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Effects of hydrocarbons on the physiology and growth of *Ulva* sp. (Chlorophyta)

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ABSTRACT

Laboratory experiments were performed to investigate the physiological responses of an *Ulva* species to oil pollution. *Ulva* thalli were exposed at 16 °C for up to 12 days to emulsions in seawater of one of four standard petroleum fractions (P1, d 0.7, bp 80-110 °C; P2, d 0.73, bp 100-140 °C; P3, d 0.76, bp 140-160 °C; P4, d 0.76, bp 180-220 °C), in each case at a concentration of 500, 1 000, 2 000, 4 000 or 8 000 ppm ($^{v}/_{v}$). The physiological variables determined were: photosynthesis rate, respiration rate, photochemical efficiency (Fv/Fmax), non-photochemical quenching (qN), chlorophyll *a* concentration, and growth rate. A probit analysis was used to determine 50 %-inhibition time and concentrations of the physiological responses. Our results showed that the best indicator of stress due to exposure to petroleum hydrocarbons was photosynthesis rate. The fraction P1 (low density, low boiling point) was the most toxic, with the 72-hour IC50 for rate of photosynthesis being 871 ppm.

Key words: Hydrocarbons, *Ulva,* photosynthesis, fluorescence, growth, chlorophyll, toxicity.

RESUMEN

Efectos de hidrocarburos sobre la fisiología y el crecimiento de* Ulva *sp. (Chlorophyta)

Se realizaron experimentos de laboratorio para investigar las respuestas fisiológicas de una especie de Ulva. *Los discos de* Ulva *fueron expuestos a 16 °C hasta 12 días, a emulsiones en agua de mar con cada una de las cuatro fracciones de petróleo estándar (P1, d 0,7, bp 80-110 °C; P2, d 0,73, bp 100-140 °C; P3, d 0,76, bp 140-160 °C; P4, d 0,76, bp 180-220 °C), en cada caso a una concentración de 500, 1 000, 2 000, 4 000 y 8 000 ppm ($^{v}/_{v}$). Las variables fisiológicas determinadas fueron tasa de fotosíntesis, tasa de respiración, eficiencia fotoquímica (Fv/Fmáx), disipación no fotoquímica (qN), concentración de clorofila* a *y tasa de desarrollo. Se usó un análisis de probit para determinar los tiempos y concentraciones de inhibición del 50 % de las respuestas fisiológicas. Nuestros resultados revelaron que el mejor indicador del estrés debido a la exposición de hidrocarburos de petróleo fue la tasa de fotosíntesis. La fracción P1 (baja densidad, bajo punto de ebullición) fue el más tóxico, con una concentración de 871 ppm para la inhibición del 50 % de la tasa de fotosíntesis en 72 horas.*

Palabras clave: *Hidrocarburos,* Ulva, *fotosíntesis, fluorescencia, crecimiento, clorofila, toxicidad.*

INTRODUCTION

Due to the growing consumption of petroleum products, oil pollution is increasingly frequent in aquatic ecosystems. Although massive oil spills are the most visible form of oil pollution, less dramatic forms –e.g. due to loading/unloading operations, refinery waste, urban runoff, or atmospheric deposition– are more important in most areas. Such pollution is often chronic in rivers and estuaries (Koons, 1984).

Ecotoxicity of hydrocarbons is highly variable, depending on their type and concentration, exposure time, state, environmental conditions and the sensitivity of affected species. The ecological impacts of oil on specific seaweed habitats has been studied by many authors, and clear differences are observed between sensitive taxa, such as *Fucus, Ulva* and *Pelvetia*, and resistant taxa, e.g. *Laminaria, Chondrus* and *Ascophylum* (Freedman, 1995).

In the present study, we performed laboratory experiments to investigate the effect of different hydrocarbons on the physiology of an *Ulva* species. Specifically, we monitored effects on photosynthesis and respiration rates, efficiency of photosynthetic activity, growth, and chlorophyll *a* concentration. For each petroleum fraction and each physiological variable, probit analyses were then used to determine 50 %-inhibition concentrations (IC50s) for given exposure times, and 50 %-inhibition times (IT50s) for given concentrations. The basic aim of these experiments was to investigate whether *Ulva* species are potentially useful bioindicators of hydrocarbon pollution, and if so, to identify the most reliable and readily determined physiological indicator variables.

MATERIALS AND METHODS

Ulva samples were collected from a pollution-free estuary, the Ribeira salt marsh in northwest Spain. At the laboratory, thalli were cleared of epiphytes and washed in filtered seawater. Discs (∅20 mm) were acclimatised in a cold chamber at 16 °C in diluted seawater (25 mS/cm) for 24 h before the assays.

