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Limitation phenomena induced by biofilm formation during hydrocarbon biodegradation

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ABSTRACT

Limiting factors induced by the formation of a biofilm on hydrocarbon's surface during *in-situ* bioremediation were identified. A liquid-liquid system (hydrocarbon-aqueous solution) and a device made of six fixed-bed columns packed with sand were successively used on laboratory-scale. In the liquid-liquid system, it was shown that biofilm growth was limiting by the diffusion of both oxygen and hydrocarbon within the biofilm. At the end of growth, the biofilm thickness reached $80\pm 20\mu\text{m}$. In the fixed-bed columns, additional difficulties were observed. First, distribution of oxygen in the deepest part of the sand was difficult: from 84 to 100% of the biofilm growth occurred in the upper 2/3 part of the sand. Then, the surface of the hydrocarbon available for microorganisms was found to be a limiting factor for the biofilm's development.

KEYS WORDS biofilm, hydrocarbon, bioremediation, diffusion limitation, *Pseudomonas alkanolytica*.

INTRODUCTION

Developments in the chemical industry have resulted, over time, in increasing incidences of soil contamination. Hydrocarbon pollution, resulting from the closure of old gas supply companies and oil tank leaks, has made a major contribution to this contamination causing extensive damage to the ecosystem. In the past 20 years, methods have been developed to clean up such contaminated soils¹. Among them, *in-situ* biological methods are being considered more frequently since they are able to decontaminate soil in depth. So, these methods are relatively cheap with less disruption of the ecosystem than chemical or physical processes². *In-situ* bioestimulation consists of enhancing the growth of indigenous microorganisms, that are able to use hydrocarbons as source of carbon and energy by injecting into the soil an aqueous solution containing nitrogen, phosphate, mineral salts and oxygen. Under favourable conditions, decontamination of several soils has been successfully carried out^{3,4,5,6}. The main difficulty of this type of process resides in the presence of two non-miscible phases, hydrocarbon and the aqueous solution, which must be in contact with the microorganisms. Moreover, some microorganisms may excrete polymers and form a biofilm on the hydrocarbon surface⁷. Biofilms are usually composed of two major components: microbial cells and extracellular polymers (essentially polysaccharides and glycoproteins)⁸. Biofilm formation includes 5 steps: (i) conditioning of a surface or interface, (ii) adhesion of cells, (iii) formation of micro-colonies, (iv) biofilm formation and growth and (v) possible

sloughing of pieces of the biofilm due to flow rate dynamics or shearing effects of flowing fluids. If strong shear forces are applied to biofilms, thickness does not exceed more than a few micrometers whereas for free development, thickness reaches several millimetres. Thus, biofilms should strongly influence mass transfer by limiting the diffusion of nutrient or oxygen within the biofilm⁹. If the biofilm thickness exceeds the depth of substrate penetration, an “inactive” area could appear in the inner biofilm. For instance, when the oxygen transfer is limited by diffusion, an aerobic area could develop near the support surface. Thus, biofilm could considerably modify the bioremediation process by forming an additional barrier between the two phases and the micro-organisms. However, the presence of biofilm is rarely taken into account in bioremediation studies¹⁰.

This paper describes the effect of the development of a biofilm on a hydrocarbon surface during bioremediation process. First, growth kinetics were established in a liquid-liquid system, composed of an emulsion between a hydrocarbon and an aqueous solution, in order to identify factors limiting growth. Then, biofilm growth was studied in a six-column laboratory-scale system to reproduce the complexity of the *in-situ* bioremediation process.

MATERIALS AND METHODS

Micro-organisms and culture conditions

Bacteria

Pseudomonas alkanolytica ATCC 21034 was used for all experiments. The strain was maintained on an agar Petri dish at 4°C. The storage medium was composed of digested soy peptone: 5 g, meat extract: 1 g, yeast extract: 2 g, NaCl: 5 g, agar: 15g in 1 dm³ of reverse osmosis purified water. The pH was adjusted to 7.2 with 1 mol.dm⁻³ NaOH.

