of 6 hours. Then the sand was washed with mineral salt medium until no further bacteria came out. The experiments were run by trickling down 1 liter of the aqueous phase into each column through polyethylene tubing (Figure 1); the sand was kept saturated. This aqueous phase was aerated with air at 0.9 vvm, and recycled through each column at a flow rate of 0.36 $ml.min^{-1}$. The six columns were run simultaneously at room temperature (close to 20°C).

Biofilm quantification

Biofilm quantification was difficult due to the high heterogeneity of the system: a solid support, two non-miscible liquid phases and bacteria embedded in a polymer. Among some of the methods proposed for quantification of biofilm growing on dodecane (Joannis et al., 1998), dry weight determination was chosen for this study. The biofilm was extracted from the sand by adding water to it and mixing vigorously. Five successive extractions were made with respectively 1.67 ml (twice), 0.67 ml (twice) and 0.33 ml of water per gram of sand for a period of 30 minutes. The total volume extracted, which contained dodecane and biofilm, was stirred to obtain a homogeneous emulsion. Five samples (24.7 ml each) were withdrawn and centrifuged (10 min - 20,000 rpm). The aqueous phase was removed; the pellet and the supernatant were mixed with a solvent (acetone-petroleum ether; 3v/1v) and centrifuged again. The resulting pellet was suspended in sterile water, filtered through acetate cellulose filter (0.2 µm) and dried to a constant weight. The biofilm dry weight was the arithmetic mean of these five dry weight values.

The total amount of sand was analysed in two ways: either the whole quantity was taken into account, or the sand was divided into three slices (top, middle, and bottom) analysed separately in order to study the biofilm distribution in the column. In the latter case, the total biofilm quantity was the sum contained in all three slices.

A fraction of the biofilm detached from the porous medium was also found in the aqueous medium of the 1 liter flask. It could be provided from biomass detached from the biofilm or biofilm weakly bound to the hydrocarbon. This quantity was estimated by the filtration of a 20-ml sample through acetate cellulose filters and drying to a constant weight.

Thus, the total formed biofilm dry weight was the sum of the quantity recovered from the porous medium and the fraction present in the aqueous medium.

Proteins content

Proteins were extracted from the biofilm by hydrolysing 0.5 cm^3 of the sample with 0.5 cm^3 of 0.25 $mol.l^{-1}$ H_3PO_4 at 100°C (Joannis et al., 1998). The protein concentration was estimated with Lowry's method (Lowry et al., 1951) using a solution of BSA (Bovine Serum Albumin) containing an equal volume of H_3PO_4 as reference.

Ammonium concentration

The $(NH_4)_2SO_4$ concentration in the aqueous phase was determinated by the Nessler method (Greenberg et al., 1992)

Statistical test

Student's t-test

The Student's t-test was used to determine the homogeneity of the biofilm distribution between the columns or between the different slices of a single column. This test allows comparison of two arithmetic means of sets of n values. For this study, each dry weight measurement was the average of n=5 measurements. So the Student parameter $t'_{i,j}$ was calculated for each pair of columns by:

$$t'_{i,j} = \frac{m_i - m_j}{\sqrt{\frac{s^2}{n_i} + \frac{s^2}{n_j}}} \qquad (i)$$

where m_i et m_j are the arithmetic mean of n_i and n_j measures (n_i=n_j=5) of dry weights contained in the columns i and j respectively. $s^2 = s^2_i + s^2_j$, where s_i and s_j are the standard deviation of the dry weight estimated from n_i and n_j values (Schwartz, 1963). The standard deviation was found to be linked with the dry weight by the relation (Joannis, 1998):

$$s_i = 0.0458\, m_i^{0.03} \qquad (ii)$$

The difference between the biofilm dry weights of the two columns was not considered significant if $\left|t'_{i,j}\right|$ was less to the tabulated value of the Student's t parameter for a confidence interval of 95% (α = 0,05) and a degree of freedom $n_i + n_j - 2 = 8$ (Schwartz, 1963). This tabulated t-value is 2.31.

Confidence intervals

As the dry weight was determined from five measurements, the 95% confidence interval for a dry weight measurement was: $m \pm \frac{1.776}{\sqrt{5}} \cdot s$. The intervals were estimated for each experimental value and they were presented with bars on the graphs.

