RESULTS OF 12 YEARS OF RESEARCH IN SPILLED OIL BIOREMEDIATION: INIPOL EAP 22

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ABSTRACT: *Oil biodegradation is a natural but slow process, limited mainly by the scarcity of nitrogen and phosphorus. To enhance hydrocarbon breakdown by indigenous microbial communities, a fertilizer formulation that would keep nutrients in contact with oil was sought. A research project established for this purpose 12 years ago has resulted in the development of an oleophilic fertilizer known as Inipol EAP22. The product is an oil-soluble fertilizer formulated as an oleophilic microemulsion. It contains not only nitrogen and phosphorus, but also an easily biodegradable carbon source.*

Numerous experiments, in both laboratory and field, have shown that, even in cold climates, application of this fertilizer increases the number of hydrocarbon-degrading organisms and the extent and rate of biodegradation. Kinetic studies show that the amount of nitrogen and phosphorus in the fertilizer is sufficient to allow the indigenous bacteria to consume all of the carbon source contained in Inipol EAP22. Once this carbon is consumed and the number of hydrocarbon degrading bacteria has increased, oil biodegradation can begin. In addition, adding fertilizer has a marked effect on the oil's consistency and increases its bioavailability.

The recent large bioremediation project in Alaska has shown that it is possible to enhance the biodegradation of oil on shorelines through the application of such a fertilizer.

Enhanced oil biodegradation can be considered today as a new tool in oil spill cleanup, to be used in conjunction with other treatments.

In the last two decades, giving rise to oil spills have increased scientific knowledge of the behavior of hydrocarbons, and have led to the development of new intervention methods. When an accidental oil spill occurs, the first priority is mechanical recovery, but of equal importance are other 'problem fighters' such as dispersants.

Once in the environment, hydrocarbons undergo a natural alteration, the extent and speed of which is closely linked to the nature of the spilled product and local environmental conditions.

Several processes determine the behavior of hydrocarbons in the natural environment. Whereas some of these, such as evaporation and dissolution, are rapid, others, such as oxidation and biological degradation, are long-term processes on which depend the ultimate evolution of the hydrocarbons.

This research project was established 12 years ago, to find ways of favoring natural elimination, in particular biodegradation. It was hoped that, if natural biodegradation could be accelerated, a new operational tool in the fight against hydrocarbons would be gained.

Oil biodegradation. Biodegradation of hydrocarbons is a phenomenon that has been known for some time.[35] According to C. E. Zobell, more than 200 species of bacteria, yeasts, and even algae can biodegrade hydrocarbons.[33] These microorganisms can be found in a variety of different environments, such as oceans, lakes, estuaries, and sediments, and particularly in environments chronically exposed to hydrocarbons. The presence of microorganisms especially adapted to the degradation of hydrocarbons was shown by Atlas and Barthal in the early 1970s.[4,5]

Although many studies have concentrated on degradation of certain hydrocarbons by isolated strains. It seems obvious that the biological oxidation in a mixture as complex as crude oil occur only under the influence of a group of microorganisms.[11,19]

Hydrocarbon-specific microflora exist naturally. The experimental approach chosen was to use those microorganisms present in the environment that showed good adaptability to the degradation of hydrocarbons over several days.[6] This method was preferred to the use of specific cultures of oil-degrading bacteria. In the natural environment, these specific culture cannot improve hydrocarbon degradation, since they disappear rapidly from the environment once in competition with the dominant microflora.[18]

However, hydrocarbon degradation is a slow process; temperature, oxygen content,[7] and above all the availability of the nutrients nitrogen and phosphorus[2,3,13] are the main limiting factors.

The importance of this limiting effect has been shown in different ecosystems, in both tropical[23,29] and Arctic zones.[2,3]

Various low-temperature experiments have illustrated the effect of temperature on degradation, namely a slowing of degradation kinetics[2,3] and inhibition of the microorganisms due to the presence of certain volatile compounds in the petroleum. This microorganism inhibition causes latency periods, which delay biodegradation.[2,3]

Many authors have shown that hydrocarbon biodegradation can be accelerated by the addition of nitrogen- and phosphorus-containing fertilizers, both in aqueous environments[32] and in sediments.[10,30] Nutrients containing mineral salts have sometimes been used successfully for soil treatment.[17]

However, the nitrogen and phosphorus elements of these compounds cannot be retained when in contact with hydrocarbons, due to their high solubility.

