Comparison of methods for measuring denitrifying activity in marine sediments from the Western Mediterranean coast

Denitrification
C_2H_2 blockage
Marine sediment
Mediterranean

Dénitrification
Blocage C_2H_2
Sédiment marin
Méditerranée

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ABSTRACT

Different types of denitrification rates in coastal marine sediments from the Western Mediterranean coast (France) were simultaneously determined by various C_2H_2 inhibition techniques: *in situ* denitrifying activity in intact cores or sediment slurries; DEA (Denitrifying Enzyme Assays); and potential activity. Denitrifying activity was also estimated by the use of kinetic parameters (Km and V_{max}). Values for denitrifying activities in the top two centimetres ranged from 0 to 50 µmoles of nitrous oxide produced per litre of sediment per day, and were similar when intact core or sediment slurries were used. DEA showed comparable values for all the study sites. The potential activity ranged from 298-600 µmoles per litre per day. Estimated activity based on kinetics parameters was between 0.4 and 2.5 % of the denitrification potential. Estimation of denitrifying activity based on kinetic parameters and *in situ* NO_3^- concentration thus seemed impossible.

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RÉSUMÉ

Comparaison de méthodes de mesure de l'activité dénitrifiante dans des sédiments marins de la côte occidentale méditerranéenne

Les taux de dénitrification ont été déterminés dans différents types de sédiment prélevés sur la côte méditerranéenne occidentale. Les différentes techniques d'inhibition à l'acétylène ont été utilisées simultanément : activité dénitrifiante *in situ* dans des carottes intactes ou dans des boues, DEA (dosage des enzymes impliquées dans la dénitrification), activité potentielle. L'activité dénitrifiante a été également estimée en utilisant les paramètres cinétiques (Km et V_{max}). Les valeurs d'activité dénitrifiante dans les deux premiers centimètres varient de 0 à 50 µmoles d'oxyde nitreux produit par litre de sédiment et par jour, et sont comparables à celles trouvées dans les carottes de sédiment ou les boues. La DEA montre des valeurs comparables pour les sites étudiés. L'activité potentielle est comprise entre 298 et 600 µmoles par litre et par jour. L'activité estimée représente 0,4 à 2,5% de l'activité potentielle. Ainsi, l'estimation de l'activité dénitrifiante basée sur les paramètres cinétiques et la concentration *in situ* du NO_3^- semble impossible.

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INTRODUCTION

Denitrification has been generally considered as a bacterial respiratory process whereby nitrogenous oxides (NO_3^-, NO_2^-) are reduced to dinitrogen gas (N_2, N_2O). These nitrogenous oxides can be used as alternate electron acceptors when oxygen is absent. In recent years, a number of reviews have been devoted to denitrification (Tiedje *et al.*, 1982; Payne, 1973; Tiedje, 1988).

Various methods have been used to measure denitrifying activity:

1) indirect methods, *e. g.* nitrate disappearance (Andersen, 1977); nitrogen balance;

2) direct methods, *e. g.* appearance of $^{15}N_2O$ or $^{15}N_2$ by ^{15}N technique (Siegel *et al.*, 1982); acetylene inhibition technique (Balderston *et al.*, 1976; Yoshinari *et al.*, 1977). Denitrification methodology has been the subject of detailed papers (Knowles, 1982; Seitzinger, 1988). Recently Tiedje *et al.* (1989) compared the acetylene and ^{15}N methods, and found no significant differences between them.

The difficulty of quantifying denitrification lies not in the technique itself, but in the interpretation of the rates measured; this is partly dependent on the method used. Natural substrate concentrations and distribution must be maintained in order to obtain realistic values for denitrifying activities. The major advantage of the acetylene method, developed in this study, is that it uses the natural substrate pool. This technique is based on the inhibition of the final stage of denitrification. The accumulated N_2O is easily measured with high sensitivity by electron capture gas chromatography. However, acetylene also inhibits nitrification (Hynes and Knowles, 1978; Walter *et al.*, 1979); consequently, in sediments where *in situ* nitrate concentration are low, the lack of the coupled nitrification/denitrification leads to an underestimation of denitrification.

