Phosphorus requirements for bacteria that metabolize hydrocarbons in the sea

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Phosphorus Requirements for bacteria that metabolize hydrocarbons in the sea

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Introduction

The possibility of artificially stimulating the purification of marine zones polluted by hydrocarbons has been considered by Le Petit and Barthelemy, 1968; Atlas and Bartha, 1972 and Cobet et al, 1972. However, if this method is to be used, it is necessary to identify accurately the requirements that micro-organisms involved in biodegradation have for essential elements such as phosphorus. The proportion of salts and particularly bivalent cations (Ca\(^{2+}\), Mg\(^{2+}\), Sr\(^{2+}\)) determines the extent to which phosphates are soluble in seawater. The phosphates precipitate beyond a concentration that depends on the chemical properties of the water; changes in which can be traced from the open sea to the littoral zones that receive land water from watercourses and runoff.

The effect of variation in phosphate concentration was analysed, first on microflora present in seawater and growing on gas oil, and second on bacterial strains isolated from such microflora and cultivated for experiments with simple, chemically pure hydrocarbons. Decane and hexadecane were the only carbon sources used, while acetate was used as a control substrate.

Material and Methods

Bacterial strains used

The main properties of five of the six bacterial strains used in this work (Achromobacter sp 1, Alcaligenes sp 2, Alcaligenes sp 5, Acinetobacter sp and Arthrobacter sp) have been described by Le Petit et al, 1975 and for this reason the generic names Achromobacter and Alcaligenes have been used although they no longer conform with the 8th edition of Bergey's Manual (Buchanan and Gibbons, 1974).

A strain of Pseudomonas aeruginosa sol 20 that can grow as well in freshwater as in seawater was also used.

Culture media

These were divided into 400 ml quantities for each 2-litre toxin flask. The composition of the basal saline solutions was as follows:

Artificial Seawater (ASW) (Burkholder, 1963) - NaCl, 23.476 g; Na\(_2\)SO\(_4\), 3.917 g; NaHCO\(_3\), 0.192 g; KCl, 0.664 g; KBr, 0.096 g; MgCl\(_2\cdot6\)H\(_2\)O, 10.643 g; SrCl\(_2\cdot6\)H\(_2\)O, 0.040 g; H\(_3\)BO\(_3\), 0.026 g; CaCl\(_2\cdot2\)H\(_2\)O, 1.459 g; enough distilled water for 1000 ml. The ionic strength of the solution, supplemented with 2.5 g/litre of NH\(_4\)Cl as a source of nitrogen, is 0.73.

Modified artificial seawater (mASW) - NaCl, 23.476 g; (NH\(_4\))\(_2\)SO\(_4\), 3.57 g; KCl, 0.664 g; KBr, 0.096 g; MgCl\(_2\cdot6\)H\(_2\)O, 0.3 g; SrCl\(_2\cdot6\)H\(_2\)O, 0.024 g; H\(_3\)BO\(_3\),
0.026 g; CaCl₂·2H₂O, 0.1 g; enough distilled water for 1000 ml.
The ionic strength of the solution is 0.50.

Medium A- NaCl, 0.5 g; NH₄Cl, 2.5 g; solution of trace elements,
2.5 ml (for 500 ml of distilled water: MgCl₂·6H₂O, 2 g; FeCl₃·6H₂O,
0.628 g; ZnCl₂, 0.4 g; MnCl₂·4H₂O, 0.4 g; CaCl₂·2H₂O, 0.2 g; C₆H₅Cl·2H₂O,
0.02 g; Na₂SO₄·10 g; Na₂MoO₄·2H₂O, 0.020 g); enough distilled water
for 1000 ml. The ionic strength of the solution is 0.057.

Medium B- NaCl (concentrations ranging from 0.2 to 0.8 M); KCl, 0.5 g;
NH₄Cl, 2.5 g; solution of trace elements, 2.5 ml; enough distilled water
for 1000 ml. The ionic strength of the solution is 0.255, 0.455, 0.655
and 0.855 for 0.2 M, 0.4 M, 0.6 M and 0.8M NaCl respectively.

Media A and B are modifications of the medium described by Imelik (1948).