Culture medium

The culture medium consisted of a mineral salt solution and a hydrocarbon added at 2 % v/v. The mineral salt solution composition was (NH₄)₂SO₄: 2 g, Na₂HPO₄: 3.61 g, KH₂PO₄: 1.75 g, MgSO₄·7H₂O: 0.2 g, CaCl₂: 50 mg, FeSO₄·7H₂O: 1 mg, CuSO₄·5H₂O: 50 mg, H₃BO₃: 10 mg, MnSO₄·5H₂O: 10 mg, ZnSO₄·7H₂O: 70 mg, (NH₄)₆Mo₇O₂₄·4H₂O: 10 mg in 1 dm³ of deionised water. The pH was adjusted to 7 using 1 mol.dm⁻³ NaOH and the medium was sterilized for 20 minutes at 121°C. The hydrocarbon was n-dodecane (Fluka). Before being added to the mineral salt solution, the dodecane was checked for sterility under a microscope (Olympus, 400×magnification). For each test, possible contamination was regularly checked by observing the morphology of the cells under a microscope (Olympus, 400×magnification).

Preparation of the starter cultures

Two different starter cultures were used for the inoculations: one for the liquid-liquid reactor and another for the soil six columns system. To obtain reproducible initial conditions, the starter cultures were prepared in two steps. First, a preculture, inoculated from the storage medium, was grown for 5 days in a flask containing 100 cm³ of the culture medium. The flask was shaken at 250 rpm and incubated at 30°C.

Then, for the two-phase reactor, 1 cm³ of the aqueous phase of the preculture was introduced in 100 cm³ of fresh culture medium. After a 3-day growth at 30°C, an appropriate volume of the aqueous phase was introduced into the liquid-liquid system to have 0.01g.dm⁻³ (dry weight) of biofilm at the beginning of the experiments.

For soil experiments, 3 cm³ of the preculture were introduced in 300 cm³ of medium culture. After a 3-day growth at 30°C, the culture was centrifuged 5 minutes at 20,000 rpm. The pellet was washed and suspended in an appropriate volume of the aqueous solution to obtain a concentration of 0.5 g.dm⁻³ (dry weight). This suspension was used to inoculate the sand.

Experimental devices

Liquid-liquid system

The liquid-liquid system was a 4 dm³ working volume cylindrical reactor (200 mm in diameter, 300 mm in high). The emulsion between the aqueous solution and dodecane (2% v/v) was obtained using a Rushton turbine (75 mm in diameter, 6 flat blades) located halfway up the culture medium (Figure 1-a). To study oxygen limitation, tests were carried out by pumping air through the culture medium. The air was sterilized by passing through a 0.22 µm filter before being introduced into the reactor at a flow rate of 8.6 cm³.s⁻¹. Unless otherwise stated, the reactor was stirred at 250 rpm and the temperature was set at 30°C. The growth kinetics were determined by withdrawing samples from the reactor every 12 hours.

Bioremediation system

Bioremediation experiments were carried out in 6 glass columns (Amersham Pharmacia Biotech, Saclay, France) (40 cm in length, 2.4 cm in diameter) simultaneously. A sand (Prolabo), with standardised size (400 µm in diameter) was used as a soil model. The porosity was 0.34 and the hydraulic conductivity was estimated at 8.10⁻⁵ m.s⁻¹. The permeability was 8.2 10⁻¹² m². Each column was packed with 150 g of sterile sand (Figure 1-b). Dodecane was first introduced into the sand from the bottom of the column. The sand was then washed with sterile water for 12 hours. The quantity of capillary-trapped dodecane in the columns was estimated by weighing the quantities introduced and recovered. The hydrocarbon was

homogeneously distributed throughout the sand, as a Sudan red coloration proved. Biological inoculation was carried out by loop circulation from the top of the column of 300 cm³ of the starter for 6 hours. The sand was then washed with the aqueous solution to recover the free bacteria.

The bioremediation process was run by trickling down 1 dm³ of the aqueous solution into each column; the sand was kept saturated. The aqueous phase was recycled through each column with a peristaltic pump at a flow rate of 0.36 cm³.min⁻¹, so the interstitial rate was 2 mm.min⁻¹. Experiments were carried out at room temperature, which was 20°C ± 2°C. The growth kinetics were determined by sampling the sand of an entire column at different times since a preliminary study proved that both initial inoculation and biofilm growth were statistically similar in the 6 columns¹¹. The last column was divided into three parts equal in volume (top, middle, and bottom) which were separately analysed to determine the biofilm distribution in the sand. At the end of the experiment, the biofilm sloughing from the porous medium was estimated by measuring the dry weight of biofilm in the aqueous phase.

