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Effect of crude oil on denitrification and sulfate reduction in marine sediments

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Abstract. The denitrifying activity was measured in different types of sediment from the Mediterranean coast of France before, and after, a massive contamination (30–100 g kg^{-1} sediment) of hydrocarbons. A closed system was used in order to maintain anoxic conditions and to control substrates and gaseous products concentrations. We have demonstrated that the respiratory metabolism was inhibited in all cases following an incubation time of 20 to 50 days. At this time, the addition of lactate restore the denitrifying activity. The inhibitory effect of crude oil was not related to an alteration of bacterial cells, but to changes in environmental conditions allowing denitrification. The presence of hydrocarbons in the sediments causes a decrease in the redox potential and a concomitant stimulation of the sulfate reduction.

Introduction

Oil spills caused by leakage from storage tanks, offshore petroleum wells or accidents during transportation are responsible for an increasing pollution in aquatic environment. The effects of the released compounds on marine fauna and flora have been the object of numerous studies, but to our knowledge there have been few investigations of the impact of these substances on the biogeochemical cycles, especially on microbial anaerobic processes (Pfaender & Buckley 1984).

In this work, we study the effects of a high level of hydrocarbon pollution on a key step of the nitrogen cycle: denitrification. This process which produces nitrogen is involved in the mineralization of organic matter in anoxic conditions, as are the processes of fermentation, sulfate reduction and methanogenesis. (Henrichs & Reeburgh 1987).

To measure the denitrification, we used a closed system filled with sediment, in order to ensure the maintenance of anoxic conditions within the sediment, as well as to control the concentration of both the substrates and gaseous products, such as nitrous oxide. All analytical assays to determine the physical, chemical and bacterial characteristics of the sediment were performed before, and after, the enrichment with petroleum.

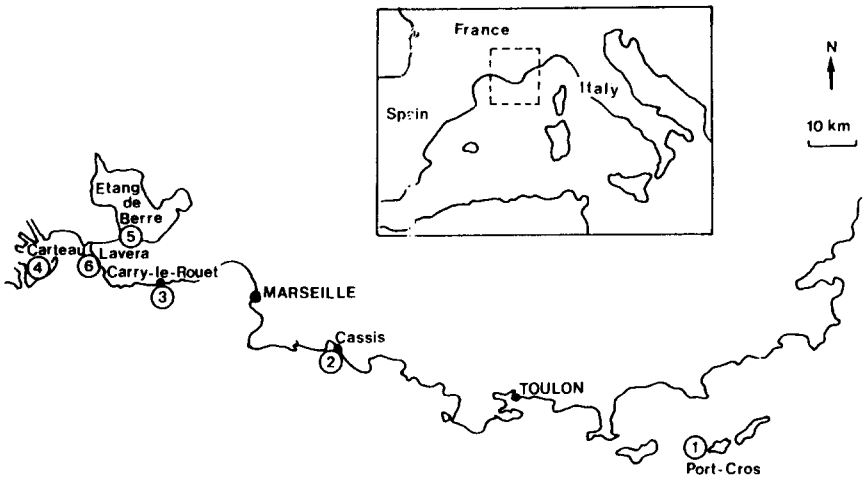

Fig. 1. Study sites on the occidental Mediterranean coast.

The aim of this work is to study the effect of short-term incubation with an Arabian light petroleum on the denitrification in marine sediment.

Materials and methods

Study area and sampling

The investigation was carried out at 6 localities on the Mediterranean coast (Fig. 1). Sediment samples were collected from the 6 locations. They were taken by hand in transparent methacrylate cores about 20 cm length, stored at 4 °C during transport to the laboratory and analyzed as soon as they arrived, as do the physical and chemical parameters, bacterial content and the processes of denitrification and sulfate reduction.

The sediment classification was performed only by sifting. The coarse fraction ($> 63\,\mu$) was sand and the fine fraction ($< 63\,\mu$) correspond to silt.