Experiments were performed with four standard petroleum fractions: P1 (petroleum benzine, density 0.7, boiling point 80-110 °C, Fluka), P2 (petroleum benzine, d 0.73, bp 100-140 °C, Fluka), P3 (petroleum benzine, d 0.76, bp 140-160 °C, Fluka), and P4 (petroleum p.a., d 0.76, bp 180-220 °C, Merck). Emulsions of each fraction were prepared in filtered seawater (25 mS/cm) at concentrations of 500, 1 000, 2 000, 4 000 or 8 000 ppm ($^{v}/_{v}$), in 1-litre dark glass bottles, with rotary shaking for 1 h at 71 rpm (see Ostgaard and Jensen, 1983). For each assay, 36 *Ulva* discs were placed in glass Petri dishes (∅140 mm) containing 150 ml of emulsion or seawater only, then maintained for 12 days in a temperature-controlled chamber (16 °C) with illumination at 5.77 kilolux and a 12/12 h photoperiod. Samples (6 discs) were taken from each dish on days 0, 1, 3, 5, 9 and 12 for determination of physiological variables (see below). Four replicates were performed for each of the 20 treatments, with four controls (seawater only) for each fraction, giving a total of 96 Petri dishes.

The six discs taken from each dish on each sampling day were first analysed to determine photosynthesis and respiration rates in filtered seawater (25 mS/cm) with the light/dark bottle technique; (90 min light, 90 min dark) (Anon., 1992), using Karlsrugger bottles; oxygen concentrations were measured with a shaking-head polarographic oxymeter (OXI92, WTW). The discs were then adapted to darkness for 60 m. Fluorescence-induction kinetics were measured *in vivo* using a modulated-pulse fluorometer (PAM-2000, H.Walz), with determination of the ratio of variable to maximum fluorescence (Fv/Fmax, i.e. photochemical efficiency) and non-photochemical quenching (qN). Subsequently, following measurement of the surface area of thalli with an AM100 scanner (ADC), growth rate (μ, day^{-1}) was determined with the following equation (Anon., 1992):

$$\mu = ln\ (X_2/X_1)/(t_2 - t_1) \qquad [1]$$

where X_1 and X_2 are surface area (mm^2) at the beginning (day t_1) and at the end (day t_2) of the time interval under consideration, respectively.

The discs were then frozen until analysis of chlorophyll concentration with the Vollenweider method (1974). Briefly, after extraction with 90 % acetone for 1 h in the dark, the extract was centrifuged for 3 min at about 3 000 rpm, and absorption over the range 400-750 nm was then determined in a Milton Roy Spectronic 3 000 Array spectrophotometer. Chlorophyll *a* concentration (µg per g dry weight of material submitted to extraction) was calculated using Vollenweider's formula (1974):

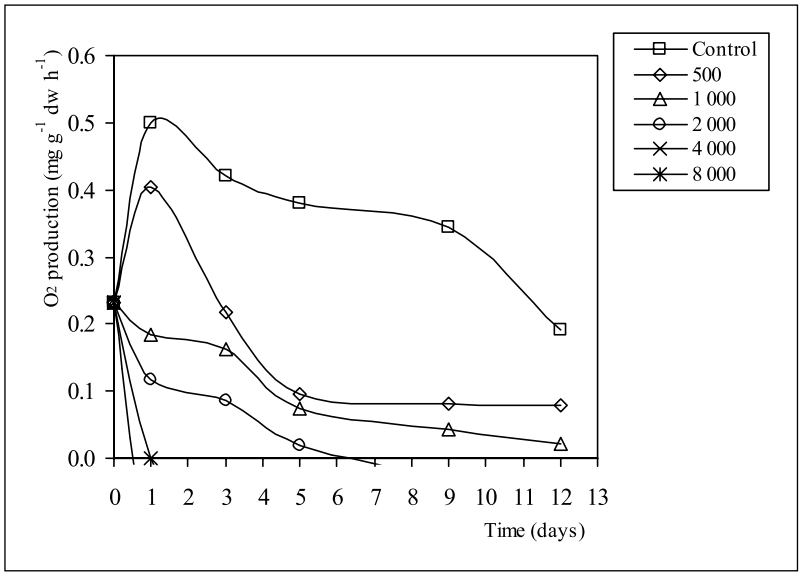

Figure 1. Photosynthesis rate (net oxygen production rate) of *Ulva* thalli exposed to the petroleum fraction P1 at different concentrations and different times

Chlorophyll *a* (µg g^{-1}) =
= (11.9 × (2.43 × (D665 – D665a))) × (V/L) [2]

D665 and D665a are optical densities at 665 nm before and after acidifying with 20 µl of 1N HCl, respectively; V is the volume of acetone (ml) used for extraction; and L is the path length (cm) of the cuvette.