Analytical methods

Biofilm quantification

Dry weight measurement by filtration. This technique is based on filtration through cellulose acetate filters (Sartorius) (0.2 µm pore size). For the two-phase culture, two samples were directly removed from the culture medium with a piston sampler (12.5 cm³) and centrifuged for 10 min at 20,000 rpm. Three parts were observed: a pellet (biofilm), an aqueous phase and a layer floating on the top (an amalgam of dodecane, biofilm and some traces of the aqueous phase). After removing the aqueous phase, a solvent mixture (acetone-petroleum ether, 3:1 v/v) was added. This solvent made it possible to separate dodecane from the biofilm by making the hydrocarbon soluble with the aqueous phase¹² and by precipitating biofilm¹³. The total amount of biofilm was recovered after centrifugation at 34,000g and removal of the supernatant. The resulting pellet was suspended in sterile water, filtered and dried at 70°C until a constant dry weight was achieved.

For the porous system, biofilm should be extracted from the porous medium. Therefore, the amalgam between dodecane and the biofilm was recovered by mixing the sand with sterile water using the following protocol. The content of the column was pushed out into a 1 dm³ flask. Then, five successive extractions were made by adding sterile water to the sand, mixing vigorously for 30 minutes and recovering the liquid phase (biofilm, hydrocarbon and water) after sand sedimentation. We checked that the total amount of biofilm was recovered by

weighing the sand of each column before and after the experiments. The total water volume used was 750 cm³ per column. The five extracts were combined. Five samples of this liquid phase were taken out with a 24.7 cm³ piston sampler to be analysed as it is described above for the liquid-liquid samples.

Dry weight estimation from optical density measurement. This second method consisted in an *in-situ* measurement of the optical density (OD) of the culture medium with a biomass probe (Model 652 cell growth monitor, Wedgewood Technology, Inc., San Carlos, USA). The biofilm dry weight was directly quantified by using the existing linear relations between OD and biofilm dry weight¹³. This method was only used for the liquid-liquid culture without air supply as air droplets led to interference with OD measurement.

Droplet size and biofilm thickness in the liquid-liquid reactor

In the stirred tank reactor, the hydrocarbon formed spherical droplets, so the surface (A) available for the micro-organisms could be calculated from:

$A = \frac{6 \cdot V_d}{d}$, where V_d is the volume of dodecane, and d the diameter of the droplets.

The specific area (a), which is the ratio of the dodecane surface and the total volume of the dispersion expressed in m⁻¹, is calculated from:

$a = \frac{6 \cdot \varepsilon}{d}$, where ε is the volumic fraction of dodecane.

The droplet diameter was determined from video pictures by comparing the droplet size with the diameter of a stainless rod (0.5 mm) immersed in the emulsion. The dodecane droplet diameter was found to be equal to the diameter predicted by the Calderbank's correlation¹⁴, which was 200 μm for an agitation rate of 250 rpm. At the end of growth, the diameter of dodecane droplets and the thickness of the biofilm were estimated from video pictures of the reactor by comparing them to the diameter of various stainless steel rods (from 0.1 to 0.5 mm).

Other analytical methods

The dissolved oxygen in the liquid-liquid reactor was measured with an O₂ probe (PO₂, Ingold, Inceltech, Toulouse, France) linked with an amplifier (Model 170, Ingold, Inceltech, Toulouse, France). The probe was standardized using an air-saturated solution (100% of dissolved oxygen).

The pH was measured with a pH probe (Ingold, Inceltech, Toulouse, France). A PID regulator (Inceltech, Toulouse, France) was used to maintain the pH at 7 with 1 mol.dm⁻³ NaOH.

The (NH₄)₂SO₄ concentration in the aqueous phase was determined by the Nessler method¹⁵.

Proteins were extracted from the biofilm by hydrolysing 0.5 cm³ of the sample at 100°C with

0.5 cm³ of 0.25 mol.dm⁻³ H₃PO₄¹³. The protein concentration was estimated with Lowry's method¹⁶ using a solution of BSA (Bovine Serum Albumin) containing an equal volume of H₃PO₄ as reference.

Statistics

A statistical analysis of the biofilm quantification methods was previously made¹³ and the standard deviation (e%) was linked with biofilm dry weight. In this paper, results are presented on the graphs in terms of 95 % confidence intervals.

RESULTS AND DISCUSSION

Factors limiting growth in the liquid-liquid system

In this system, it could be observed that the biofilm growth took place on the dodecane surface (Figure 1-a). This surface, estimated from the size of the hydrocarbon droplets, decreased during the growth due to the consumption of the hydrocarbon. In the same time, the thickness of biofilm increased due to its development.