Port-Cros (station 1), situated in the National Park, presents a fine sandy sediment. Cassis (station 2) and Carry-le-Rouet (station 3), are located near a sealing harbour, also with a sandy sediment. Carteau (station 4) is a location situated on the pathway of petroleum tankers. The sediment is composed of 95% fine sand and 5% silt. The sediment collected from Berre (station 5) was muddy with a low percentage (5%) of fine sand. The site of Lavera is located near a refinery outlet which had been emitting petroleum wastes chronically. Stations 1–5 showed either low, or undetectable, levels of hydrocarbon pollution, whereas station 6 was highly contaminated.

Analytical assays

Temperature, salinity, dissolved oxygen and redox potential were recorded for each site with the appropriate devices: salinometer (YSI33) oxymeter (YSI58) and a pH/mV meter (Schott CG817T) equipped with a combined Ag/AgCl reference and platinum electrode. Before each measurement, we wait until the reading remains constant during one minute.

All nitrogen compounds were measured in the supernatant obtained after centrifugation at 2000 *g* for 10 min. Nitrates were reduced on a Cu–Cd column adapted to Technicon II according to Treguer & Le Corre (1975). Nitrite concentrations were determined colorimetrically by the method of Bendschneider & Robinson (1972).

Ammonium was extracted from sediment with a 0.5 M KCl solution for 2 h at 5 °C. The mixture was then centrifuged at 2000 *g* for 10 min and the ammonium determined by the technique described by Slawick & Isaac (1972).

Nitrous oxide was determined by a gas chromatography Girdel series 30 equipped with an electron capture detector chromatographic operating conditions; 8-length 'Porapak Q' column (mesh 50/80); oven temperature: 80 °C; injector temperature: 180 °C; detector temperature: 250 °C. Nitrogen was used as carrier gas with a flow rate of 20 ml min^{-1}. The extraction of N_2O from the liquid phase was effected by the procedure of Chan & Knowles (1979) modified by the technique of multiple equilibrium (MacAulife 1971).

Organic matter: an aliquot of sediment was dried at 110 °C to constant dry weight. After calcination at 550 °C carbon, nitrogen and organic hydrogen were determined on an autoanalyzer CHN LECO 800.

Hydrocarbons were extracted from the sediments for 4 hours by digestion under alkaline conditions. The cooled extract was filtered under vacuum pressure on GF/C Whatman filter paper. The sediment was extracted several times with methanol, toluene and hexane. The filtrate was transferred to a separatory funnel and the organic phase was extracted three times with 100 ml of hexane. The three fractions were dried over $MgSO_4$ overnight. Hexane was removed by evaporation. The residue was weighed and represented the extractable organic matter. This residue was then partitioned on a silica column into saturated fraction (FS) and aromatic fraction (FA). Each fraction was weighed on a microbalance. The fractions were analysed by capillary gas chromatography and by UV fluorescence. (Mille et al. 1988).

Enrichment of marine sediment with oil petroleum

The top two centimetres of several cores of sediment were mixed, bubbled with nitrogen to maintain anoxic conditions and stored in capped glass vials containing Arabian light (5 to 10% w/w). In order to obtain a good homogenization, the mixture (oil-sediment) was stirred overnight at 4 °C. For each site, four experimental systems were treated.

Bacterial numeration

Five ml of sediment in 45 ml of sterile seawater was subjected to 60 min vigorous reciprocal agitation (96 rpm) at 4 °C and centrifuged at 2000 *g* for 10 min. The supernatant was used for number determination on agar plates or with the most probable number method (MPN).

Aerobic heterotrophic bacteria were prepared on agar plates with Zobell medium after an incubation time of 2, 8 and 15 days, at 20 °C (Oppenheimer & Zobell 1952).

Denitrifying bacteria: the cultures were prepared with medium containing lactate ($1\ g\ l^{-1}$), acetate ($1\ g\ l^{-1}$), succinate ($1\ g\ l^{-1}$) and nitrate ($3\ g\ l^{-1}$). Anaerobic conditions were obtained by flushing nitrogen through the sealed flask for 20 min. After blockage with acetylene (20%) and incubation at 32 °C for 24 or 48 h, we measured the production of nitrous oxide as described above.