Preliminary tests showed that it is possible to add to seawater monobasic
and dibasic phosphate buffers of pH 7.2 up to concentrations of 1.5 x 10⁻³ M
without the risk of precipitation. However, the phosphate solution must be
added under sterile conditions after autoclaving the culture medium and cooling
it completely. Furthermore, all media must be buffered. The phosphate
concentration in the ASW is insufficient, so Tris 0.05 M, pH 7.2 is required.
With mASW and media A and B, the usable phosphate concentrations are higher,
so the Tris is added only as a supplement so that the buffers will have a
final molarity of 0.05M.

The addition of 2 mg/litre of FeSO₄·7H₂O proved vital to the proper growth
of certain strains in ASW and mASW media.

The following concentrations were used for the carbon sources: sodium
or ammonium acetate 0.1 or 0.5% depending on the bacterial strain used; decane
or hexadecane at concentrations such that all the hydrocarbons are consumed
at the end of the growth phase (from 0.25 to 1%). A check was made on a
control culture by CCl₄ extraction of the residual hydrocarbons and determination
of the -CH₂ groups at 3.45 µm in a 10 mm calcium fluoride cell (Leitz infrared
spectrophotometer).

The culture media were seeded from preliminary preparations in which the
phosphate content, as low as possible, was compatible with the total
consumption of the selected carbon source. The preliminary preparation was
centrifuged and the bacteria were washed twice in the phosphate-depleted basal
medium. The bacterial suspension used was such that the inoculated medium had
an optical density close to 0.100.

In order to obtain phosphorus-deficient bacteria, the cells in a
preliminary preparation were centrifuged and washed twice, then put back in
suspension for 24 hours in a phosphate-depleted medium.

Bacterial growth and productivity by weight [?]*

Bacterial growth was measured with a Beckman DU spectrophotometer at

*metabolization of hydrocarbons - Tr
450 mm on cultures shaken at 30°C. The productivity was measured by (micro-Kjeldahl) nitrogen analysis, after the nitrogen content in each strain had been determined on a weighed quantity of dried cells. Washing in artificial seawater makes it possible to avoid lysis, a phenomenon occurring when distilled water is used.

Respiratory activity

This was measured by Warburg's manometric technique. Gaseous phase, air; temperature 30°C; total volume, 3 ml; pH, 7.2; bacterial suspension (3 to 8 mg dry weight): bacteria suspended in the basal solution + Tris 0.05 M, pH 7.2; KOH 40%; substrate 0.5 ml (sodium acetate 0.5%; hexadecane (1.3%) emulsified in the Branson (20 kc) sonic desintegrator in the presence of 1% Tween 80). Endogenous respiration was measured in the presence of Tween at the same concentration.

Determination of phosphorus in culture media

Because of the possibilities of interference with various ions of seawater, we used the technique developed by Murphy and Riley (1962) and described by Strickland and Parsons (1965).

Staining of polyphosphates

Method developed by Ebel et al (1958).

Results

Expérimental demonstration of the sensitivity of microflora present in seawater to phosphorus concentrations

Generally, when NH₄Cl as a nitrogen source, a gas oil as a carbon source and phosphate in the form of sodium buffer are added to freshly sampled seawater, the microflora in the water proliferate. Figure 1 illustrates the variations in rate of growth following variations in phosphorus concentration obtained with one of the water samples taken in a littoral zone. The cultures were retarded and partially inhibited at concentrations equal to or greater than 7 x 10⁻⁴ M. However, such inhibition does not occur systematically with all water samples.

Reaction to phosphorus of isolated bacterial strains

Artificial seawater culture with acetate and hexadecane

Acetate and hexadecane were selected because in the culture media used in these experiments they are metabolized by most of the isolated strains.

Growth rate - All strains studied had a constant, maximum growth rate on acetate starting at 2 x 10⁻⁴ M phosphate (Fig 2), but they were distinguished
by the amount of variation between $5 \times 10^{-5}$ and $2 \times 10^{-4}$ M. The variation was very slight in *Alcaligenes* sp 2, *Alcaligenes* sp 5 and *Arthrobacter* sp. In *Acinetobacter* sp 2, the growth rate was increased almost 5 times.
FIG 1. Microbial development (expressed in optical density) as a function of time in a freshly taken seawater sample to which NH₄Cl, gas oil and phosphate at varying concentrations were added: 1) seawater after autoclaving; phosphate added: 2) 0; 3) 10⁻⁴M; 4) 4 X 10⁻⁴M; 5) 7 x 10⁻⁴M; 6) 10⁻³M.