Theoretical considerations

Due to the thickness of the biofilm, diffusion limitations could occur during the biofilm development as reported by several authors for biofilms growing on solid supports^{8,17,18}. For biofilm growing on liquid hydrocarbon, diffusion limitation could concern molecules located on both sides of the biofilm: dodecane and the rate-limiting component of the aqueous phase. Figure 2 explains this theoretical phenomenon for diffusion limitation of dodecane and dissolved oxygen. For thin biofilm (Figure 2-a) a gradient of both dissolved oxygen and dodecane could be observed, even if the hydrocarbon probably poorly diffused within the biofilm due to its non-miscibility with water. However, the two components are not exhausted in the biofilm, so the biofilm could develop. When the biofilm thickness increases (Figure 2-b), both hydrocarbon and oxygen could be exhausted in some areas: oxygen near the dodecane surface and dodecane near the aqueous phase. This leads to the appearance of an active area and two inactive areas where growth can no longer occur. Beyond a critical thickness (Figure 2-c), the active zone should be reduced to one point. The biofilm development stops.

Growth kinetics

Figure 3-a presents the biofilm growth kinetics without air supply, in order to reproduce operating conditions encountered in the soil. Oxygen was transferred into the culture medium only through the liquid surface. The volumetric oxygen mass transfer coefficient ($k_L a$) was 0.0008 s⁻¹. The biofilm growth shows 4 distinct phases. After a 10 h lag phase (1), the biofilm

growth occurred at an average rate of $11.3 \text{ mg}\cdot\text{dm}^{-3}\cdot\text{h}^{-1}$ between 10 and 60 h while the quantity of dissolved O_2 decreased (2). This confirms that the consumption of dodecane by *Pseudomonas alkanolytica* is aerobic, which is common for most of the bacteria growing on a hydrocarbon substrate^{12,19}. Between 60 and 225 h (3), the growth was linear (the observed rate was $5.4 \text{ mg}\cdot\text{dm}^{-3}\cdot\text{h}^{-1}$). During this phase, dissolved oxygen was completely depleted and the growth rate should be controlled by the oxygen transfer from the surrounding air into the liquid by the impeller. After 225 h (4), the growth reached a stationary phase and the oxygen concentration increased up to 100%. During the growth, the droplet diameter reduced from 200 to 160 μm , which suggests a consumption of half the dodecane¹¹. At the end of the growth, biofilm thickness was $80 \pm 20 \mu\text{m}$, which is about half of the droplet size.

The arrest of the growth was probably caused by diffusion limitation of both dodecane and oxygen within the biofilm as it is described above. A series of experiments were carried out to verify that no other limiting factors were the cause of the growth stop, such as nutrient exhaustion, inhibition by low pH or product inhibition.

First, the final quantity of dodecane and nitrogen source was estimated. As already mentioned, about half of the dodecane was still present at the end of growth and the residual amount of $(\text{NH}_4)_2\text{SO}_4$, the only source of nitrogen, was $200 \text{ mg}\cdot\text{dm}^{-3}$. For other components of the aqueous phase, an experiment was carried out by supplying 30 cm^3 of the mineral salt solution just before the stationary phase. No difference was observed with the previous experiment, demonstrating that the limitation was not due to exhaustion of a mineral salt.

Then, limitation due to low pH value was studied, since pH linearly decreased from 7 to 5.5 during the batch experiments (Figure 4) due to the production of acids¹⁹ coupled with mineral salt consumption. Two experiments were performed with the same culture conditions except for the pH, which was either controlled or not (Figure 4). Even if the final quantity of biofilm was slightly higher when the pH was controlled, no significant difference was observed between the two cultures, and the growth reached a stationary phase in both cases.

At last, an experiment was made by supplying 30 cm^3 of dodecane to the culture medium when the growth reached the stationary phase. As can be seen on Figure 5, as soon as dodecane was supplied to the culture, the growth occurred on fresh hydrocarbon droplets at the same rate as the initial growth. Thus, no inhibiting products were present in the medium culture at the end of growth. This result supports the hypothesis of an arrest of growth caused by the diffusion limitation of both dodecane and oxygen within the biofilm and also evidences that the dodecane availability has an influence on the biofilm growth and thickness.