Hydrocarbonoclastic bacteria: the carbon source used is a crude oil and cultures were agitated at 32 °C on a reciprocal shaker. We selected only one which exhibited a turbidity compared to the control (without petroleum) (Mills et al. 1978).

Bacterial activities

The measurement of denitrification were based upon the acetylene inhibition technique (Balderson et al. 1976). Acetylene, which inhibits the reaction from N_2O to N_2, is distributed into sealed vials containing 5 ml of sediment enriched with nitrate (250 mM). The rate of N_2O accumulation is used as a measure of denitrification.

The rate of sulfate reduction was measured with a radiotracer technique described by Jørgensen & Fenchel 1974. Ten microlitres of labeled sulfate 35 SO_4Na_2, ($100\ \mu Ci\ ml^{-1}$) were injected into sediment core. The first 2 centimetres are then incubated 24 h at the *in situ* temperature. The radioactivity of sulfate and sulfite is determined as well as the chemical concentration of sulfate. The rate of sulfate reduction is calculated per unit of sediment volume (l) and time (day) (Jørgensen 1978).

Results

Analytical assays

The results given in Table 1 show that the nitrate and nitrite concentrations were low compared to those of ammonium, especially in the sediment of Port-Cros. The redox potential was found to be positive in sediment of sites 1, 2, 3 and negatives in sites 4, 5 and 6, where the sediment was rich in organic matter. Values of the C/N ratio from 3 to 9 were characteristic of organic matter assimilated by microorganisms (Walsh et al. 1981).

Table 1. Abiotic parameters, bacterial numeration and activities in 0–2 cm depth sediment. Buffer solution: 220 ± 5 mV at 25 °C, pH 7. For sediment classification: see in the text.

Parameters	Port-Cros	Cassis	Carry	Carteau	Berre	Lavera
Sediment	Fine sand	Rough sand	Fine sand	Silt/sand	Silt	Silt
NO3 μM	5.08	4.52	3.46	2.06	2.28	3.15
NO2 μM	0.75	0.68	0.55	0.47	0.56	2.11
NH4 μM	891.72	450.50	290.00	329.94	195.70	74.73
Temp. °C	17	18.5	20	20	18.4	22
Salinity	37.0	37.0	37.0	30.0	28.5	30.0
Dissolved O_2 (ppm)	9.0	8.5	9.0	6.7	4.3	3.5
pH	6.8	6.9	7.3	7	7.5	7.8
Redox potential mV	+220	+140	+180	−10	−75	−370
C/N	4.88	6.50	5.75	9.20	3.25	8.45
Total organic matter $g\,kg^{-1}$	1.3	1.5	1.6	4.4	8.7	108
Organic matter extractable $g\,kg^{-1}$	0.05	0.07	0.14	0.06	0.39	64.6
Saturated fraction $g\,kg^{-1}$	0.008	0.008	0.042	0.018	0.054	18.8
Aromatic fraction $g\,kg^{-1}$	0.007	0.012	0.012	0.02	0.027	12.3
Aerobic heterotrophic bacteria (ml^{-1})	1.1×10^8	4.8×10^8	4.5×10^8	9.5×10^8	1.5×10^9	3.0×10^8
Denitrifying bacteria (ml^{-1})	1.4×10^6	1.5×10^6	2.0×10^6	4.5×10^6	2.5×10^6	1.5×10^6
Hydrocarbonoclastic bacteria (ml^{-1})	2.5×10^4	2.5×10^4	7.5×10^5	9.5×10^5	1.5×10^5	4.5×10^7
Denitrification rate (nmol N_2O produced $cm^{-3}\,d^{-1}$)	204	196	167	294	144	210
Sulfate reduction rate (nmol reduced $SO_4\,l^{-1}\,d^{-1}$)	0	0	0	0	160	233

Table 2. Petroleum enrichment and percentage of the insertion.