RESULTS AND DISCUSSION

Biofilm formation on hydrocarbon droplet

BATH (Bacterial Adherence To Hydrocarbon) protocol (Rosenberg et al., 1980) was used to estimate the affinity of cells to dodecane. This test proved that *Pseudomonas alkanolytica* had a high affinity with dodecane as more than 95% of the cells adhered to hydrocarbon. Microscopic observations (Olympus,×400 magnification) of *Pseudomonas alkanolytica* growing in an emulsion of mineral salt solution and dodecane (2% v/v) indicated that this

bacteria formed a biofilm on the hydrocarbon droplets. Several authors also reported that alkane degrading microorganisms which had strong affinity to oil could form biofilm or flocs if air bubbles (Blanch & Einsele, 1973; Bouchez-Naïtali et al., 2001; Nakahara et al., 1977). Moreover, bacteria are usually poorly absorbed onto the sand, so the hypothesis was made that in porous media, biofilm only grew at the surface of dodecane as schematised on Figure 2. This was confirmed by microscopic observations during experiments in the porous medium; when the content of a column was withdrawn, biofilm was observed around the dodecane droplets and no adhesion of bacteria on the sand was observed.

Validation of the experimental device

The validation of the 6-column system consisted of proving that the 6 columns were identically inoculated and that the biofilm growth was similar in all of them. The distribution of dodecane and biofilm in the six columns was investigated separately.

Hydrocarbon distribution in the porous medium

The distribution of the dodecane in the column was studied prior to introduction of the bacteria. The six columns were saturated with pure dodecane and the quantity trapped was estimated by the difference between dodecane introduced and recovered after washing. The values of retained dodecane are presented in Table 1. The mean quantity was 101 ± 12 mg per gram of sand. This value was similar to pollutant concentrations in soil given in the literature ranging from 50 to 135 mg per gram of soil (Song & Bartha, 1990; Wang & Bartha, 1990, Xu et al., 1995). The experimental error was 8.3 %.

To estimate the hydrocarbon distribution, the dodecane was colored with Soudan red dye before being introduced in the column. The homogeneous coloration in the sand after washing with water indicated that the dodecane was uniformly distributed all through the porous medium. Thus, distribution of dodecane was uniform between and through the column prior to the innoculation.

Biofilm inoculation in the porous medium

First, the biofilm distribution was compared between the columns just after inoculation. Two series of three columns each were prepared according to the above-mentioned protocol.

For the first series, the biofilm content of an entire column was estimated. The Table 2 presents the mean (m_i) of biofilm dry weight determined with n=5 measurements for each column, and the $t'_{i,j}$ parameter calculated considering pairs of columns. It can be seen that the value of $\left|t'_{i,j}\right|$ was less than 2.31. This means that the columns were inoculated with equivalent quantities of biomass at the beginning of the experiments. The average initial quantity was 0.10 mg per column for this series.

For the second series, the columns were divided into three slices (top, middle, bottom) in order to find the initial biofilm distribution in the sand. The Table 3 shows the mean (m_i) of biofilm dry weight determined with n=5 measurements for each part of the column, and the Student's t-test calculated with the following combinations: top-middle, top-bottom, and middle-bottom. As can be seen in Table 3, the values of the Student's t-test parameter were less than 2.31. This result indicated that there was no concentration gradient in the packed bed at the beginning of the experiments and that the method of inoculation led to a uniform distribution all along the columns. Moreover, statistical analysis showed that the total amount in the 3 columns was similar ($\left|t'_{i,j}\right| < 2.31$). The average initial quantity of biofilm was 0.17 g for this series.

The initial quantity of biofilm in the columns of the two series (0.10 g and 0.17 g) would appear to be different. But this was due to the relatively high experimental error for such low values: about 40 % and 25 % for 0.10 g and 0.17 g respectively. The two means were however statistically equivalent since the value of the Student's t-test for these two measures was 1.82.

Biofilm growth in the porous medium

Then, biofilm development was studied after biofilm growth. Two series of three columns each were prepared according to the above-mentioned protocol. The growth duration was different for each series: 5 and 7 days respectively. The mean (m_i) of biofilm dry weight determined with n=5 measurements for each column, and the values of $|t'_{i,j}|$ for each pair of columns are presented for both series in Table 4. All the Student's parameters were inferior to 2.31 even though $|t'_{1,2}|$ for 7 days of growth was near to the limit. We can thus state that the biofilm growth was the same in each series of three columns.