Influence of the dodecane surface

Experiments were carried out with several interfacial areas by changing the impeller rotation rate. For each stirring rate, the interfacial area was calculated from the droplet diameter estimated by Calderbank's correlation. The biofilm growth kinetics obtained for initial dodecane specific surfaces of 550 m^{-1} , 600 m^{-1} and 700 m^{-1} are shown on Figure 6. An improvement of the biofilm maximum growth rate with the specific surface was observed: $4.3 \text{ mg.dm}^{-3}.\text{h}^{-1}$ for $a = 500 \text{ m}^{-1}$, $11.4 \text{ mg.dm}^{-3}.\text{h}^{-1}$ for $a = 600 \text{ m}^{-1}$ and $12.5 \text{ mg.dm}^{-3}.\text{h}^{-1}$ for $a = 700 \text{ m}^{-1}$. The acceleration of growth was probably due to the improvement of the oxygen transfer rate linked to the increase in the stirring rate²⁰. However, the amount of biofilm in the medium was almost the same at the end of growth (about 1.5 g.dm^{-3}) in all three experiments. The greater the dodecane surface, the thinner the biofilm. The increase of the shear force on the biofilm surface consequent on the impeller rotation rate could disturb biofilm development. Thus, modifying the impeller rotation rate did not clearly demonstrate the effect of the dodecane surface since it not only changed the dodecane surface area but also the surrounding conditions of growth.

Effect of oxygen supply

To improve the rate of oxygen dissolution into the culture medium, the medium was aerated at $8.6 \text{ cm}^3.\text{s}^{-1}$. The k_{LA} value increased up to 0.002 s^{-1} . The results are shown on Figure 3-b. Aeration involved an amalgam between hydrocarbon, biofilm and air droplets which could explain the dry weight values dispersion on the graph. The average rate of biofilm growth was almost four times higher than for the non-aerated culture ($2.8 \text{ mg.dm}^{-3}.\text{h}^{-1}$ without aeration versus $11 \text{ mg.dm}^{-3}.\text{h}^{-1}$ with aeration). Aeration prevented oxygen dissolution from limiting the reaction since the percentage of dissolved O_2 is almost always above zero. However, as for the non-aerated culture, growth severely slowed down as soon as the biofilm concentration reached 1.5 g.dm^{-3} , which appeared after 50h. At the same time, oxygen increased up to 100%, suggesting a non-consumption by the micro-organisms for the same reasons as explained above.

Bioremediation process

In this system, the dodecane was capillary-trapped. BATH (Bacterial Adherence To Hydrocarbon) experiments²¹ proved that *Pseudomonas alkanolytica* had a high adherence to dodecane¹¹. Moreover, it has been reported that bacteria are usually poorly absorbed onto sand²². So, the hypothesis was made that during inoculation, the bacteria adhered to the hydrocarbon rather than on the sand, and that biofilm only grew at the surface of the dodecane

(Figure 1-b).

Theoretical considerations

In comparison with the liquid-liquid system, the bioremediation system could generate additional limitation phenomena due to the presence of the porous matrix. Whereas nutrient distribution was spatially homogeneous in the liquid system, feeding the column from the top could lead to a concentration gradient through the sand. Oxygen exhaustion through the column has already been observed^{23,24}. Moreover, the biofilm development could be limited by the size of the sand pores.

Effect of oxygen supply

Two experiments were carried out to study the effect of O₂ on biofilm growth. In the first test, no air was supplied in the aqueous phase which circulated through the column. Table 1 (line 1) presents the dry weight of the biofilm produced and the biofilm distribution throughout the column at the end of the growth. It can be seen that 89.1 % of the biofilm developed in the upper 1/3 part of the sand. As dodecane was homogeneously distributed in the column and as a significant quantity of the nitrogen source was found in the outlet of the column, the biofilm spatial distribution seems to be due to a depletion of the dissolved oxygen in the upper third part of the column. A second test was undertaken with an aeration of the aqueous phase at a rate of 15 cm³.s⁻¹. The results in Table 1 (line 2) show that the total biofilm quantity was almost double than without aeration. The biofilm located in the middle of the column reached 42.3% of the total biofilm, which indicates a better distribution of the oxygen at this depth. No growth was observed at the bottom of the column due to the depletion of oxygen. Although oxygen distribution was improved by the aeration of the aqueous phase, it was still difficult to bring oxygen to the deepest parts of the sand. On large-scale *in-situ* systems, the oxygen limitation is one of the most frequently quoted reasons for bioremediation failures^{25,26}.