FIG 2a, b. Change in growth rate (r) as a function of phosphate concentration, for Achromobacter sp 1 (○), Alcaligenes sp 2 (●), Alcaligenes sp 5 (x), Acinetobacter sp (△) and Arthrobacter sp (▲) in ASW medium; a on acetate; b on hexadecane.
between these concentrations. *Achromobacter* sp 1 takes an intermediate position in this respect; its growth rate is doubled. With hexadecane as a substrate the following results are obtained (Fig 2b). Three strains (*Achromobacter* sp 1, *Alcaligenes* sp 2 and *Acinetobacter* sp) have a constant, maximum growth rate starting at a phosphate concentration of $10^{-4}$ M. There is little variation between $5 \times 10^{-5}$ and $10^{-4}$ M. One strain (*Alcaligenes* sp 5) has a constant maximum growth rate between $5 \times 10^{-5}$ and $8 \times 10^{-4}$ M, and an appreciable decrease at $1.2 \times 10^{-3}$ M. Another strain (*Arthrobacter* sp) has a constant maximum growth rate starting at $2 \times 10^{-4}$ M; for this concentration, the rate is three times what it is at $5 \times 10^{-5}$ M phosphate.

**Productivity by weight** - By using a hexadecane concentration such that the substrate is completely consumed at the end of the growth phase, we were able to note slight differences in the way the various strains reacted to phosphorus (Fig 3). *Achromobacter* sp 1 and *Arthrobacter* sp had maximum productivity for a $10^{-4}$ M phosphate concentration; *Alcaligenes* sp 2 and *Acinetobacter* sp for $4 \times 10^{-4}$ M and *Alcaligenes* sp 5 for $6 \times 10^{-4}$ M.
FIG 3 Change, as a function of phosphate concentration, in productivity by weight (K%) for growth of Achromobacter sp 1 (●), Alcaligenes sp 2 (○), Alcaligenes sp 5 (×), Acinetobacter sp (△) and Arthrobacter sp (▲), in an ASW medium with hexadecane.

TABLE 1. Consumption of PO₄-P as a function of its concentration in ASW medium

*Pi, initial phosphate; Pc, consumed phosphate.

+P/C₁₆ = consumed phosphate/consumed C₁₆ (expressed in moles).
Ratio of consumed phosphorus to consumed hexadecane - Table 1 shows that Achromobacter sp 1, Alcaligenes sp 2 and Alcaligenes sp 5 are characterized by a maximum value of the ratio of phosphorus to hexadecane consumed, for 4 x 10^{-4} M phosphate. For Acinetobacter sp, there is very little variation in the ratio beyond 10^{-4} M, while for Arthrobacter sp, the ratio continues increasing beyond 4 x 10^{-4} M. After characteristic staining, microscopic examination reveals a synthesis of polyphosphates that disappear after resting 24 hours in a phosphorus-depleted medium. The growth observed is thus not necessarily proportional only to hexadecane consumption. Except in the case of Achromobacter sp 1 for phosphate concentrations between 5 x 10^{-5} and 4 x 10^{-4} M, none of the strains ever consumed all the available phosphorus in the medium.

Respiratory activity - For all strains, no change in Q_{O_2} was noticed when, in the respiratory systems, the phosphate concentration was made to vary from 0 to 1.2 x 10^{-3} M; the same thing was seen after the strains were left for 24 hours in a phosphate-depleted medium.

Phosphorus requirements of Pseudomonas aeruginosa sol 20 in freshwater and in seawater

The potential of P aeruginosa sol 20 to develop in either freshwater or seawater allowed us to identify, in both types of medium, which phosphorus concentrations corresponded to maximum growth. The growth of P aeruginosa was compared with that of Alcaligenes sp 5, in ASW and on decane, an n-paraffin that was metabolized by both strains.

For these two strains, maximum productivity was obtained with 4 x 10^{-4} M in ASW (Fig 4a and b); this productivity declined with higher concentrations for Alcaligenes sp 5. In medium A, the maximum was attained for P aeruginosa with 2 x 10^{-2} M phosphate and was greater than the maximum obtained in ASW.