In the present study, an attempt was made to measure, by a simple and rapid method, the denitrification rate in marine sediment collected from six localities on the Mediterranean coast. The measurements were compared with those obtained by various other methods: profiles of denitrification activity in undisturbed sediment cores (Jorgensen and Sorensen, 1988); potential activity (rate under anaerobic and nitrate saturated concentration); and values estimated from kinetic parameters (Oren and Blackburn, 1979). To our knowledge, this is the first report on a comparison of C_2H_2 methods relative to the microbial denitrifying activity in marine sediments.

MATERIALS AND METHODS

Study area and sampling

The investigation was carried out at six localities on the Mediterranean coast (Fig.1). Sediment samples were taken by hand in transparent methacrylate cores about 20 cm in length, stored at 4°C during transport to the laboratory and analyzed upon arrival for their physical and chemical parameters. Cores used to determine denitrifying activity were stored overnight at *in situ* temperature under aerated and circulating water from the sampling site (Jorgensen and Sorensen, 1988).

Sediment classification was performed by sifting. The coarse fraction (> 63 µm) was sand and the fine fraction (< 63 µm) was silt.

The sampling sites have been described previously by Bonin *et al.* (1990).

Analytical assays

Temperature, salinity and redox potential were recorded for each site with the appropriate devices: salinometer (YSI33) and a pH/mV (Schott CG817T) equipped with a combined Ag/AgCl reference and platinum electrode. Before each measurement, a delay of one minute was allowed for the reading to become constant.

All nitrogen compounds were measured in the supernatant obtained after centrifugation at 2 000 xg for 10 min. Nitrates were reduced on a Cu-Cd column adapted to Technicon II according to Treguer and Le Corre (1975).

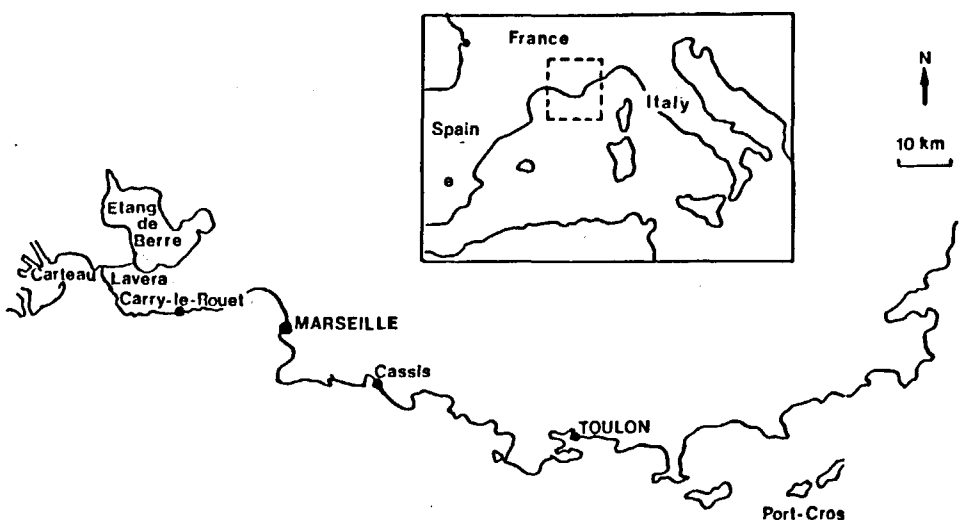

Figure 1

Study sites on the Western Mediterranean coast.

Sites d'étude sur la côte ouest méditerranéenne.

Nitrite concentrations were determined colorimetrically by the method of Bendschneider and Robinson (1972).

Nitrous oxide was determined by a gas chromatography Girdel series 30 equipped with an electron capture detector. Chromatographic operating conditions : 8 ft-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/mn.

Organic matter

An aliquot of sediment was dried at 110°C to constant dry weight. After calcination at 450°C, carbon, nitrogen and organic hydrogen were determined with an autoanalyzer CHN Leco 800 (Kristensen and Anderson, 1987).

Denitrification activity

The various methods used for measuring denitrification were based upon the acetylene inhibition technique (Balderston *et al.*, 1976). All experiments were performed at 22 ± 2° C.