RESULTS

Physiological responses to the four petroleum fractions were basically similar, though differing in intensity. The following section presents detailed results for fraction P1; summarised results for the other fractions are given at the end.

Photosynthesis rate decreased with increasing P1 concentration (figure 1). In the control and 500 ppm assays, accelerated metabolism was observed during the first few days, presumably reflecting a transplant effect, since photosynthesis rate subsequently dropped back to normal. No such accelerated metabolism was observed in assays with higher P1 concentrations, which clearly inhibited photosynthesis from the first day onwards. Respiration rate was affected from the first day onwards by all P1 concentrations, and especially by concentrations above 500 ppm (figure 2); in all cases, oxygen consumption was very high during the first three days, and then dropped steadily.

The results obtained for Fv/Fmax (photochemical efficiency) and qN (non-photochemical quenching

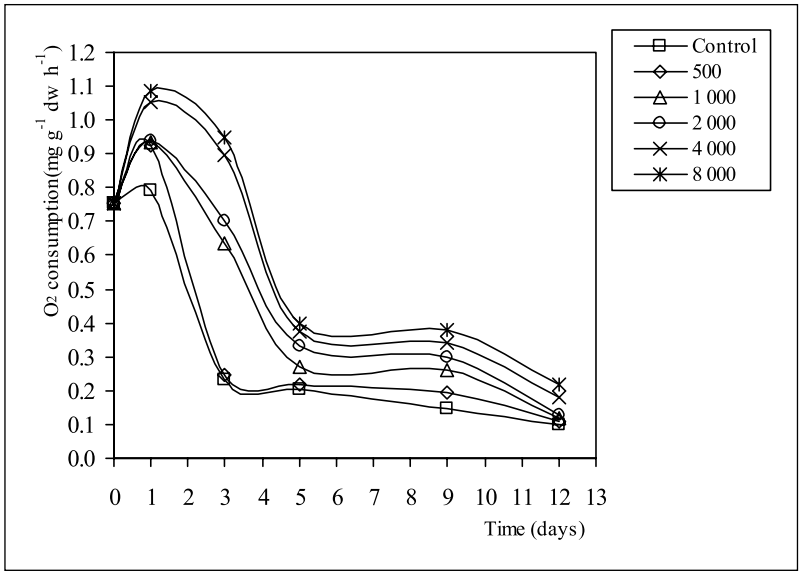

Figure 2. Respiration rate (net oxygen consumption rate in the dark) of *Ulva* thalli exposed to the petroleum fraction P1 at different concentrations and different times

index) clearly reflect the stress caused by exposure to hydrocarbons (figures 3 and 4). At high P1 concentrations, Fv/Fmax declined and qN increased. At the highest concentration (8 000 ppm), however, qN dropped to zero by day 12. These results indicate that P1 caused an increase in chlorophyll fluorescence.

In general, increasing P1 concentrations caused increasingly marked reductions in both growth rate and chlorophyll *a* concentration. Total inhibition of growth was not observed. At P1 concentrations of 4 000 and 8 000 ppm, retarded growth rates were observed from the first day of the assays onwards (figure 5).

All P1-exposed algae had a lower chlorophyll *a* content than the controls. The lowest chlorophyll contents were those obtained in assays with the highest P1 concentrations (figure 6).

For each petroleum fraction and each physiological variable, probit analyses were performed to determine 50 %-inhibition concentrations (IC50s) for given exposure times, and 50 %-inhibition times (IT50s) for given concentrations. The resulting curves for IT50 for photochemical efficiency and for IC50 for photosynthesis rate (in both cases following exposure to P1) are illustrated in figures 7 and 8, respectively. These curves make it possible to estimate minimum inhibitory concentration, maximum-effect concentration, and minimum response time. Summary statistics for the toxicities of the four petroleum fractions, considering effects on photosynthesis rate and on photochemical efficiency, are listed in tables I and II. These results indicate that photosynthesis rate is the best indicator of stress due to hydrocarbon pollution, and that P1 (density 0.7, boiling point 80-110 °C) is the most toxic of the four petroleum fractions tested. The mean concentration of P1 required for 50 % inhibition of the photosynthesis rate after 72 h of exposure was 871 ppm.