Sites	Added petroleum $g\,kg^{-1}$	Petroleum incorporated $g\,kg^{-1}$	Percentage insertion
Port-Cros	100	9.3 ± 2.52	9.3
Cassis	100	9.1 ± 2.04	9.1
Carry	100	10.8 ± 2.65	10.8
Carteau	50	9.8 ± 2.18	19.6
Berre	30	9.42 ± 2.15	31.4

The Arabian light petroleum used was analyzed by gas chromatography in order to determine the hydrocarbon initially present in the sediment. Chromatograms of the saturated hydrocarbon fraction (FS) allow the determination of the biogenic or anthropogenic origin according to some selective tests: the presence or absence of unresolved complex mixture (UCM), the determination of carbon preference index (CPI), the ratio between light ($< C20$) and heavy ($> C20$) compounds, the concentration of isoprenoic compounds (pristane and phytane), and the presence or absence of alkenes (Saliot 1981).

Our results show that sediment sampled near Port-Cros was lightly contaminated with biogenic hydrocarbons and those from Lavera revealed an anthropogenic origin. The results are not so clear for the four other sites which revealed a complex mixture of both origins. A high number of microorganisms was observed in each location. The estimation of heterotrophic aerobic bacteria was about 10^8–10^9 cells ml^{-1}.

We note that the denitrifying microflora is well represented and about constant (10^6 bacteria ml^{-1}) whatever the nature of the sediment. We found aerobic hydrocarbonoclastic bacteria in all sediment, with a very high number in the sediment collected from Lavera.

Chemical analysis of petroleum mixed to sediment

Different quantities of petroleum (30–100 $g\,kg^{-1}$) were added to the sediments in order to obtain the same weight of incorporated petroleum: 1% (w/w) (Table 2). Thus, the percentage of the insertion of petroleum in sediment is highly-dependent on the granular nature of the sediment. It reached 10% in a sandy sediment (sites 1, 2, 3) and reached 30% in a muddy sediment (sites 4, 5). Chromatograms of the saturated fraction before, and after, the petroleum contamination show the same chemical composition (Fig. 2). The concentration of hydrocarbon initially present in the sediment (Table 1) was very low and their presence does not modify the composition of the incorporated products.

Evolution of denitrifying activity in the artificially polluted sediment

For each site, the denitrification was measured in each of four systems and their control (without contamination). After one week of incubation, the production of N_2O was at a maximum and the added nitrate had almost disappeared. At

