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EFFECTS OF PETROLEUM BIODEGRADATION PRODUCTS ON PHYTOPLANKTON GROWTH

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RÉSUMÉ - Les effets des produits de la dégradation bactérienne d'un pétrole brut ont été étudiés sur la croissance de deux microalgues marines. Sucres, lipides et acides aminés sont les principaux constituants issus de la dégradation du pétrole et sont présents dans le milieu de culture des bactéries aux concentrations de 660 mg.l^{-1}, 46 mg.l^{-1} et 72.4 μM.l^{-1} respectivement. La croissance des algues est inhibée lorsque les produits sont suffisamment concentrés dans leur milieu de culture. Elle est stimulée lorsque leur concentration est faible. Parmi ces composés, les lipides semblent les plus toxiques. L'inhibition est plus ou moins importante suivant l'espèce étudiée. *Prorocentrum micans* est plus sensible que *Phaeodactylum tricornutum* vis-à-vis de ces substances.

Mots clés : bactéries, produits de dégradation, pétrole brut, biosurfactants, toxicité, phytoplancton.

ABSTRACT - The effects of products resulting from petroleum biodegradation in continuous culture, have been studied on the growth of two marine algae. Sugars, lipids and amino acids are the main components resulting from petroleum degradation in the bacteria used medium. Their concentrations are respectively 660 mg.l^{-1}, 46 mg.l^{-1} and 74.2 μM.l^{-1}
The algae growth is inhibited when products are enough concentrated ; it is stimulated by low concentration. Among these products, lipids are more toxic than others. The two species show different sensitivity towards the used medium. *Prorocentrum micans* growth is more inhibited than that of *Phaeodactylum tricornutum*.

Key words : bacteria, petroleum biodegradation products, crude oil, biosurfactants, toxicity, phytoplankton.

INTRODUCTION

Petroleum biodegradation is a naturally occurring process. Actually, it is considered as a tool against oil pollution in marine environment. There is a dearth of information concerning the impact of compounds produced by bacterial degradation of crude oil on marine biota. Such biodegradation products are available in our laboratory as work is being done to optimise crude oil biodegradation (Bertrand *et al.*, 1983 ; Rambeloarisoa *et al.*, 1984) in fermentor. They are a complex mixture of degraded hydrocarbons, residual petroleum and compounds excreted by bacteria. Bacteria excretion products are mostly tensio-active substances (biosurfactants) including sugars and lipids, and amino acids. These products cannot be isolated as a whole because they are part of the used medium in which bacteria were grown.

Consequently, in this study, the effects of petroleum biodegradation products on phytoplankton have been investigated *via* the effects of the used medium on the growth of two microalgae. Experiments have been made to test separately compounds that are present in the used medium, with a special emphasis on lipids.

MATERIALS AND METHODS

Degradation of petroleum in continuous culture.

A crude oil (7.5 $g.l^{-1}$) Asthart type is degraded continuously in fermentor containing filtered sea water supplemented in PO_4 (0.4 mM) at pH 8 and 30°C, with a dilution rate of 0.05 h^{-1}. The fermentor is inoculated with a mixed bacterial culture which is composed of 8 strains isolated from sea water foams in hydrocarbons polluted areas. In such experimental conditions, the percentage of degraded crude oil is 83 %. The used medium is centrifuged at 15 000 rpm for 15 mn. Remaining bacteria and most of residual petroleum are removed from the used medium through 0.2 μm Millipore membrane before its introduction in the culture medium of the test algae.

Test algae

Both algae are common species from the Gulf of Marseille : *Phaeodactylum tricornutum* has been chosen because much is known about its physiology and its growth, even with respect to hydrocarbon experiments (Lacaze and Villedon de Naide, 1976) ; (Lacaze, 1978 ; Kusk, 1981 a,b) : *Prorocentrum micans* is a Dinoflagellate often found in surface slicks and possibly in connection with high lipid level in surface water (Kattner and Brockmann, 1978 ; Cassie, 1981 ; De Souza Lima, 1981). Its growth is slower than that of *P. tricornutum.*

Culture medium of algae

Cultures are grown in 40 ml test tubes closed with cotton plugs allowing volatiles to escape. Tubes are successively filled up with :
- nutrients (Antia and Cheng algae medium)
- various percentages of the fermentor used medium (adjusted to 100 % in filtered sea water). When required, lipids have been extracted from the used medium. This allows lipids, lipid free used medium and untreated used medium to be tested separately on phytoplankton. Each test is carried out with suitable control. The lipids are extracted using diethyl ether after acidification of the medium (pH = 2). Thus, most lipids are recovered without harmful effects on algae as diethyl ether is quickly evaporated from the aqueous phase.

- an aliquot of the algae preculture in exponential growth, such that the initial cellular concentration are about 5 000 cells. ml^{-1} for diatoms and 100 cells.ml^{-1} for dinoflagellates.

Temperature is set at 18°C. Irradiance is 70 μeinstein $m.^{-2}s^{-1}$ with a 14 $h.d^{-1}$ photoperiod. All experiments are run under sterile conditions. Tests are performed until the end of exponential growth.