The growth rate for P aeruginosa decreased very slightly in ASW between 10^{-4} M (0.13) and 1.2 x 10^{-3} M (0.11), while the maximum (0.33) was attained with 5 x 10^{-3} M in medium A.

Growth on acetate and hexadecane in mASW

Growth rate - On acetate (Fig 5a), the growth rate of Acinetobacter sp, constant up to 6 x 10^{-3} M phosphate, doubled with 10.5 x 10^{-3} M. This strain was distinct from the other four, where the rates were nearly constant between 1.5 x 10^{-3} and 10.5 x 10^{-3} M. The individual properties of each strain were also visible on hexadecane (Fig 5b).
Fig 4a, b. Productivity (x%) on decane with different phosphate concentrations in the culture medium, for: Alcaligenes sp 5 (x) and P aeruginosa sol 20 (x) in ASW medium; P aeruginosa sol 20 in medium A.
The growth rate for *Alcaligenes* sp 5 remained constant while that of *Acinetobacter* sp increased between $1.5 \times 10^{-3}$ and $6 \times 10^{-3} M$ and then stabilized. For *Alcaligenes* sp 2 and particularly *Achromobacter* sp 1, a decline in the growth rate was observed at these values. The sensitivity of the latter strain to the phosphorus concentration was revealed by an experiment the results of which are shown in Figure 6. In a medium initially containing $1.5 \times 10^{-3} M$ phosphate, the sudden increase, during the exponential phase, to a concentration of $10.5 \times 10^{-3} M$ resulted, about two hours after the addition of phosphorus, in a change in the slope of the curve that did not occur in the control culture. The growth rates, measured before and after the addition of PO$_4$-P were identical to those obtained for initial concentrations of $1.5 \times 10^{-3}$ and $10.5 \times 10^{-3} M$.

*Arthrobacter* sp proved unable to develop on this medium with hexadecane as a carbon source.

**Respiratory activity** - This did not vary with any of the sources when the phosphorus concentration varied, even after the strain was left for 24 hours in a phosphate-depleted medium.

**Influence of NaCl concentration**

In medium B, the physiological responses of *Alcaligenes* sp 5 and *P aeruginosa* were examined when NaCl and phosphorus concentrations respectively varied; the objective was to determine a possible relation between these parameters. Acetate was selected as a carbon source because it was consumed by both these strains, whereas paraffins were metabolized in this medium only by *P aeruginosa*. 
Growth rate - Variations in the growth rates of *P. aeruginosa* and *Alcaligenes* sp. 5, with different NaCl and phosphorus concentrations, are shown in Figures 7 and 8.
FIG 5a, b Change, as a function of phosphate concentration, in the growth rate ($r$) of Achromobacter sp 1 (O), Alcaligenes sp 2 (Φ), Alcaligenes sp 5 (x), Acinetobacter sp (A) and Arthrobacter sp (A) in a mASW medium: (a) on acetate; (b) on hexadecane.

FIG 6 Influence of the addition of phosphate during the exponential phase on the growth rate of Achromobacter sp 1 (mASW + hexadecane): (1) control growth (initial phosphate concentration $6 \times 10^{-3} M$); (2) growth of the culture supplemented with phosphate ($4.5 \times 10^{-3} M$).
As regards *P. aeruginosa*, no effect on the PO$_4$-P content was observed in the medium supplemented with 8.5$^4$x 10$^{-3}$ M NaCl (according to Imelik, 1948), as the growth rate had already reached its maximum. For higher NaCl molarities, an increase in phosphate concentration brought about an increase in the growth rate, readily visible for 0.2 M NaCl, with the maximum attained at 10$^{-2}$ M PO$_4$-P. For 0.4 and 0.6 M NaCl, the maximum was obtained at 5 x 10$^{-3}$ M phosphate. *Alcaligenes* sp 5 had a maximum growth rate for phosphate concentrations of 3 x 10$^{-3}$ M and 1.5 x 10$^{-3}$ M, with 0.2 M and 0.4 and 0.8 M NaCl respectively. With low phosphate molarities (1.5 x 10$^{-4}$ M) and particularly with very high ones (10.5 x 10$^{-3}$ M), this rate is considerably lessened, regardless of the NaCl molarities.
These results were confirmed by the following experiment (Fig 9): in a medium initially containing $6 \times 10^{-3}$ $M$ phosphate, a sudden increase, during the exponential phase, to a concentration of $10.5 \times 10^{-3}$ $M$ immediately produced a change in the slope of the curve that did not occur in the control culture.
FIG 7 Change, as a function of phosphate concentration, in the growth rate \((r)\) of \(P\ aeruginosa\ sol\ 20\) in medium B supplemented with \(8.5 \times 10^{-3}\ M (\square); 0.2\ M (\bullet);\ 0.4\ M (\bigcirc);\ 0.6\ M (\Delta)\ NaCl.\)