Since the objective of all such work is to favor development of microorganisms capable of degrading hydrocarbons at the water-hydrocarbon interface, a number of studies have focused on the development of oleophilic nutrients.

Various approaches have been used to create this oleophilic character: development of dispersants containing oleophilic nutrients,[9] encapsulation of mineral salts in paraffin,[4,21] and the use of a mixture of soya lecithin and ethyl allophanate.[22] Elf Aquitaine has focused on the formulation of a microemulsion.

Oleophilic fertilizers. After testing more than 30 compounds in the laboratory, it was possible to define precise specifications for the desired characteristics of such a fertilizer. Most important the nutrient had to be oleophilic with nitrogen and phosphorus present in an optimal ratio. There also had to be an easily assimilable source of carbon for the microorganisms. These objectives (to which also had to be added low cost and nontoxicity) were realized in the development of a microemulsion containing a solution of urea encapsulated in an external oleic acid phase.[27,28]

The main characteristics of this nutrient—called Inipol EAP 22—are as follows:

- Marked oleophilic character
- Optimal nitrogen/phosphorus ratio
- Delaying effect on release of nitrogen and phosphorus
- Nontoxicity to flora and fauna and good biodegradability

Other than this oleophilic character, another important parameter intervenes in the biodegradation of hydrocarbons: the oil-water interface. The chemical heterogeneity and the insolubility of crude oil in water present particular problems to these microorganisms which feed on these hydrocarbons. Although the microorganisms produce biotensioactive agents during degradation of crude oil,[25] it is favorable to increase the water-hydrocarbon interface to enhance microbial oxidation.

This is particularly important for a "Chocolate mousse" emulsion, since biological degradation is much reduced in this case, probably due to the limitations of the interface for microbial attack.[1] This phenomenon is particularly frequent in the case of coastal pollution resulting from an ocean spill.

In addition to the biological fertilizer properties of Inipol EAP 22, it also acts chemically by modifying the rheological properties of the crude oil being treated. Thus, the microemulsion can inhibit the formation of water-in-oil emulsions, and more particularly, partially break down these emulsions, thus favoring bioavailability. After treatment of a crude oil with Inipol EAP 22, rapid changes have been observed both in the viscosity of the crude oil and in the interfacial tension.

Figure 1 shows the net reduction in the surface tension between the crude oil and seawater in the presence of Inipol EAP 22. The microemulsion contains a surfactant (lauryl phosphate). Figure 2 shows clearly that the reduction in interfacial tension is linked to the oleic acid, and not to the lauryl phosphate (added in exactly the same proportions as in Inipol EAP 22). In this way, this oil-softening effect allows the water-hydrocarbon interface to be increased.

Materials and Methods

Microbial analysis. It is possible to apply various different techniques to count the microorganisms that degrade hydrocarbons.[34] The total number of bacteria was counted after staining with acridine orange, using a microscope with fluorescence equipment. The sample was transferred to a nutrient solution and homogenized. From 1 to 15 mL of the solution was transferred to 100 mL of Sorensen's phosphate buffer (pH 7.5, 0.1 M). The buffer solution was stained for 10 minutes with AO and filtered through a 0.22-micrometer polycarbonate filter (nucleopore). The filters were dried and embedded on microscope slides in a nonfluorescing immersion oil. The number of metabolically active bacteria was counted with the use in fluorescein diacetate (FDA). The sample preparation was identical to that described for the total number of cells, except that the staining time was about 3 minutes.

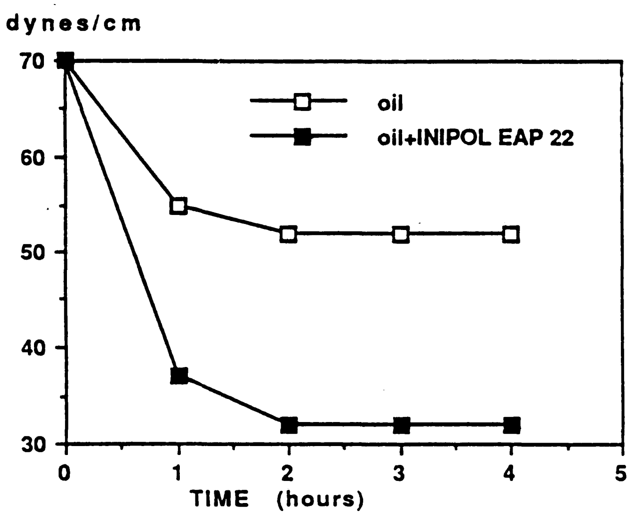

Figure 1. Evolution of oil-seawater interfacial tension in presence of Inipol EAP 22 (oil, topped 300° F Arabian light crude oil; 20 mg oil per liter of seawater)