DISCUSSION

Crude oil is a complex mixture of hydrocarbons, with four to 26 or more carbon atoms per molecule. The constituents may be straight, branched or cyclic chains, including aromatic compounds (i.e. with benzene rings). Some polycyclic aromatic hydrocarbons (PAH) are known to be potent carcinogens. The exact composition of crude oil varies from one oilfield to another, and over the life of a single oilfield (Clark, 1989). Water-soluble components of crude oils include a variety of compounds that are toxic to a wide spectrum of marine plants and animals. Aromatic compounds are more toxic than aliphatic compounds, and middle-molecular-weight compounds are more toxic than high-molecular-weight compounds. Low-molecular-weight compounds are generally unimportant because they are volatile, and are rapidly lost to the atmosphere (Clark, 1989); however, they tend to be more water-soluble than other hydrocarbons (Anon., 1985). As well as being more toxic, aromatic compounds are generally more soluble in water than aliphatic compounds: for example, benzene has a water solubility of 1.8 g/l (McAuliffe, 1966). The aromatic hydrocarbon fraction is, therefore, that which poses the greatest threat to fish and planktonic organisms (Howarth and Marino, 1991).

Due to the complex responses of oil-water mixtures, and to the variability of the composition of crude oil, it is difficult to compare the results of studies in this field. The chemical composition of such mixtures is highly dependent on the proportion of dispersed to dissolved constituents. The aqueous phase is, furthermore, physical and chemically variable, losing components of low molecular weight by evaporation, and experiencing chemical changes over time. In assays of the toxicity of crude oil or petroleum fractions, emulsions should thus be prepared by slow agitation in the dark to avoid increases in toxicity (Clark, 1989; Freedman, 1995).

Photosynthesis involves reactions at five different functional levels: processes at the pigment level, primary light reactions, thylakoid electron-transport reactions, dark-enzymatic stroma reactions, and regulatory feedback processes (Heinz Walz GmbH, 1993). Chlorophyll fluorescence can be an indicator of activity at all these levels. Chlorophyll is the major antenna pigment, funnelling absorbed light energy into reaction centres, where photochemical conversion of the excitation energy takes place (Bolhàr-Nordenkampf *et al.*, 1989). The indicator function of chlorophyll fluorescence arises from the fact that fluorescence emission is complementary to the alternative pathways of de-excitation, which are primarily photochemistry and heat dissipation. Fluorescence yield is highest when the yields of photochemistry and heat dissipation are lowest. Hence, changes in fluorescence yield also

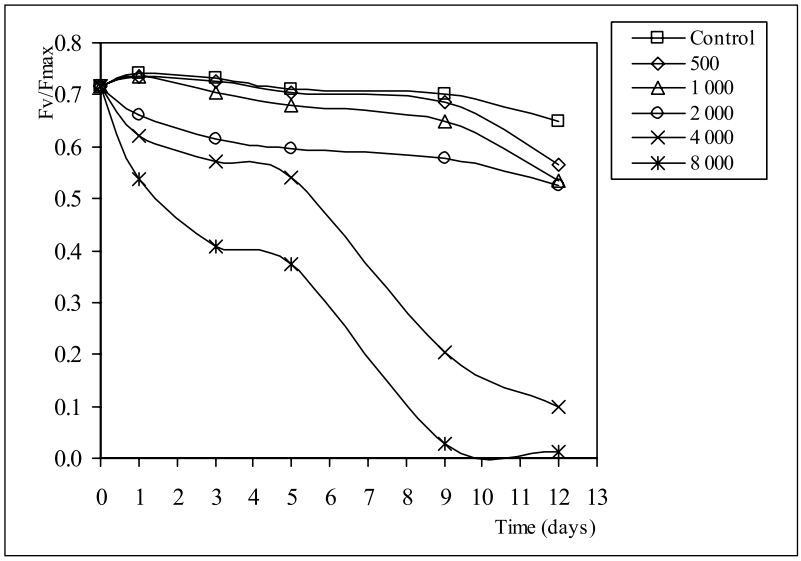

Figure 3. Mean photochemical efficiency (Fv/Fmax) of *Ulva* thalli exposed to the petroleum fraction P1 at different concentrations and different times. Means (± SE) in table III