Since, on one hand, bacterial and dodecane distribution was uniform at the beginning of the experiment, and on the other hand biofilm growth was similar in the columns, the kinetics of biofilm formation in the porous medium could be determined by sampling the content of an entire column at different times.

Biofilm growth kinetics

Figure 3 presents the growth kinetics obtained for 15 g of trapped dodecane and 0.19 g of biomass at the initial time. The experiment lasted 392 h. The error bars on Figure 3 had quite the same values. This confirms that per cent experimental error decreases when dry weight increases. Thus except for initial value, the experimental error was less than 15 %, which could be considered as good for such a complex system.

A linear growth phase was observed with a rate of 1.8 $mg.h^{-1}$. This linear phase was observed with almost microorganisms growing on n-alkane (Blanch & Einsele, 1973; Bouchez-Naïtali et al., 2001; Chakravarty et al., 1975; Gutierrez & Erickson, 1977; Joannis, 1998; Mallee & Blanch, 1977; Miura et al., 1977) whatever the mechanism of uptake (interfacial accession or biosurfactant-mediated hydrocarbon uptake). For interfacial uptake, growth was exponential until the interfacial area was covered with cells, then the onset of the linear phase occurred. When biofilm or flocs were observed, the exponential phase was very short and the formation

of biofilm determined the onset of the linear phase due to the limitation of substrate or oxygen transport (Blanch & Einsele, 1973; Bouchez-Naïtali et al., 2001, Joannis-Cassan et al., 2005). In this study, a limitation in transport of both limiting-nutrient of the aqueous phase (probably dissolved oxygen) and dodecane to the cells within the biofilm could explain the linear growth.

After 250 h, the growth stopped even though half of the initial quantity of dodecane was still present. The cessation of biofilm growth appeared to be linked to dodecane conversion stop as no more consumption of nitrogen was observed and quantity of produced proteins reached a maximum (Figure 3). At the end of growth, the biofilm thickness could reach up to 80 µm (microscopic observation, Olympus, ×400 magnification). So diffusion limitation of both dissolved oxygen and hydrocarbon through the biofilm should explain this stop. Indeed their concentration within biofilm was not constant: oxygen concentration decreased from the aqueous phase to the hydrocarbon surface whereas dodecane concentration in biofilm decreased from the dodecane surface to the aqueous phase (Figure 4). When biofilm grew, each compound became exhausted in each area located near the surfaces, so growth could only occurred in the part of biofilm where both oxygen and dodecane were available, called active area. Beyond a critical thickness, the active area could disappear, so the biofilm development stopped even though all nutrients were available in the liquid phase. Nakahara already observed a decrease in the specific growth rate while considerable hydrocarbon remained (Nakahara et al., 1977). This was attributed to the alkane transport limitation. We comforted this hypothesis with an experiment carried out in a flask with an emulsion of mineral salt solution and dodecane (2% v/v of dodecane). After growth stop, dodecane was added to the medium. Immediately, the growth started again at a significant growth rate on fresh dodecane droplets. This experiment confirmed that the availability of dodecane rather than quantity was the key of biofilm growth.

Influence of dodecane surface

Considering the importance of dodecane surface, an experiment was made in the porous medium by introducing a water-dodecane emulsion (2% v/v) to inoculate the columns. In this case, 2g of dodecane remained in the columns just after inoculation. Even if the surface of dodecane could not be precisely estimated, the specific surface should be higher with emulsified dodecane than with pure dodecane. The growth kinetics is presented on Figure 5. The initial quantity of cells was higher than for the experiment with pure dodecane (0.36g against 0.19g for pure dodecane), probably due to the higher hydrocarbon surface available for the cells immobilisation. The mean growth rate was 1.1 mg/h. Contrary to the experiment with pure dodecane, the growth did not slow down after 400h. The total amount of biofilm produced was the same (0.48 g) for the two experiments. As the dodecane specific surface was higher, the biofilm thickness should be smaller in the second experiment. Thus, even if the growth rate could be limited by nutrients diffusion as previously, the duration of the growth was longer. Another phenomenon could explain this difference as space for biofilm growth was higher with emulsified dodecane. Indeed the hydrocarbon took about 9 % of the empty volume of the sand against about 66% for pure dodecane. So the biofilm development should be less limited by space during the experiment with emulsified hydrocarbon. This seemed to be confirmed by the amount of biofilm sloughed of the column which was 3.5 times less for emulsified dodecane than for pure dodecane.