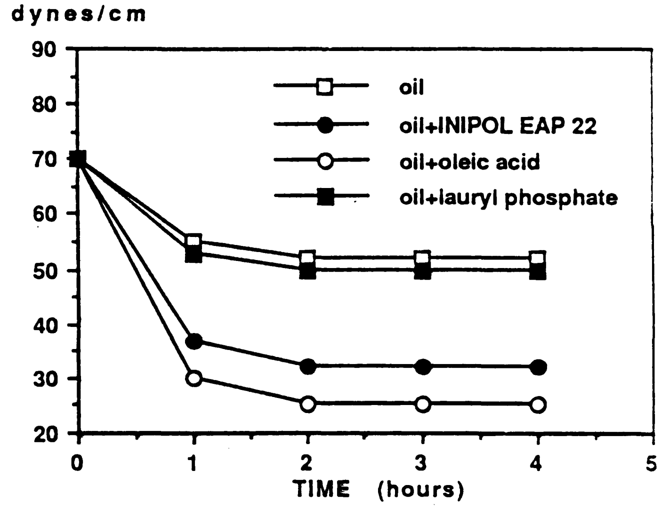

Figure 2. Evolution of oil-seawater interfacial tension in presence of Inipol EAP 22 and some of its components

Chemical analysis. In the aliphatic fraction, *n*-alkanes are generally more susceptible to biodegradation than isoprenoids.[8] The decrease in the C_{17}/pristane and C_{18}/phytane ratios (in which C_{17} and C_{18} are *n*-alkanes and pristane and phytane are isoprenoids) is used to quantify the biodegradation.

The analysis was affected on a Hewlett Packard 5890 gas-phase chromatograph equipped with a 50 meter CP SIL 5 capillary column. The operating conditions were: temperature program 50° to 320° C at 7° C/min; FID: 350° C.

Although isoprenoid markers have to date been very frequently used, it seems that in the future these should be replaced by compounds from the steranes and hopanes series. These latter compounds are reputedly more difficult to biodegrade. In fact, various studies have shown that branched alkanes such as pristane can be degraded by a marine bacterial community.[24,26] This finding has been confirmed by United States Environmental Protection Agency (EPA) researchers in the course of their bioremediation project following the *Exxon Valdez* oil spill.

Results

Many experiments were conducted, in the laboratory and in field trials, in both aqueous environments and sediments.

Oil-on-water experiments. Many tests were conducted in the laboratory with the dual aim of selecting the fertilizer and of developing the oleophilic formulation. Tests were also conducted in laboratory closed environments, and in 18-m^3 tanks built at the Ricard Oceanographic Foundation, Ile des Embiez, France, to test the yield of biodegradation. Table 1 summarizes the results and the test conditions of these experiments, conducted in natural seawater. All experiments were conducted in conditions of optimum oxygenation. This explains the high rate (60 to 80 percent) of hydrocarbon disappearance in the presence of Inipol EAP 22.

In low-energy systems of salt water, characterized by low oxygenation, the oil degradation kinetics are much slower. Figures 3 and 4 show the decrease of the rate with time in the aliphatic fraction of crude oil. Although a more substantial elimination of the aliphatic fraction occurs with treatment by addition of biodegradation, in this case oxygen appears to be an important limiting factor.