Influence of the dodecane surface

The influence of the initial dodecane surface was investigated in the porous media. Contrary to the previous experiments, where pure dodecane was used to inoculate the columns, a water-dodecane emulsion (2% v/v) was introduced into the sand. In this case, 2 g of dodecane remained capillary-trapped in each column. Even if the surface of dodecane could not be precisely estimated, the specific surface should be higher for emulsified dodecane than for pure dodecane. The aqueous phase was aerated at 15 cm³.s⁻¹. The results are presented in Table 1 (lines 3). The total biofilm produced (0.48 g) was the same whether 15 g of pure dodecane or 2 g of emulsified dodecane were introduced into the sand. This was probably due

to a lower dodecane surface availability for pure dodecane coupled with a lack of pore space for the biofilm development as 15 g of dodecane took about 2/3 of the empty volume of the sand. The comparison of the percentage of biofilm sloughed of the column (Table 1) for pure and emulsified dodecane consolidated this hypothesis as the percentage of detached biofilm was 3.5 times higher for pure dodecane.

These results confirmed the importance of the dodecane distribution in the polluted soil. However, for dispersed dodecane, the bioremediation process was still limited by oxygen distribution in the column as only 16.3% of the growth occurred in the deepest third part of the sand.

Comparison of the growth kinetics in the liquid-liquid and the bioremediation systems

The biofilm growth kinetics in porous medium, with aeration of the aqueous phase and dispersed dodecane is presented on Figure 7. The development of biofilm in the liquid-liquid system at the room temperature¹¹ is presented on the same graph. It can be seen that the biofilm development occurred at almost the same growth rate for both experiments. However, contrary to the liquid-liquid system, the growth continued after 300 h in the porous system. This difference is probably the consequence of the difference of surrounding conditions for biofilms (shear stress, dodecane accessibility). The protein content of the biofilm was determined for each experiment: the percentage was 51% in the liquid-liquid system and 12% in the sand device. As proteins are partially responsible for the cohesion of the biofilm, this suggests that the operating conditions should be more favourable for the bacteria attachment in the porous medium: the cells should be more easily in contact with dodecane and the biofilm cohesion should not need to be as strong as in the stirred tank. Thus, the depth of nutrients diffusion should increase and the limitation due to diffusion transport of oxygen and nutrient would appear later than in the liquid system. However, beyond a critical thickness, the bioremediation process could fail because of the limitation of hydrocarbon and oxygen diffusion within the biofilm formed on the dodecane surface.

CONCLUSION

This study shows the importance of taking into account the biofilm formation on hydrocarbon's surface during bioremediation processes. A diffusion limitation of oxygen and dodecane within the biofilm was observed in the liquid-liquid reactor. This phenomenon adds to other difficulties encountered in the soil during bioremediation processes: oxygen distribution in the soil's depth and hydrocarbon surface availability. As diffusion limitations

could not be removed without destroying the biofilm, future work should aim at improving substrate diffusion, for example by researching a consortium composed of bacteria growing on hydrocarbon and micro-organisms consuming the polymer of the biofilm.

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Table 1 Effect of the aqueous phase aeration and the dodecane distribution on the biofilm growth during bioremediation process.

Initial quantity of dodecane	Aeration of the aqueous phase / flow rate	Biofilm at the end of the growth					
		Total dry weight (g)	% of biofilm in the column	% of biofilm out of the column	Distribution in the column (% of dry weight)		
					Top ^a	Middle ^b	Bottom ^c
15 g (pure)	No	0.27	80	20	89.1	2.2	8.7
15 g (pure)	Yes / 15 cm ³ .s ⁻¹	0.48	79	21	57.7	42.3	0
2 g (emulsified)	Yes / 15 cm ³ .s ⁻¹	0.48	94	6	43	40.7	16.3

^a Between 0 and 6 cm below the surface

^b Between 6 and 12 cm below the surface

^c Between 12 and 18 cm below the surface

Figure 1. Experimental devices and biofilm location in liquid-liquid system (a) and bioremediation system (b).

Figure 2. Theoretical schedule of diffusion of substrates in the biofilm in case of: thin biofilm (a), middle-thickness biofilm (b) and thick biofilm (c).

Figure 3. Effect of aeration on the biofilm growth in the liquid-liquid system. The results show biofilm dry weight (■) and dissolved oxygen (□) when the system was not aerated (a) or aerated at $8.6 \text{ cm}^3 \cdot \text{s}^{-1}$ (b).

Figure 4. Influence of pH on the biofilm growth in the liquid-liquid system with no aeration.

Figure 5. Effect of dodecane supply on the biofilm growth kinetics in the liquid-liquid system.

Figure 6. Influence of the surface of dodecane on the biofilm growth in the liquid-liquid system.

Figure 7. Comparison of biofilm growth kinetics in the liquid-liquid system and in the bioremediation process with aeration and dispersed dodecane at room temperature.