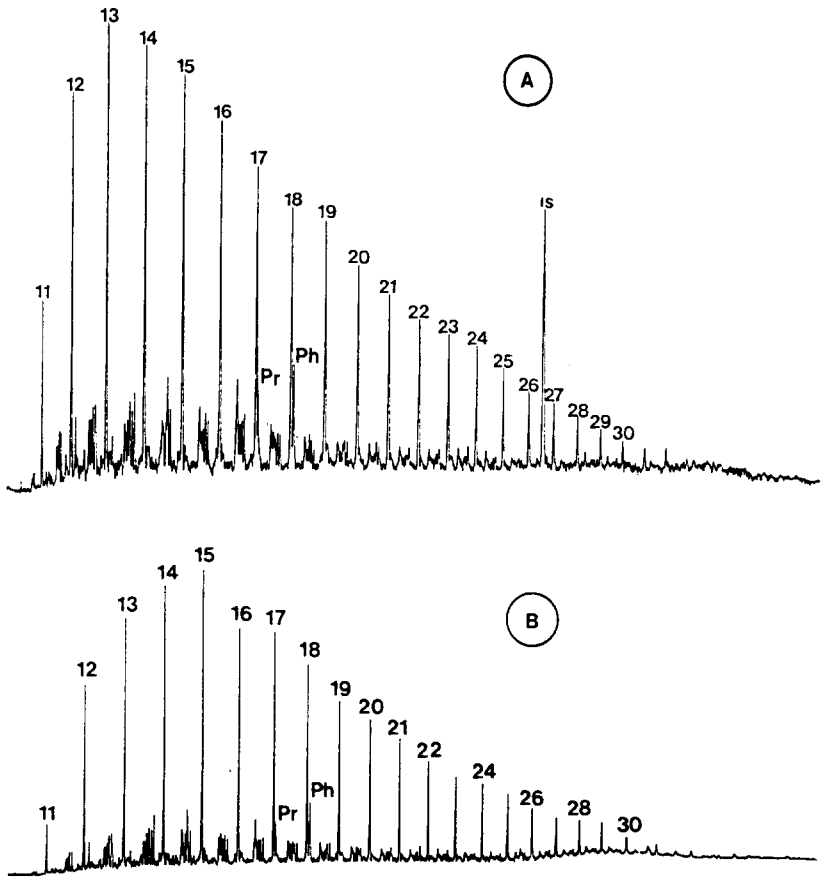

Fig. 2. Chromatograms of the saturated hydrocarbon fraction (FS) of Arabian light petroleum, before (*A*) and after the enrichment with this petroleum: Port-Cros (*B*).

this time, the N_2O was removed by 30 min of nitrogen bubbling, and nitrate was added (250 μM). This step was repeated until the denitrifying activity had completely disappeared. In contrast with denitrifying activity measured in initially contaminated sediment which remains constant, during 58 days incubation (Lavera Fig. 3), the addition of petroleum inhibits the denitrifying activity and the degree of inhibition depended upon the time of incubation and the origin of the sediment (Table 3 and Fig. 4). In the sediment from Carteau & Berre, the activity decreased the first week and disappeared after 22 days, whereas in the sediment of Port-Cros, Cassis and Carry the activities were maintained for longer periods, respectively 64, 57 and 50 days.

Addition of Na-lactate ($3\,g\,l^{-1}$) restored the denitrifying activity in each system (Fig. 4). In the control system of each sediment sampled, the activity remained constant. No change was observed in the sediment from Lavera which was initially contaminated by hydrocarbon. Nitrate determination was performed in the pore water after the inhibition of the denitrification; in each case we observed a decreasing of the nitrate concentration.

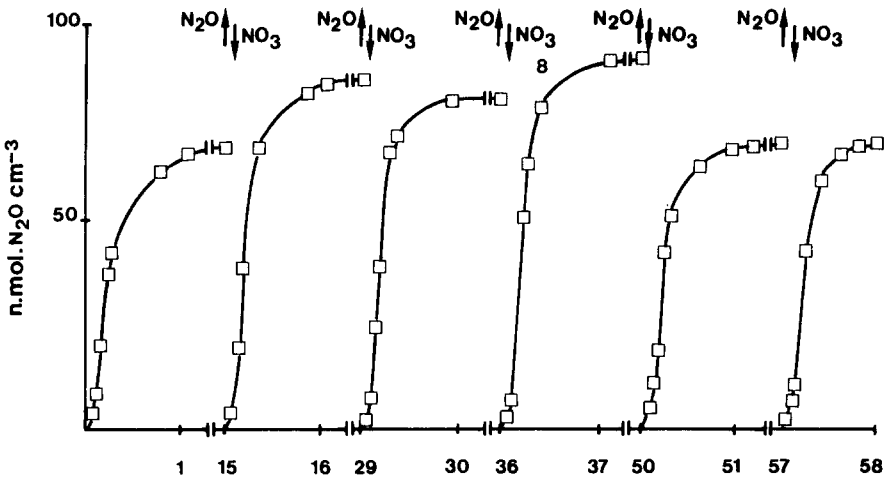

Fig. 3. Effects of Arabian light petroleum on N_2O production rate in sediments. Lavera (initially contaminated) $N_2O\uparrow$: N_2O removed by nitrogen bubbling, $NO_3\downarrow$: NO_3 added, both at the end of each step of the experimentation.