FIG 8 Change, as a function of phosphate concentration, in the growth rate \((r)\) of \(Alcaligenes\ sp\ 5\) in medium B supplemented with \(0.2\ M (\bigcirc); 0.4\ M (\bigcirc); 0.8\ M (\Delta)\ NaCl.\)

FIG 9 [1] hours
Effect of the addition of phosphate during the exponential phase on the growth rate of \(Alcaligenes\ sp\ 5\) (medium B + acetate): (1) control growth (initial phosphate concentration \(6 \times 10^{-3}\ M);\) (2) growth of the culture supplemented with phosphate \((4.5 \times 10^{-3}\ M)\)

TABLE 2: Effect of NaCl and phosphate concentrations on the \(Q_{O2}\) of \(P\ aeruginosa\) and \(Alcaligenes\ sp\ 5\) cultivated on acetate in medium B.
The growth rates, before and after the addition of P$\textsubscript{04}^{-}$P, were identical to those obtained for initial concentrations of $6 \times 10^{-3}$ M and $10.5 \times 10^{-3}$ M.

Respiratory activity - The increased P$\textsubscript{04}^{-}$P concentration (Table 2a,b) stimulated the respiratory activity of the strains, particularly *Alcaligenes* sp 5. This effect was independent of the NaCl content for this strain while for *P aeruginosa* the effect was much clearer with 0.6 M NaCl.

It is worth while mentioning that for 0.4 M NaCl the respiratory activity of the other two strains, *Achromobacter* sp 1 and *Alcaligenes* sp 2 was not stimulated by the increased phosphate content, under identical experimental conditions.

**Discussion**

The examination of the six strains used showed that the effect of phosphorus concentration on the strains' development depended on at least three experimental factors: the ionic composition of the medium; the carbon source used; and the taxonomic position of the bacteria.

1. The culture medium itself determines the concentration range within which phosphorus affects bacterial growth. For *P aeruginosa*, the P$\textsubscript{04}^{-}$P concentration making it possible to obtain maximum productivity on decane, was 200 times greater in medium A than in ASW. The examination of Figures 2a and b shows that in an ASW medium, where the ionic strength is 0.73, the phosphorus content required for the optimum development of all strains studied is between $2 \times 10^{-4}$ and $8 \times 10^{-4}$ M. Below and above these values, the optimum growth rate for some strains is not assured. These results agree with those obtained by Atlas and Bartha (1972) with water having a salinity of 28%: their experimental system was saturated with 3.5 $x 10^{-4}$ M phosphate. Finally, in *Alcaligenes* sp 5 in medium B (Fig 8), cell division was greatly reduced beyond a phosphate concentration of $3 \times 10^{-3}$ M and this was accentuated when the molarity of NaCl in the culture medium increased. Simonis and Urbach (1963) observed, in an alga of the Chlorophyceae Class (order Chlorococcales), *Ankistrodesmus braunii*, a specific effect of sodium on phosphorus uptake and its incorporation into organic compounds. Siegenthaler et al (1967) also observed on a marine fungus, *Dermocystidium* sp, that phosphorus uptake increased linearly with the NaCl concentration up to 0.2 M and remained stable from 0.2 to 0.4 M. Beyond that, uptake decreased as the NaCl concentration increased. The authors attributed this effect to osmotic pressure, because at identical pressures, sucrose also has an inhibitory action.