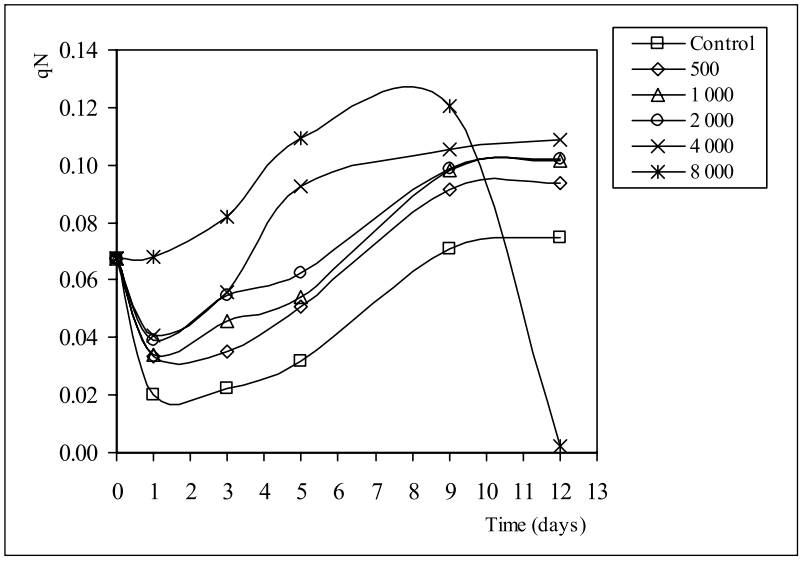

Figure 4. Mean non-photochemical quenching (qN) by *Ulva* thalli exposed to the petroleum fraction P1 at different concentrations and different times. Means (± SE) in table III

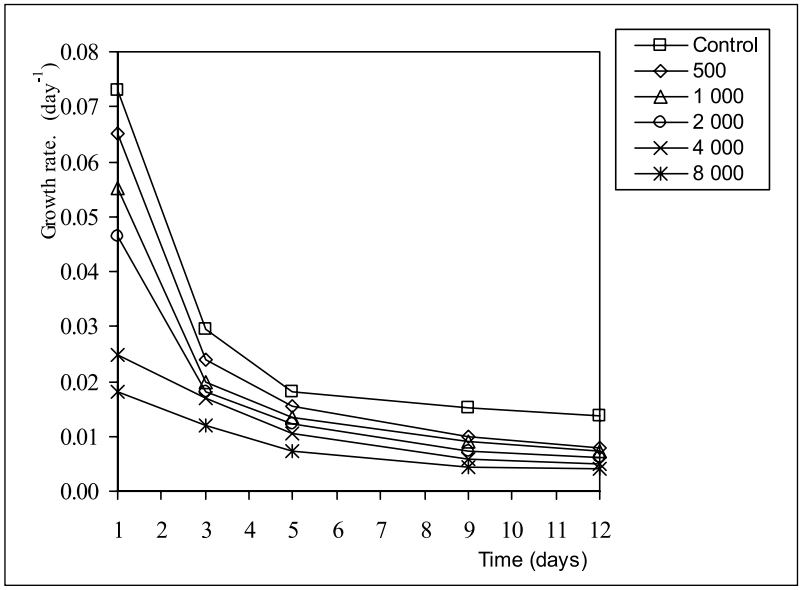

Figure 5. Mean growth rate of *Ulva* thalli exposed to the petroleum fraction P1 at different concentrations and different times. Means (± SE) of surface area (mm^2) in table III

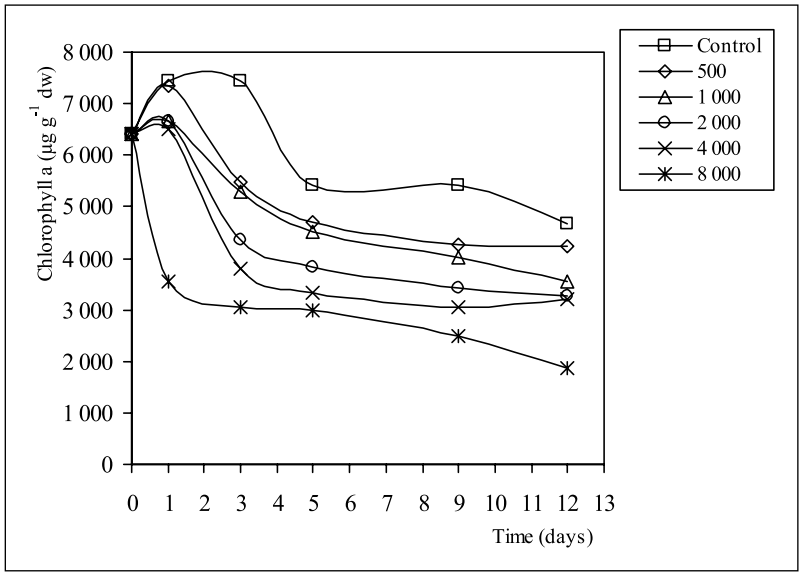

Figure 6. Mean chlorophyll *a* concentration (µg per g dry weight) in *Ulva* thalli exposed to the petroleum fraction P1 at different concentrations and different times. Means (± SE) in table III