Biofilm repartition in the porous medium

The spatial biofilm distribution in the sand was studied by dividing the column in three slices. The biofilm quantities in each part, at the initial time and after about 400h of the growth, are presented on Figure 6. Biofilm distribution was homogenous at the beginning of the experiments as shown previously. When pure dodecane was introduced in the columns, biofilm development after 392h of growth took place in the upper 2/3 part of the column and

was even more important at the top (Figure 6a). This indicates a limitation in oxygen distribution in the column since sufficient quantities of dodecane and mineral salts were still present at the end of growth. The dissolved O_2 introduced at the top of the column was consumed by the bacteria located in the upper layer and was no longer available for the micro-organisms located above 11.2 cm. Below this level, a lysis or a detachment of the biofilm occured since the final quantity of biofilm was less than the initial value. The cells located at the bottom of the column were not able to survive the period of oxygen deprivation. This was correlated to an increasing quantity of unbound biofilm from the porous medium during growth that reached a maximal value of 20 % of the total dry weight (Figure 3). This quantity represented the part of the biofilm weakly bound to the hydrocarbon, which was washed out by the aqueous phase.

For the experiment with emulsified dodecane (Figure 6b), the growth was the same in the 2/3 upper part of the column. A development was observed in the deeper part, even though it was inferior to the upper parts. In this case, few quantity of detached biofilm was found (Figure 5). These results confirmed the difficulty to bring oxygen to the deepest parts of the soil during bioremediation processes even though the aqueous phase was saturating with oxygen (Downey & Elliot, 1990; Irvine et al., 1993).

CONCLUSION

This work proposed the use of several identical packed columns to study the biofilm growth in a porous medium under biostimulation conditions. Use of the six-column system provided representative samples of soil by avoiding problems caused by heterogeneity of the biofilm distribution inside one column. Reliable values of biofilm dry weight were obtained, as experimental error was less than 15%. This work confirmed the limitation in oxygen distribution in the column, and pointed out a diffusion limitation of substrates within the

biofilm. So during bioremediation process, hydrocarbon consumption could be uncompleted, even though sufficient nutrients are supplied to the soil, due to the presence of biofilm on the surface of hydrocarbon. Future practical approach to bioremediation process should take into account the biofilm formation on hydrocarbon as it could be a reason which explains some failures of these processes.

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Table 1 Quantity of dodecane introduced in the columns

Assay number: i	1	2	3	4	5	6
Quantity of trapped dodecane per quantity of sand ($mg.g^{-1}$): m_i	107	113	106	78	101	103
Arithmetic mean ($mg.g^{-1}$): m	101					
Standard deviation ($mg.g^{-1}$): s	12					
Experimental error (%): s / m	8.3 %					

Table 2 Quantity of biofilm in the columns just after inoculation (Biofilm dry weight was the mean of $n_i = n_j = 5$ measurements, $s^2 = s_i^2 + s_j^2$)

Column number: i		1	2	3
Biofilm dry weight mean (g): m_i		0.09	0.08	0.12
$t'_{i,j} = \frac{m_i - m_j}{\sqrt{\frac{s^2}{n_i} + \frac{s^2}{n_j}}}$	$\lvert t'_{1,2} \rvert$		0.26	
	$\lvert t'_{2,3} \rvert$		1.04	
	$\lvert t'_{1,3} \rvert$		0.78	

Table 3 Distribution of the biofilm in the columns just after inoculation (Biofilm dry weight was the mean of n_i = n_j = 5 measurements, $s^2 = s_i^2 + s_j^2$)