Effect of petroleum on microbial communities

In system where the denitrifying activity disappeared, a bacterial count was performed. No significant changes were observed in the composition of the community. We found the same number of heterotrophic, denitrifying, and hydrocarbonoclastic bacteria as before the contamination with petroleum hydrocarbon.

The decrease in the rate of denitrification after exposure to petroleum was not correlated with bacterial death. The following experiment explains this fact. Bacteria removed from analyzed sediment were incubated with petroleum at the same concentration as that introduced into the sediment (10%) and with nitrate (250 μM). In these experimental conditions the denitrifying activity was always maintained (Fig. 5). Thus, petroleum has no effect on free cells. Similar results were obtained for every sediment site tested.

Redox potentials and sulfate reduction activity

It was important to observe the evolution of the redox potential with the anaerobic conditions maintained in each experimental system. To measure redox potential, the electrode was slowly pushed into the sediment. The redox potentials were found to decrease and become negative (Table 4).

Prior to petroleum contamination a sulfate reduction activity was detected only in sediments collected from Berre (site 5) and Lavera (site 6). After addition of petroleum this activity increased in sediments from Berre, appeared in sediments from Cassis, Carteau and Carry.

Table 3. Effects of Arabian light petroleum on N_2O production rate in different sediments. (Incorporated petroleum: $10\,g\,kg^{-1}$ sediment, incubation in the dark and at 20 °C.)

Days	Port-Cros	Cassis	Carry	Carteau	Berre	Lavera
1	204 (± 47)	196 (± 38)	157 (± 35.7)	284 (± 67)	144 (± 45)	210 (± 38.7)
8				222 (± 86)	84 (± 27)	
15	255 (± 57)	210 (± 39.11)	149 (± 34.9)	132 (± 45)	16 (± 8)	240 (± 42.8)
22		186 (± 38.7)	98 (± 31.6)	0	0	
29	248 (± 36)	195 (± 27)	66 (± 29.3)			336 (± 59)
36	177 (± 24)	46 (± 26)	26 (± 19.6)			324 (± 57.9)
50	58 (± 25)	0	0			312 (± 58.5)
57	0					300 (± 49.8)

Values in N_2O produced $cm^{-3}\,d^{-1}$.
Standard deviation is given in parenthesis.

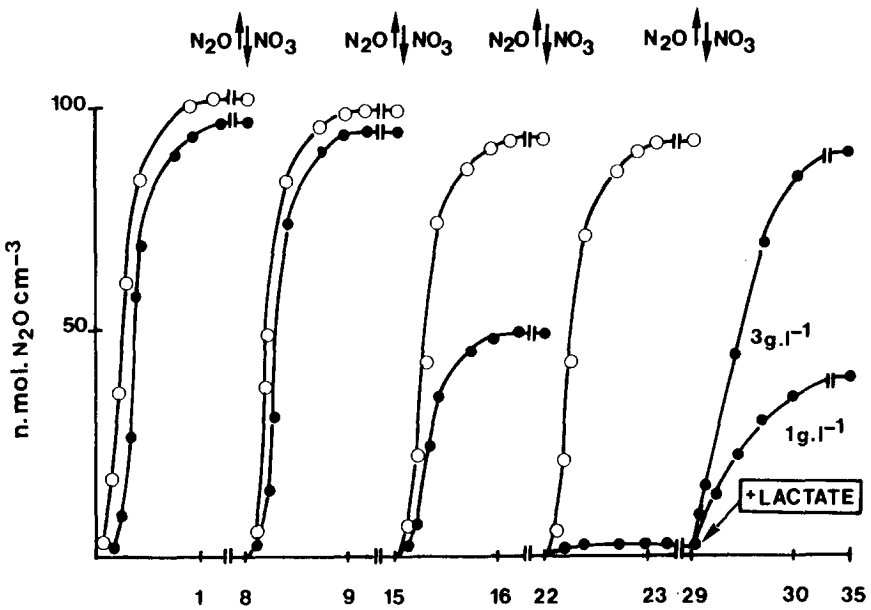

Fig. 4. Effects of Arabian light petroleum on N_2O production rate in sediments. Carteau: Control o—o; Incorporated sediment (10 g kg^{-1} sediment) ●—●; Lactate: 3 g l^{-1}, $N_2O\uparrow$; $NO_3\downarrow$, see Fig. 3.