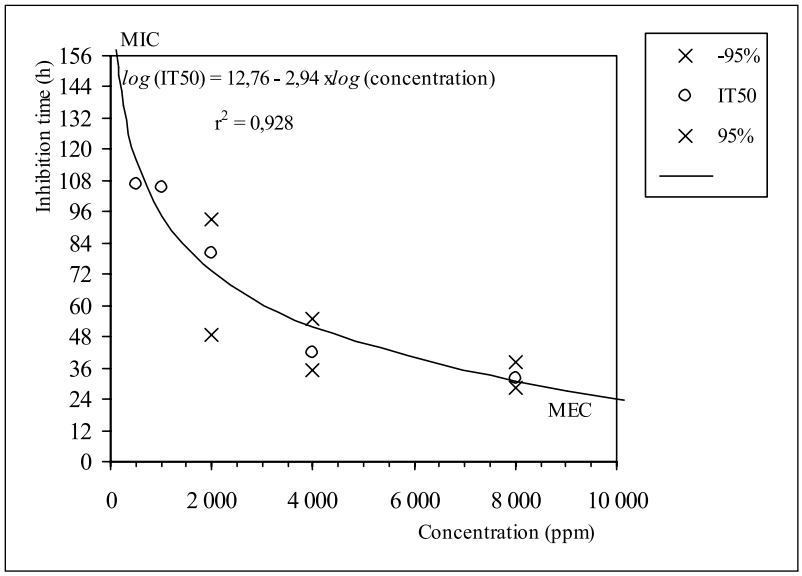

Figure 7. Toxicity curve for the estimation of 50 %-inhibition times (IT50s) for photochemical efficiency (Fv/Fmax) following exposure to petroleum fraction P1. 95 % confidence limits are shown. each IT50 estimate, for (MIC): minimum inhibitory concentration; (MEC): maximum-effect concentration; (MRT): minimum response time

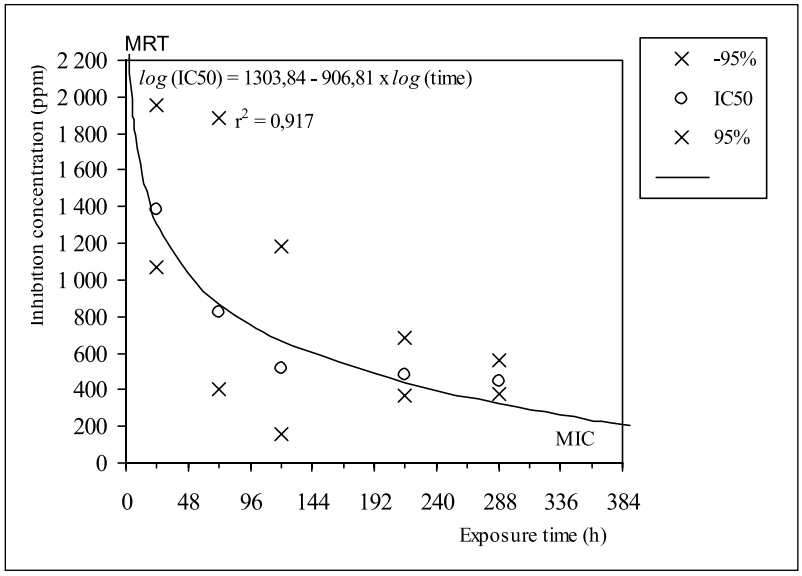

Figure 8. Toxicity curve for the estimation of 50 %-inhibition concentrations (IC50s) for photosynthesis rate following exposure to petroleum fraction P1. 95 % confidence limits are shown each IC50 estimate, for (MIC): minimum inhibitory concentration; (MEC): maximum-effect concentration; (MRT): minimum response time

Table I. Summary statistics describing the toxicity of the four petroleum fractions for *Ulva* thalli, considering effects on photosynthesis rate. (MIC): minimum inhibitory concentration; (MEC): maximum-effect concentration; (MRT): minimum response time. IC50s (ppm) for photosynthesis rate

	–95 %	IC50 24h	+95 %	–95 %	IC50 72h	+95 %	MIC	MEC	MRT
P1	1 069	1 304	1 951	399	871	1 888	238	8 000	12 h
P2	2 183	3 694	8 064	2 021	2 685	8 486	533	8 000	12 h
P3	1 839	2 606	4 629	674	1 792	3 608	440	8 000	12 h
P4	2 089	3 222	5 021	1 544	2 736	4 712	1 881	8 000	12 h

Table II. Summary statistics describing the toxicity of the four petroleum fractions for *Ulva* thalli, considering effects on photochemical efficiency (Fv/Fmax). (MIC): minimum inhibitory concentration; (MEC): maximum-effect concentration; (MRT): minimum response time. Note that the values shown were calculated from probit analysis of the data expressed as IT50s. IC50s (ppm) for photochemical efficiency