Column number: i		1			2			3	
	Top	Middle	Bottom	Top	Middle	Bottom	Top	Middle	Bottom
Biofilm dry weight mean (g) for 50g of sand: m_i	0.068	0.064	0.070	0.065	0.058	0.050	0.040	0.042	0.046
$t'_{i,j} = \frac{m_i - m_j}{\sqrt{\frac{s^2}{n_i} + \frac{s^2}{n_j}}}$	$\lvert t'_{t,m}\rvert^*$	0.11			0.19			0.05	
	$\lvert t'_{m,b}\rvert^*$	0.16			0.21			0.11	
	$\lvert t'_{t,b}\rvert^*$	0.05			0.40			0.16	
Total mass in the column : m_i (g)		0.20			0.18			0.13	
$t'_{i,j} = \frac{m_i - m_j}{\sqrt{\frac{s^2}{n_i} + \frac{s^2}{n_j}}}$	$\lvert t'_{1,2}\rvert$				0.51				
	$\lvert t'_{2,3}\rvert$				1.29				
	$\lvert t'_{1,3}\rvert$				1.80				

*t : top; m: middle, b: bottom

Table 4 Quantity of biofilm in the columns after 5 and 7 days of growth (Biofilm dry weight was the mean of $n_i = n_j = 5$ measurements, $s^2 = s_i^2 + s_j^2$)

		5 days of growth			7 days of growth		
Assay number: i		1	2	3	1	2	3
Biofilm dry weight mean (g): m_i		0.20	0.17	0.12	0.28	0.19	0.24
$t'_{i,j} = \frac{m_i - m_j}{\sqrt{\frac{s^2}{n_i} + \frac{s^2}{n_j}}}$	$\lvert t'_{1,2} \rvert$		0.77			2.30	
	$\lvert t'_{2,3} \rvert$		1.29			1.27	
	$\lvert t'_{1,3} \rvert$		2.07			1.02	

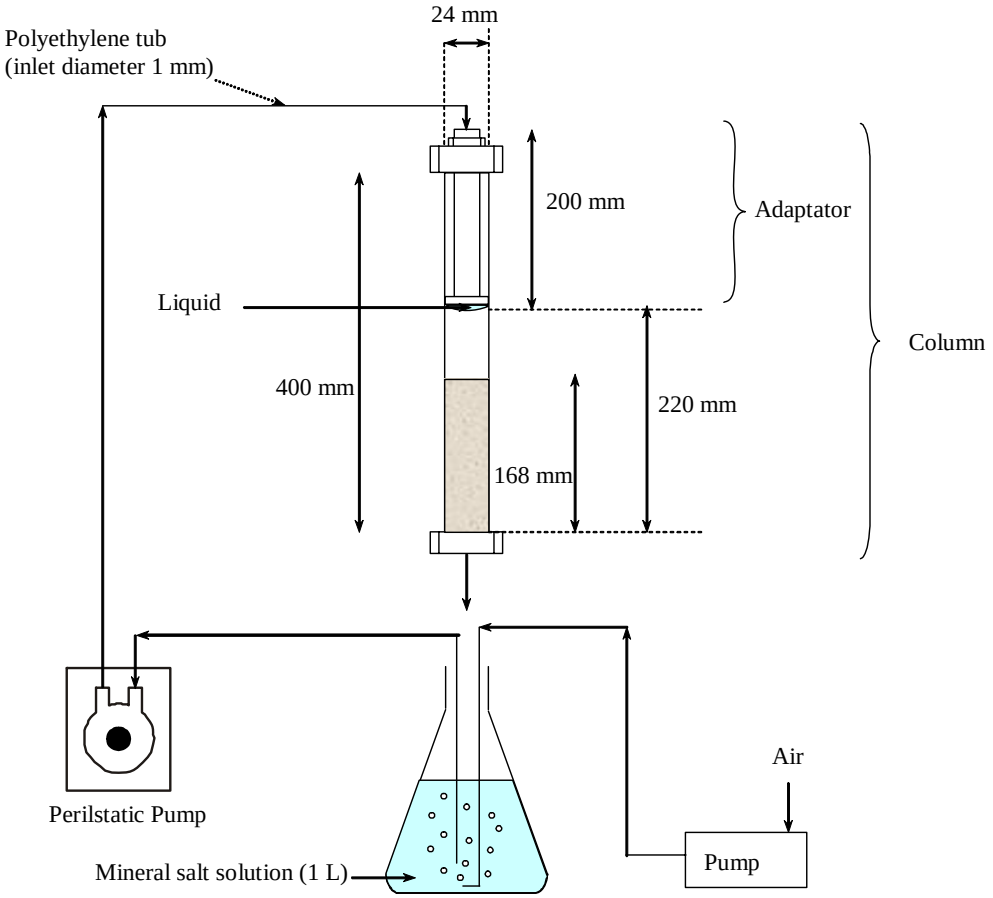

Fig. 1 Diagram of a packed bed column.