Discussion

The hydrocarbon concentration originally present in sediments collected from the five sites of the Mediterranean coast is so low that it is possible to study the impact of a strong exposure of petroleum (30–100 g kg^{-1} sediment) on microbial activities such as nitrate reduction, denitrification and sulfate reduction.

There was no observed difference in nitrate reduction between oil-treated, and control samples, and 47 to 82% of activity was maintained during time-incubation. Such data were also indicated by Delaune et al. (1979). On the contrary, the denitrification decreases for 4 to 7 weeks to total inhibition.

There have been some reports on the effect of crude oil on microbial processes especially that of denitrification. Haines et al. (1981) found that, in surface sediment samples collected in the Alaskan continental shelf (Beaufort sea), crude oil inhibits the 'natural' denitrification (i.e. without added nitrate); however it has no effect on the 'potential' denitrification measured with added nitrate. Similar studies in sediments also collected from the Alaskan shelf (Kasitsna Bay) reported by Griffiths et al. (1982) have shown that both 'natural' and 'potential' denitrification were altered by exposure to oil.

Our results are not completely comparable with those reported in the literature. Our experiments were conducted in closed systems, thus under *in vitro* conditions, and exposure to oil was only for short periods of time (about 1 month). In contrast, Haines et al. (1981) and Griffiths et al. (1982) have studied the problem of a long-term effect (up to 18 months) of crude oil on microbial

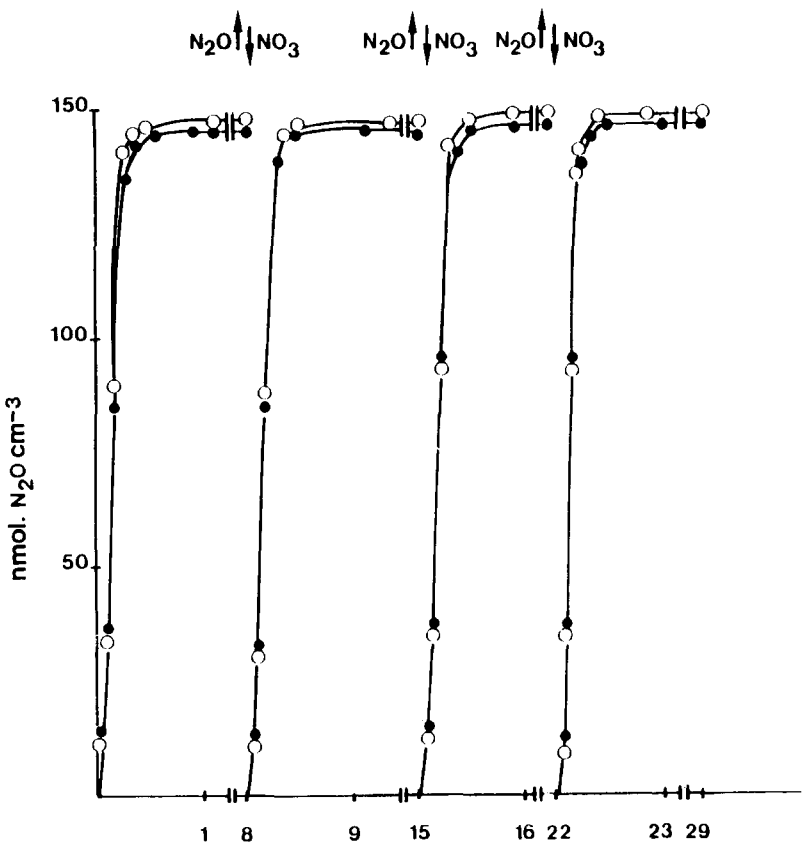

Fig. 5. Effects of Arabian light petroleum on N_2O production rate in bacteria isolated from sediment of Carteau. Control o—o; Added petroleum ($10\,g\,l^{-1}$) ●—●; $N_2O\uparrow$; $NO_3\downarrow$, see Fig. 3.