The effects on algae growth are evaluated using *in vivo* fluorescence (on a Turner Designs fluorometer) or by direct cells counting on hemacytometer. *In vivo* fluorescence is highly correlated to cellular concentrations during the exponential growth of algae (Raimbault, 1982). This parameter is suitable to determine the general trends of pollutants effects on phytoplankton.

Each point of the growth curves represents the mean value of three experiments.

Chemical analysis

Procedures used for sugars, amino acids and phosphates quantification are those described by Dreywood (1946), Mimura and Delmas (1983) and Strickland and Parsons (1972), respectively.

Lipids have been extracted according to Folch *et al.* (1956). Resulting chloroform and diethyl ether extracts are weighted separately. Each extract is analysed using the Iatroscan Thin layer chromatography - flame ionization detector system. Lipid classes are separated on chromarods using double development in hexan : benzene : acetic acid (80 : 20 : 1) and hexan : diethyl ether : acetic acid (97 : 3 : 1) solvents systems.

RESULTS

Chemical composition

The used medium contains sugars (660 mg.l^{-1}), amino acids (74.2 μM.l^{-1}), lipids (46 mg.l^{-1}) and phosphates (560 uatg.l^{-1}). The dissolved amino acids/free amino acids ratio is 10.3, that is similar to the ratio usually found in natural sea water.

The chloroform extract of the used medium represents 21.7 % of total lipids separated on Iatroscan. 75 % of the chloroform extract are polar lipids and 10 % are hydrocarbons. 78 % of lipids are recovered with diethyl ether after acidification of the used medium among which 40 % are fatty acids and 45 % Co chromatography with monoglycerides.

The composition of organic matter in the used medium is about 100 folds as much as that of sea water and about 10 time as much as that of the surface film. Both our used medium and the surface film show large amount of lipids and carbohydrates (Garrett, 1967a ; Daumas *et al.*, 1976 ; Hunter and Liss, 1981). In addition the surface film is enriched in bacteria and phytoplankton (Harvey, 1966 ; Brockman *et al.*, 1976 ; De Souza Lima, 1981). Thus we decided to dilute the initial used medium down to the range of values expected to be found in the surface film and in sea water (concentrations of 10, 1, 0.1 and 0.01 %) and to test the effects of the so-diluted used medium on phytoplankton growth.

Effects of increasing concentrations of the used medium and P. tricornutum and P. micans growth

The highest concentration (10 %) of the used medium induces a marked inhibition of the algae growth during the first 48 h. It then depresses the algae growth with a 60 % reduction of its specific growth rate (Fig. 1 and Tab. 1).

The intermediate concentration (1 %) inhibits its growth during the first 24 h. Afterwards, its growth is not different from that of the control. Its specific growth rate is, even slightly higher (Tab. 1).

The lowest concentrations (0.1 %, 0.01 %) stimulate the algae growth (Fig.1, Tab. 1).

Similar results are obtained on *P. micans* growth : total inhibition occurs at high concentration (10 %) and stimulation at low concentration (0.01 %).

Percentage of used medium in the culture medium of algae	0	0.01	0.1	1	10
Specific growth rate division. d^{-1}	1.46	2.35	2.32	1.56	0.59

Table. 1 : Effects of the total used medium on P. tricornutum growth.

We checked that the contribution of phosphate to the observed stimulation at low concentrations of the used medium is unlikely. We also checked that if some crude oil is still remaining in the used medium, it has no effects as regards the stimulation of growth at 0.1 % and 0.01 % used medium in algae cultures. Besides, we demonstrated that the possible remaining crude oil is not responsible of the growth inhibition at concentration 10 %.

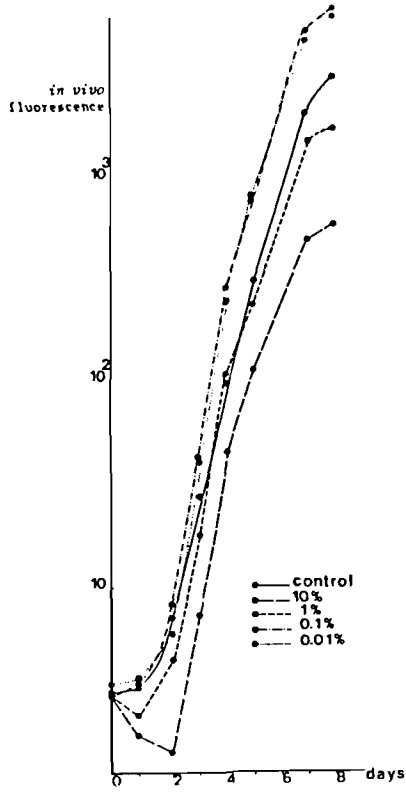

Figure 1 : Effects of increasing concentrations of fermentor used medium on P.*tricornutum* growth.

This means that metabolites only, either degraded hydrocarbons or tensioactive substances, are implicated in the stimulation- inhibition effects of the used medium on the algae growth. These metabolites might act either separately or through any combination. However, the role of the concentration factor seems to be preponderant.