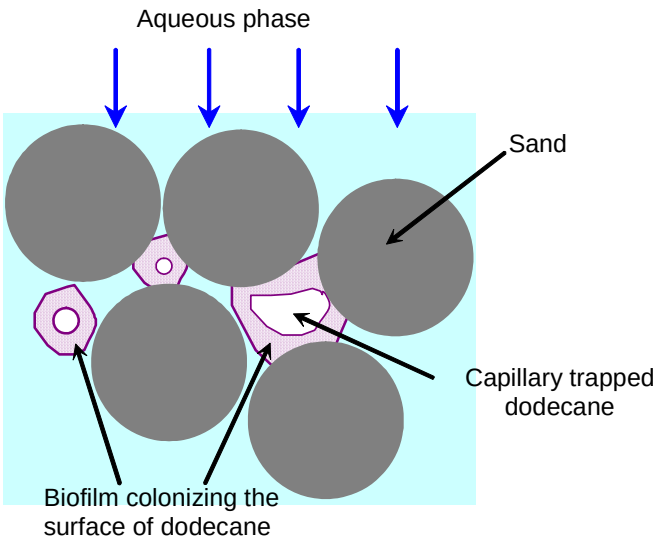

Fig. 2 Biofilm formation in the porous system.

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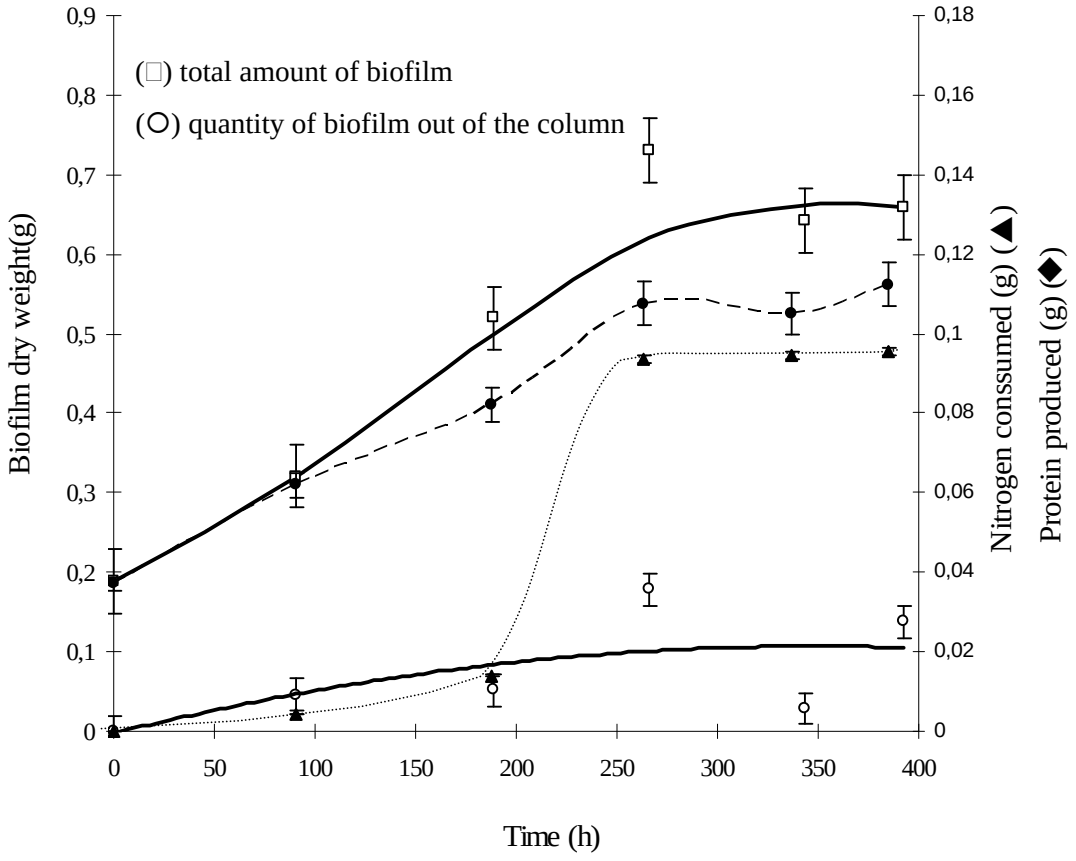

Fig. 3 Biofilm growth kinetics in terms of total amount of biofilm (□), quantity of unbounded biofilm out of the column (○), nitrogen consumption (▲) and protein produced (◆). Initial conditions: 15 g of pure dodecane, 0.19 g of biofilm. The aqueous phase was aerated at 0.9 vvm.