	–95 %	IC50 72h	+95 %	MIC	MEC	MRT
P1	4 564 (47.9 h)	2 081	1 176 (89.5 h)	450	10 000	24 h
P2	7 012 (45.1 h)	1 121	638 (80.3 h)	205	13 000	24 h
P3	4 740 (49.0 h)	1 803	517 (101.7 h)	230	13 500	24 h
P4	14 266 (46.8 h)	1 623	350 (89.8 h)	220	12 750	24 h

Table III. Means (± SE) for several determined physiological variables

Fv/Fmax; n = 24 discs

	Control	(±SE)	500	(±SE)	1 000	(±SE)	2 000	(±SE)	4 000	(±SE)	8 000	(±SE)
0	0.715	0.045	0.715	0.045	0.715	0.045	0.715	0.045	0.715	0.045	0.715	0.045
1	0.740	0.015	0.736	0.048	0.736	0.016	0.660	0.111	0.622	0.153	0.536	0.138
3	0.731	0.024	0.725	0.025	0.703	0.034	0.616	0.095	0.570	0.086	0.407	0.325
5	0.710	0.053	0.705	0.088	0.681	0.079	0.598	0.157	0.539	0.169	0.373	0.143
9	0.701	0.045	0.685	0.071	0.650	0.100	0.577	0.155	0.204	0.251	0.029	0.043
12	0.649	0.042	0.564	0.051	0.533	0.107	0.524	0.142	0.098	0.190	0.011	0.003

qN; n = 24 discs

	Control	(±SE)	500	(±SE)	1 000	(±SE)	2 000	(±SE)	4 000	(±SE)	8 000	(±SE)
0	0.067	0.086	0.067	0.086	0.067	0.086	0.067	0.086	0.067	0.086	0.067	0.086
1	0.020	0.018	0.034	0.052	0.034	0.041	0.039	0.013	0.041	0.030	0.068	0.067
3	0.022	0.018	0.035	0.033	0.046	0.034	0.055	0.038	0.056	0.029	0.082	0.077
5	0.032	0.028	0.051	0.055	0.054	0.034	0.063	0.042	0.093	0.021	0.110	0.063
9	0.071	0.070	0.091	0.033	0.098	0.094	0.099	0.068	0.106	0.087	0.120	0.055
12	0.075	0.040	0.094	0.057	0.101	0.082	0.102	0.062	0.109	0.095	0.002	0.000

Surface area (mm^2); n = 24 discs

	Control	(±SE)	500	(±SE)	1 000	(±SE)	2 000	(±SE)	4 000	(±SE)	8 000	(±SE)
0	282.63	0.00	282.63	0.00	282.63	0.00	282.63	0.00	282.63	0.00	282.63	0.00
1	304.06	9.04	301.64	12.57	298.67	8.25	296.06	9.48	289.71	7.21	287.78	5.89
3	308.65	12.00	303.60	13.08	300.05	10.48	298.32	4.57	297.30	5.90	293.01	14.69
5	309.51	12.51	305.35	13.79	302.34	13.88	300.56	12.61	297.69	7.70	293.03	8.63
9	323.59	10.08	309.01	10.95	306.25	11.15	301.61	13.57	297.83	6.67	293.87	7.02
12	333.75	9.69	310.18	9.42	308.69	8.02	304.73	14.13	299.80	10.69	296.49	9.82

Chlorophyll *a* (μg/g × h); n = 3 (each of a 150 mg sample)

	Control	(±SE)	500	(±SE)	1 000	(±SE)	2 000	(±SE)	4 000	(±SE)	8 000	(±SE)
0	6 397.2	214.9	6 397.2	214.9	6 397.2	214.9	6 397.2	214.9	6 397.2	214.9	6 397.2	214.9
1	7 451.0	187.8	7 344.1	199.7	6 674.6	176.8	6 659.1	161.9	6 504.0	159.9	3 557.6	213.0
3	7 448.7	166.0	5 486.6	205.8	5 292.2	167.7	4 360.0	195.9	3 788.8	178.8	3 035.5	204.8
5	5 428.9	184.9	4 693.6	179.6	4 526.7	156.9	3 841.9	167.9	3 330.4	186.8	2 975.4	180.0
9	5 401.6	202.8	4 252.6	175.7	4 008.6	164.0	3 427.0	185.9	3 062.4	194.7	2 495.5	181.5
12	4 673.7	179.9	4 242.2	150.8	3 556.3	184.9	3 281.6	203.9	3 218.7	185.0	1 871.9	186.8

reflect changes in photochemical efficiency and heat dissipation. In practice, the variable part of chlorophyll fluorescence originates mainly in photosystem II, and excitation transfer to photosystem I may be considered an additional competitive pathway of de-excitation (Bolhàr-Nordenkampf *et al.*, 1989; Heinz Walz GmbH, 1993; Mathis and Rutherford, 1994).