In water experiments at the largest scale (18 m^3), the nutrient added to the oil is leached relatively rapidly from the oil slick and is quickly diluted in the volume of water. This explains why the oil degradation kinetic is much lower. In experiments undertaken in 1985, in open

Table 1. Results of oil-on-water experiments in natural seawater

Experiment	Crude oil	T(°C)	Treatment with Inipol EAP 22	Duration (days)	Oil removal
A1. Flask, laboratory	Ashtart[1]	12	3	7	69
A2. Flask, laboratory	Ashtart	12	0	7	11
B1. Flask, laboratory	M'Wengui[2]	18	5	7	78
B2. Flask, laboratory	M'Wengui	18	0	7	17
C1. Flask, laboratory	Arabian Light	18	10	7	70
C2. Flask, laboratory	Arabian Light	18	0	7	20
D1. 18-m^3 tank	Arabian Light, topped at 150° C	18	10	10	65
D2. 18-m^3 tank	Arabian Light, topped at 150° C	18	0	10	20

1. Ashtart crude oil from Tunisia
2. M'Wengui crude oil from Gabon

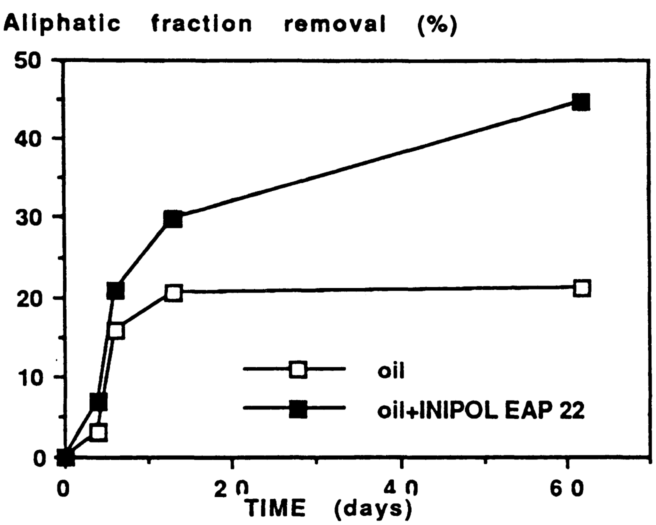

Figure 3. Aliphatic fraction removal (oil, Arabian light crude oil; low-energy system in 40-L tank)

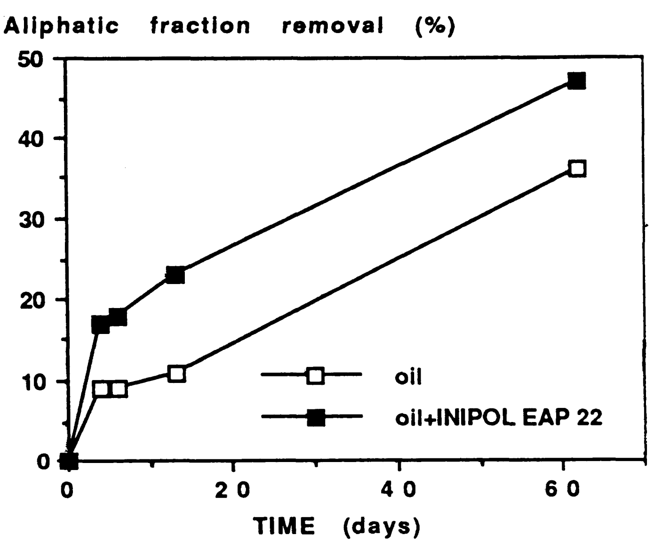

Figure 4. Aliphatic fraction removal (oil, Arabian light crude oil; low-energy system in 18-m^3 tank)

Arctic marine water systems, a low level of biodegradation occurred, despite the addition of the oleophilic nutrient. This result can be explained partly by dilution of the nutrients in the column of water, but particularly by the interaction of the photooxidation of the crude oil. This photooxidation occurs particularly intensely in the Arctic zone and produces substances that inhibit the enzymatic mechanisms of hydrocarbon degradation.[30]

Oil-on-sediments experiments. Uncontestably the best results have been obtained using Inipol EAP 22 on polluted sediments, both inland and coastal. During a trial effected in France on soil polluted by crude oil essentially localized on the surface (the top 0.5 cm), addition of the oleophilic fertilizer allowed the contamination to be reduced by 60 percent within 60 days. A reduction of only 15 to 20 percent in the hydrocarbons was obtained on the control area in the same period of time. Figures 5 and 6 show the decrease in the nC_{17}/pristane and nC_{18}/phytane ratios, which is particularly clear in the case of treated areas.