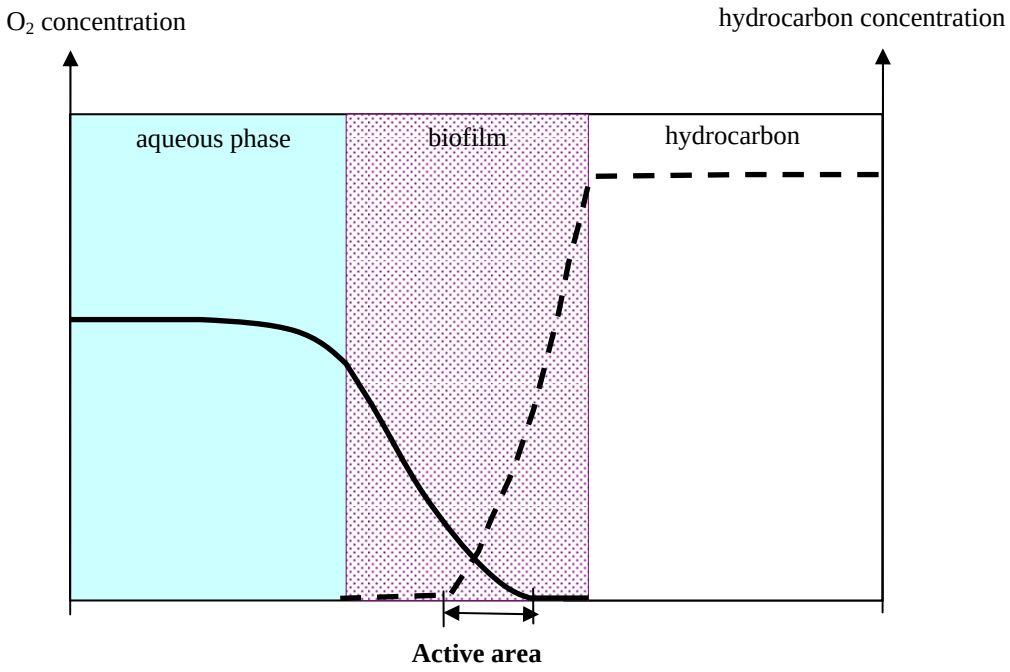

Fig. 4 Theorical dissolved ogygen (▬) and hydrocarbon (• • • •) concentration in biofilm.