The efficiency with which light energy is transformed by the photosynthetic apparatus is a good index of stress. The higher the Fv/Fmax value (proportional to the quantum yield of photochemical reactions), the lower the stress. In the present study, the potential photochemical efficiency of photosystem II was reduced as hydrocarbon concentrations were increased, clearly indicating that hydrocarbons affect photochemistry in *Ulva* sp. Similar responses have been observed in other plants under adverse environmental conditions (Bolhàr-Nordenkampf *et al.*, 1989; Moustakas and Ouzounidou, 1994).

The lowest petroleum-fraction concentration tested (500 ppm) reduced photosynthesis rate by 50 % in less than four days, and the highest concentrations in less than one day. Photosynthesis rate of *Ulva* sp. thus appears to be a good indicator of stress due to pollution by hydrocarbons.

In ecotoxicology, experiments are frequently conducted to establish the dose-response relationship for a certain compound and organism. The results of these experiments are often analysed by a logistic model and summarised as an IC50, the concentration that causes a 50 % reduction in the variable under study. The logistic model can be applied to dichotomous data, such as survival or death, and continuous data, such as weight or biomass (Van Ewijk and Hoekstra, 1993). Of the different physiological variables considered in the present study, including growth rate and chlorophyll concentration, photosynthesis rate and photochemical efficiency were the most sensitive to the pollutants considered. Of the four petroleum fractions used in the experiment, P1 proved to be the most toxic. This fraction is composed of hydrocarbons of low molecular weight, including aromatic compounds that can be expected to penetrate the thallus easily.

The toxic effects of oil on algae fall into two categories: those associated with the coating of the organism, and those due to uptake of hydrocarbons and the subsequent disruption of cellular metabolism (Lobban and Harrison, 1997). Coating reduces CO_2 diffusion and light penetration into the plant. Schramm (1972) observed that, in *Porphyra umbilicalis, Fucus vesiculosus* and *Laminaria digitata*, reductions in photosynthesis rates correlated with the thickness of the oil layer. He also found that, during exposure to the air, the oil reduced desiccation of the blades, allowing photosynthesis to occur for longer than normal, but at a reduced rate. In addition, oiled kelp fronds may break because of the weight of oil adhering to the fronds; this is largely due to higher molecular weight, water-insoluble hydrocarbons (Nelson-Smith, 1972).

The second category, disruption of cell metabolism, has been examined primarily by monitoring changes in photosynthesis, respiration, growth, pigment content, morphology, and ultrastructure (Lobban and Harrison, 1997). Disruption of cellular metabolism is usually a result of changes in the rates of photosynthesis or respiration. North, Neushul and Clendenning (1965) observed complete inhibition of photosynthesis in young blades of *Macrocystis* following 3 days of exposure to a 1% emulsion of diesel oil in seawater. In another study, as little as 10-100 ppm of unspecified fuel oil reduced photosynthesis by 50 % after 4 days of exposure (Clendenning and North, 1960). More detailed recent studies have shown that reductions in photosynthesis rates vary with the type of crude oil, its concentration, the length of exposure, the method of preparation of the oil-seawater mixture, irradiance, and the algal species (Hsiao, Kittle and Foy, 1978).

Oil may interfere with any of various steps in respiration, such as gas diffusion, glycolysis, and oxidative phosphorylation. Mechanical blockage of gas diffusion is thought to be less pronounced for oxygen than for carbon dioxide (Schramm, 1972). Lipid-soluble pigments, such as chlorophylls, may be leached out of cells by oil (O'Brien and Dixon, 1976). The compounds that penetrate the thallus most easily, and hence are most toxic, are the lower-molecular-weight, lipophilic compounds, including aromatic compounds. The least toxic components, and the least water-soluble, are the long-chain alkanes. Intermediate in toxicity are the cycloalkanes, followed by olefins. The aromatics and other toxic hydrocarbons appear to exert their toxic effects by entering the lipophilic layer of the cell membrane, disrupting its spacing. As a result, the membrane ceases to properly control the trans-

port of ions in and out of the cell (Lobban and Harrison, 1997).

In the laboratory, the concentrations at which oil will be toxic depend on the type of oil, how the extract was prepared, and how it was used, as well as on the water temperature and the presence of other pollutants or dispersants.

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