A number of trials have also been made of the treatment of contaminated shorelines. Enhancement of biodegradation of crude oil with Inipol EAP 22 had earlier been studied on a smaller scale on shorelines in Norway.[16]

Operations. In 1985, in collaboration with SINTEF, a Norwegian research organization, a beach in Spitsbergen that had been contaminated by an accidental spill of 80 metric tons of marine gas oil was treated with Inipol EAP 22.[30] Under these rigorous climatic conditions the use of the oleophilic nutrient allowed a 90-percent reduction of the sediment contamination over less than one year, compared with only 15 percent reduction without treatment.

In this experiment a maximum increase of 60 percent in the biodegradation of the aliphatic fraction was obtained. During this treat-

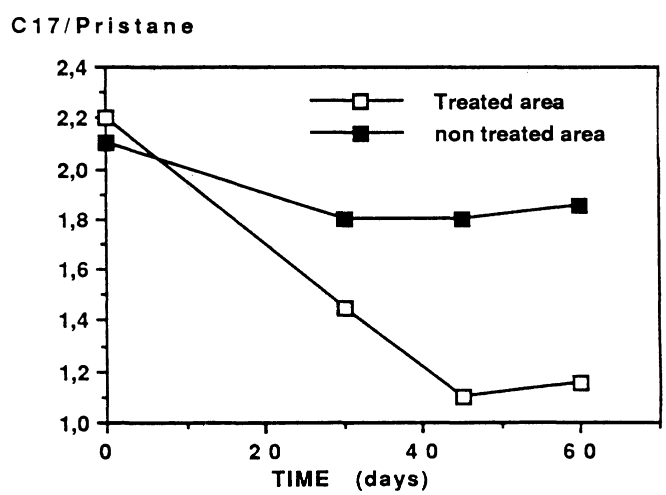

Figure 5. Biodegradation of oil, evaluated as decrease in n-C_{17}/pristane

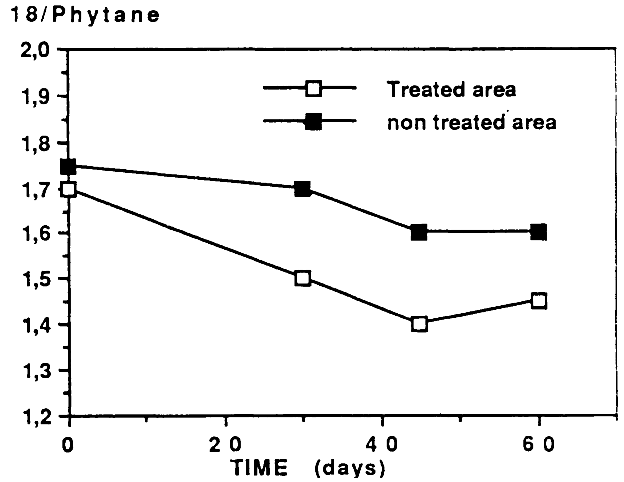

Figure 6. Biodegradation of oil, evaluated as decrease in n-C_{18}/phytane

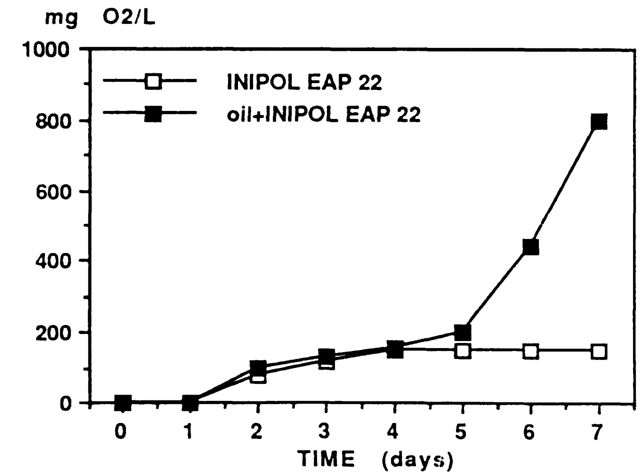

Figure 8. Cumulative oxygen uptake in seawater, with Inipol EAP 22 alone and mixture of 5 percent Inipol EAP 22 in Arabian light crude oil

ment, the microbial response to fertilizer addition was evaluated by determining the total number of bacteria and the total number of bacteria specific by hydrocarbon degradation. Figure 7 shows that the addition of fertilizer increases not only the total number of cells during the first week, but also the number of specific bacteria.