function under *in situ* conditions. In addition, these authors did not determine the concentration of oil actually mixed into the sediment. We have shown that the percentage of insertion of oil is dependent on the nature of sediment.

Data obtained from our experiments clearly indicate the inhibitory effect of petroleum on denitrification. Although the exact mechanism involved in denitrification inhibition by crude oil is not known, we can suggest two explanations: either the bacteria were affected directly by the petroleum or some of its components, or the crude oil alters the environmental conditions necessary to denitrification. The first hypothesis can be discarded for two reasons: first, the denitrification was maintained when the bacteria were separated from the sediment and they were kept in the same conditions as those where the denitrifying activity was inhibited in sediment; second, the number of bacteria stayed the same in both control and oiled sediment. The perturbation of natural environmental conditions by petroleum could explain changes and decrease of the

Table 4. Determination of bacterial number, redox potentials, residual nitrate concentration and sulfate reduction activity in each system at the end of the experiment. Buffer solution: 220 ± 5 mV at 25 °C, pH 7.

	Port-Cros	Cassis	Carry	Carteau	Berre	Lavera
Aerobic heterotrophic bacteria (nml^{-1})	9.5×10^8	6.5×10^8	7.5×10^8	4.5×10^8	9.5×10^8	2.5×10^8
Denitrifying bacteria (nml^{-1})	9.5×10^5	5.5×10^5	6.5×10^5	1.5×10^6	2.5×10^6	2.0×10^6
Hydrocarbonoclastic bacteria (nml^{-1})	3.0×10^4	1.6×10^5	7.5×10^5	9.5×10^5	1.6×10^5	2.0×10^8
Redox potential mV	−30	−180	−95	−130	−180	−540
Residual nitrate μM	89.2	43.2	81.6	67.2	43.2	22
Sulfate reduction rate nmol SO_4 reduced l^{-1} d^{-1}	60	90	78	58	388	1980

denitrification. The results of our studies on microbial processes shows a correlation between the inhibition of denitrification and the appearance, and increase, of sulfate reduction.

Sørensen et al. (1979) previously found that the relative importance of these two processes was dependent upon the concentration of available electron acceptors. The sulfate reduction overshadows the denitrification when the quantity of nitrate decrease (e.g. marine sediments are rich in sulfates). In our experiments the nitrate was added steadily, thus we can suppose that the inhibition of denitrification was caused by a decrease of redox potential, a supply of sulfates, or a deficiency of some organic substrates preferentially used by sulfate-reduction or/and fermentative bacteria. The restoration of denitrification after the addition of lactate confirms this latter suggestion. Lactate, a non-fermentable substrate has been used at $3\,g\,l^{-1}$, a sufficient concentration to serve as electron donor for both respiratory metabolisms. Jenneman et al. (1986) reported that addition of nitrate (59 mM) inhibits sulfide production in sewage sludge. This inhibition was attributed to an increase in oxidation-reduction potential due to biogenic production of nitrous oxide. In our experiment, the crude oil contamination decrease the redox potential until negative values. In these conditions sulfate reduction takes place and denitrification was slowly inhibited. The addition of nitrate (0.25 mM) was too low to induce the reoxidation of the sediment.

In conclusion, the results presented in this study, show the inhibitory effect of petroleum on denitrification in marine sediments. Our findings, taken in conjunction with those obtained on nitrification which is also blocked by petroleum (Delaune et al. 1979), indicate that an extensive oil contamination alters a large part of nitrogen cycle, but has no effect on sulfate reduction.

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