Comparative effects of the total used medium, the lipid free used medium and the lipid extract on P. tricornutum and P. micans growth at concentration 10%.

Both the lipid extract and the lipid free used medium stimulate the algae growth during the first days of the cultures (Fig. 2 and Fig. 3).

Lipid extract general effect is to depress the cultures growth (after 48 h for *P. tricornutum* and 5 days for *P. micans*). However, *P. micans* is more sensitive to the lipid extract.

The lipid free used medium which contains carbohydrates, amino acids and vitamins has no effect on the exponential growth of the algae.

The general pattern of growth inhibition due to the total used medium (10 %) and to the lipid extract (10 %) is not similar for the two algae. The total used medium inhibits much more *P. tricornutum* growth than the lipid extract does, whereas it is the opposite concerning *P. micans*. Moreover, some of the lipids present in the used medium (22 %), acting on the algae growth, are denaturated by the extraction process. Thus their effects cannot be evaluated at the present stage of the study.

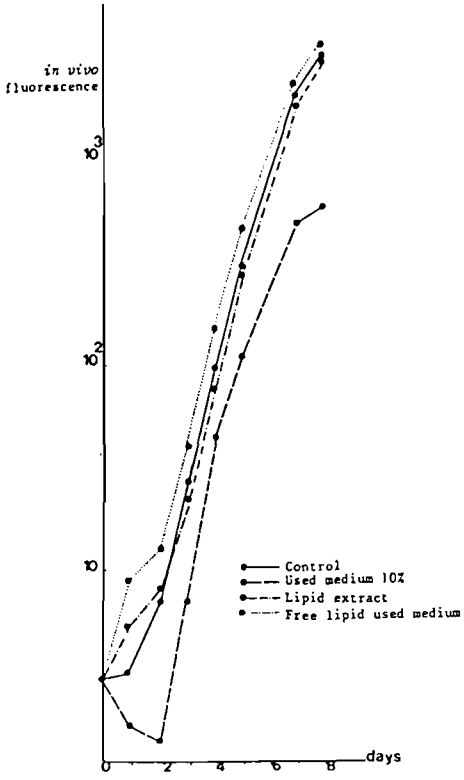

Figure 2 : Effects of the used medium (10 %), the free lipid used medium and the lipid extract on P. *tricornutum growth.*

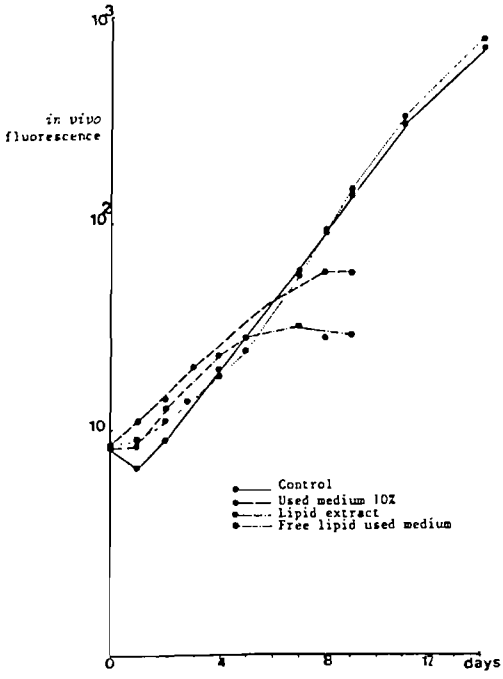

Figure 3 : Effects of the used medium (10 %), the free lipid used medium and the lipid extract on P. *micans growth.*

DISCUSSION, CONCLUSION

The effects of compounds resulting from petroleum biogradation in continuous cultures have been studied on the growth of two marine microalgae.

Effects of inhibition and stimulation of the algae growth are observed ; they are essentially dependent on the concentration factor. 10 % of the used medium in the culture medium of both algae depresses or inhibits totaly their growth, whereas 0.01 % stimulates it. *Prorocentrum micans* appears to be more sensitive to petroleum degradation products than *Phaeodactylum tricornutum* is.

The different compounds cannot be isolated separatly from the total used medium. Nevertheless, tests carried out simultaneously allow us to ensure that the observed stimulation-inhibition effects of the used medium are due to compounds resulting from petroleum biodegradation.
The observed toxicity might be due to lipid compounds. Particularly, as regards to *Prorocentrum micans* the growth of which is highly inhibited by the lipid extract of the used medium.

Lacaze (1978) intensively studied the effects of petroleum on algae growth. He demonstrated that the toxicity of most petroleum is enhanced after intensive irradiation of petroleum. This has been attributed to photooxidation products of petroleum which are

characterized by a higher polarity than the initial products. In the same way, we observed that the water soluble fraction (wsf) of Asthart is less harmful to algae than the wsf after biodegradation of this petroleum which contains large amounts of polar compounds (Bertrand *et al.*, 1983). Thus, the transformations of petroleum via physico-chemical or biological processes, have comparable effects (*in vitro*) on algae growth. High concentrations of petroleum degradation products may be more toxic to algae than the petroleum itself.

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