In 1989, after the *Exxon Valdez* oil spill, a field demonstration project established by the EPA and Exxon in Prince William Sound, Alaska, to evaluate the use of fertilizers for enhancing biodegradation of spilled oil. In laboratory and on-site tests, the oleophilic fertilizer Inipol EAP 22 showed dramatic results during the shoreline cleanup, and 500 tons were purchased by Exxon for application on a large scale during the summers of 1989 and 1990. This subject is discussed by several authors at this conference.

Kinetics

A laboratory study was conducted to investigate the mechanism by which the Inipol EAP 22 fertilizer enhanced oil degradation. This study was made with oxygen uptake measurements in seawater, with Inipol EAP 22 alone and with a mixture of oil and Inipol EAP 22 (a ratio of Inipol EAP 22 to Arabian light crude oil of 5 percent), in the presence of the indigenous bacterial flora. The results, shown in Figure 8, highlight a phenomenon in which the oleic acid in the fertilizer is degraded in the first three days, inducing biological degradation of the

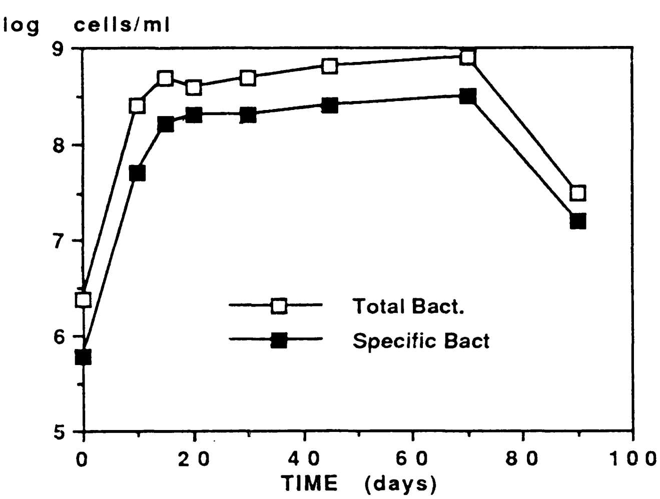

Figure 7. Microbial cell development in the fertilized sediment in field test (shoreline treatment)

hydrocarbons. Oxygen consumption begins in the first day and increases strongly after four days. During the first days, not only is the oleic acid consumed but also several fractions of the hydrocarbons. This shows that the oleophilic fertilizer does not inhibit the hydrocarbon degradation even during the first two to three days.

Biological degradation of oleic acid leads to the accumulation of oleic acid degraders, some of which can probably degrade hydrocarbons. This kinetic study confirms the starter role of the hydrocarbon chain present in the Inipol EAP 22.

Conclusions

The experiments described in this paper, together with the recent use of this technique in cleaning the polluted coasts in Alaska, show that it is possible to enhance the biodegradation of oils through the application of an oleophilic fertilizer. Effectively, bioremediation is now a new operational tool in the fight against hydrocarbon pollution.

This technique would seem particularly well suited to the treatment of polluted sediments on coastal sites.[12] However, bioremediation should be considered not a "miracle cure," but a technique complementary to more conventional methods of treating pollution. The acceleration of biodegradation by the addition of fertilizers to the polluted area is effective only after the level of contamination has been reduced, either by natural elimination mechanisms or by mechanical recovery.[35]

Bioremediation is cheap, labor-saving, and easy to put into operation and allows hydrocarbons that would otherwise persist in the environment to be eliminated. Although wave action and sediment movement act as natural self-cleaning mechanisms for polluted coasts, hydrocarbons can persist for decades, particularly in areas of low energy or those with fine sediments.[20,31]

So far as sediment treatment is concerned, the hydrocarbon/soil/microorganism system is very complex. The parameters for this system are grain size, specific surface and water content, oxygen partial pressure, and fertilizer partitioning within the sediment. Consideration of these parameters and the limitations on biodegradation that such phenomena as diffusion and adsorption can cause, together with a good understanding of the biodegradation mechanism in the presence of the oleophilic fertilizer, should lead us to the definition of an optimal nutrient formulation.

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