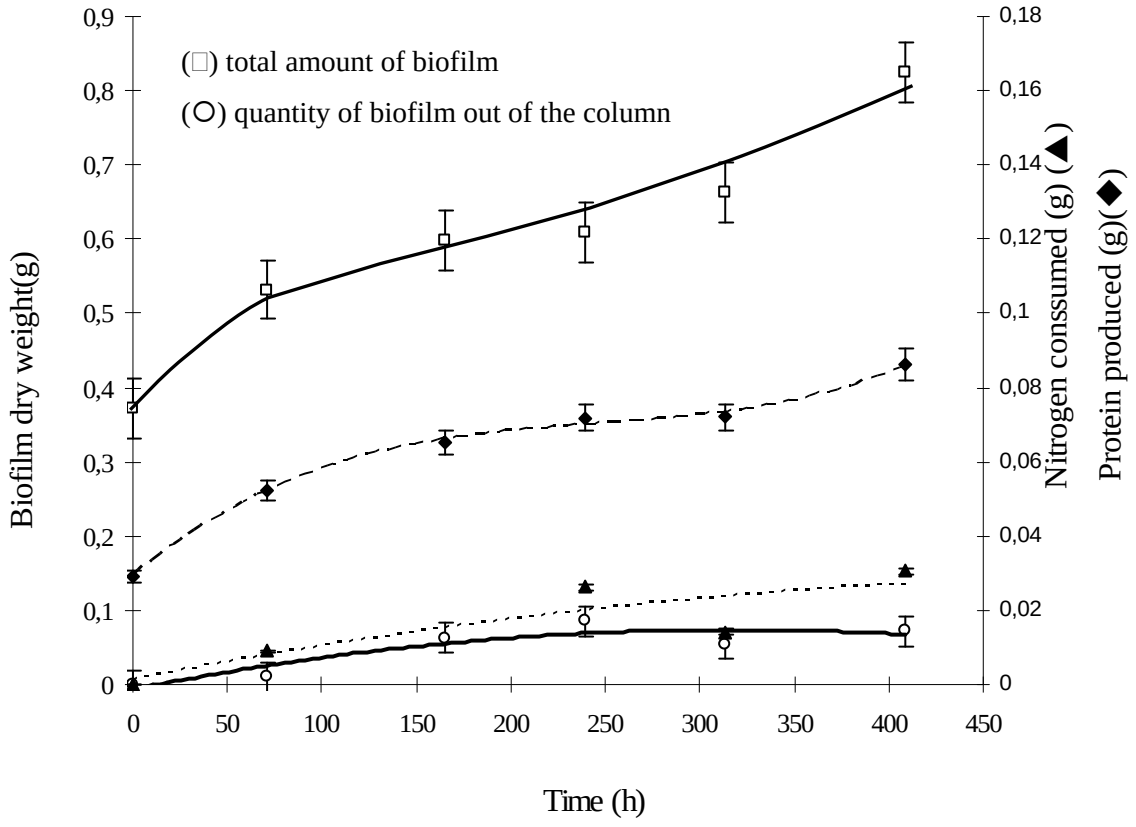

Fig. 5 Biofilm growth kinetics in terms of total amount of biofilm (□), quantity of unbounded biofilm out of the column (○), nitrogen consumption (▲) and protein produced (◆). Initial conditions: 2 g of emulsified dodecane, 0.36 g of biofilm. The aqueous phase was aerated at 0.9 vvm.

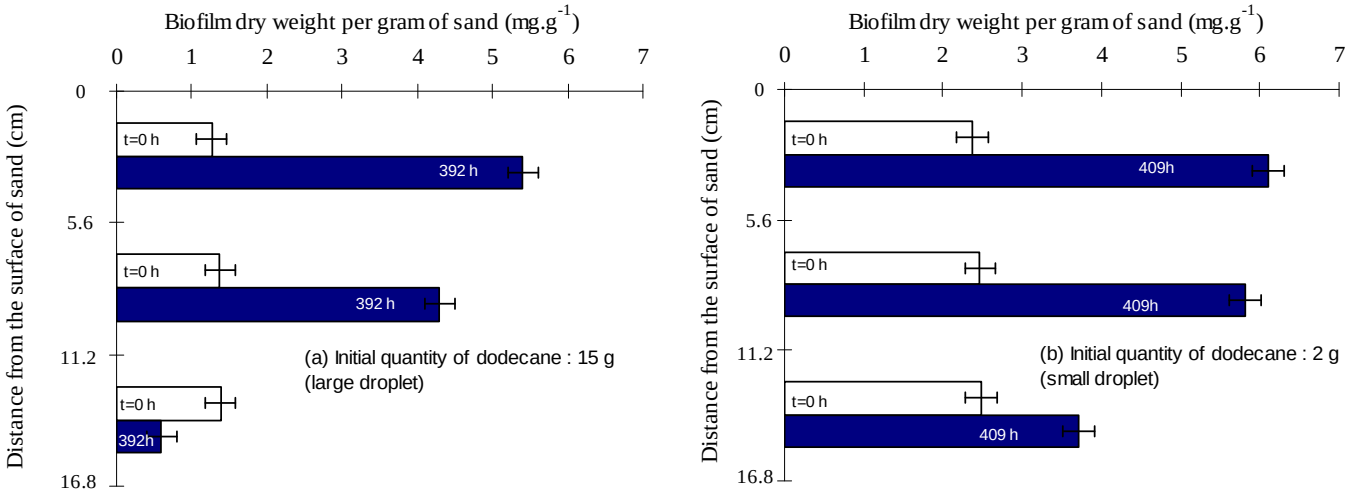

Fig. 6 Distribution of the biofilm in the porous medium just after inoculation (□) and after about 400h of growth (■) when pure dodecane (a) or emulsified dodecane (b) was introduced in the columns.