2. The limits on the influence of phosphorus concentration on the growth of the bacteria studied also depend on the carbon source used. In ASW, between $4 \times 10^{-4}$ and $1.2 \times 10^{-3}$ M phosphate, the productivity of *Alcaligenes* sp 5 decreased on decane (Fig 4a) but increased and then stabilized on hexadecane (Fig 3). On hexadecane (Fig 2b), growth slowed down at $1.2 \times 10^{-3}$ M of phosphate; this did not happen with acetate (Fig 2a). Similarly, for *Achromobacter* sp 1 in a mASW medium (Fig 5a,b) the decrease in the growth rate was very marked on hexadecane but did not show on acetate. Finally, the growth rates of *Achromobacter* sp 1 and *Acinetobacter* sp were more
sensitive on acetate than on hexadecane to variations in the phosphate concentration beyond $2 \times 10^{-4}$ M; conversely, Arthrobacter sp showed greater sensitivity on hexadecane than on acetate.

3. This leads us to consider the relations between the taxonomic position of the bacteria and their phosphorus requirements. The most characteristic result is illustrated by Figure 5b; as the phosphorus concentration increased the cell division process in Achromobacter sp 1 slowed down quite clearly, while it speeded up in Acinetobacter sp. From Figure 3 it can be seen that the productivity maxima were attained for phosphorus concentrations varying with the species considered between $10^{-4}$ and $6 \times 10^{-4}$ M. On the other hand, the increased respiratory activity noted for Alcaligenes sp 5 (Table 2b), starting at $6 \times 10^{-3}$ M phosphate, and corresponding to a decreased growth rate was not detected in Achromobacter sp 1 or in Alcaligenes sp 2. In the control strain
P aeruginosa sol 20, only a slight increase in $Q_{o2}$ occurred. The high phosphorus concentrations which, in Alcaligenes sp, correspond to increased respiratory activity and to a greatly diminished growth rate, bring about the physiological conditions for a release of energy, the cause of which is rather obscure.

The study of Table 1 shows that the phosphorus requirements of the strains used vary with the species and the phosphate concentration in the medium. Beyond $2 \times 10^{-4}$ M for Acinetobacter sp and $4 \times 10^{-4}$ M for the other strains, ever greater amounts of phosphorus remain unused, nearly 50 to 70 per cent of the original amount. In order to metabolize 1 mol of hexadecane, the number of gram-atoms of phosphorus consumed varies with the bacterial species from 0.10 to 0.14 for a concentration of $5 \times 10^{-5}$ M and from 0.20 to 0.57 for $4 \times 10^{-4}$ M. Within concentration ranges that vary from species to species, and for a given amount of hexadecane metabolized, bacteria apparently consume more phosphorus the more of it is available.

In a marine environment, the ranges of bacterial phosphorus requirements vary with ionic strength. Within these ranges, and at a given ionic strength, the bacteria develop at maximum rates of growth and productivity, regardless it seems of the taxonomic position or the carbon source metabolized. For water with a salinity equal to the mean salinity of ocean waters (Peres and Deveze, 1963) phosphorus requirements are met at concentrations between $2$ and $8 \times 10^{-4}$ M. The boundary values of the range become higher (1.5 to $3 \times 10^{-3}$ M) when these waters receive a significant supply of freshwater or when salinity is naturally weak.

In unpolluted marine zones, PO$_4$-P content is always very low. Coste et al (1972) found values of 0.1 and even 0.05 microgram-atoms per litre in the surface waters of the western Mediterranean. Such values are insufficient for bacterial growth, but the intensity of respiratory activity, measured in ASW and in mASW on hexadecane with the various strains used, proved to be independent of the phosphorus concentration. In seawater, biological oxidation of hydrocarbons must therefore occur to a greater or lesser extent depending on how rich such water is in micro-organisms that can be involved in this process. Oxidation likely helps these compounds become more soluble in water and then disappear from the water's surface.

Finally, the inhibition of bacterial development, observed in some cases with excessively high phosphate concentrations, is akin to a phenomenon noticed in algae. Fogg (1963) indicated that some species of Chlorophyceae, Chrysophyceae and Rhodophyceae would not tolerate phosphate contents equal to or greater than $2 \times 10^{-3}$ M. He specified that the biological bases for this inhibition were unknown. Whatever causes this phenomenon, any treatment at sea of polluting hydrocarbons by stimulation of microbial attack must in practice take into account the possibility of inhibition by an excessively high concentration of local phosphorus; at any point in the treatment, this concentration must be compatible with the growth of micro-organisms at the water-hydrocarbon interface.