Experiment with sediment slurry

Sediment cores were sectioned to 2 cm thick segments from the top to 10 cm. Four millilitres of each segment were distributed into 13 ml tubes containing 4 ml of natural seawater. The tubes were sealed with rubber stoppers and anaerobic condition obtained by flushing N_2 through the tube. Acetylene (15 kPa), which inhibits the reaction from N_2O to N_2, was distributed into the tube. Two tubes were sacrified for analysis after 0, 0.5, 1 and 2 hours of incubation at 20° C. The linear initial rate of N_2O accumulation is considered as a measure of *in situ* denitrification activity. After incubation, each tube was vigorously shaken by hand for 2 minutes and harvested at 2 000 xg for 3 mn. Three millilitres of gas phase were injected into a pre-evacuated venoject tube for later N_2O analysis. The extraction of N_2O from the liquid phase was effected by the procedure of Chan and Knowles (1979) modified by the technique of multiple equilibrium (Mac Aulife, 1971).

The potential activity is measured under the same conditions with sea water supplemented with nitrate (300 to 3 000 µM). The maximal rate of denitrification (V_{max}), *i. e.* the potential activity, is calculated from the Lineweaver-Burk plot by least square regression analysis. Assuming that the Michaelis-Menten kinetics were followed, the estimated denitrification rates were calculated taking into account the nitrate concentration measured *in situ* (C) from:

$$V\ est = \frac{V_{max}\ .C}{Km + C}$$

Denitrifying enzyme activity was measured according to Tiedje's procedure (1989).

Experiment with sediment cores

Denitrifying activity in intact sediment cores was measured by the procedure of Jorgensen and Sorensen (1988).

The cores were closed with rubber caps, the water phase was slowly mixed with the aid of a motor stirrer mounted on the top cap. Acetylene-saturated distilled water (200 µl) was injected in the sediment through a vertical series of silicone rubber inserts (*id.* 3 mm) placed at 5 mm intervals along the side of the tube. Two cores were sacrificed for analysis after 0, 1, 2 or 3 hours of incubation. The sediment was cut into 2 cm segments. Four millilitres of sediment collected in each segment were placed in 13 ml tube containing 4 ml of 2 N KCl solution. The N_2O was extracted and its concentration was determined as previously described for the experiment carried out with sediment slurries.

RESULTS AND DISCUSSION

Measurements of denitrification, as N_2O accumulation after blockage of the N_2O reduction step with acetylene, were undertaken on coastal marine sediment. The first part of our investigations was carried out on sediment samples collected during winter (1989) at Carteau cove in the gulf of Fos, located 12 miles east of Marseilles near the mouth of the river Rhône. The station at 5 m depth presented a muddy sand sediment.

The depth profiles of denitrifying activity were determined simultaneously without NO_3^- amendment by using sediment slurries or whole cores (Jorgensen and Sorensen, 1988). The kinetics of N_2O accumulation were monitored for three hours. These assays were realized on short term incubation. No significant difference between the rates determined after 0.5 hour incubation and those recorded after 1 hour was observed.

The results obtained by experimental procedures are reported in Table 1. In the top two centimetres, the denitrifying activity was maximum. The values obtained are the same whatever the method used. Conversely, below the uppermost segment, comparison of profiles reveals that when sediment slurries were used, the activities were up to ten times higher than their corresponding values measured in intact core. In fact, in undisturbed sediment very little activity was observed.

Table 1

Comparison of denitrifying activities measured in intact core and slurries of sediment collected from Carteau cove.

Comparaison des activités dénitrifiantes mesurées dans des carottes de sédiment intact ou mélangé prélevées dans l'anse de Carteau.

Sediment depth (cm)	NO_3^- (µM)	NO_2^- (µM)	Denitrification slurries µmol.l^{-1}.d^{-1}	Denitrification core µmol.l^{-1}.d^{-1}
0-2	6.24	3.22	94.8	90.89
2-4	3.5	2.31	24.24	2.4
4-6	1.43	5.46	30.72	3.22
6-8	2.34	1.27	8.28	0

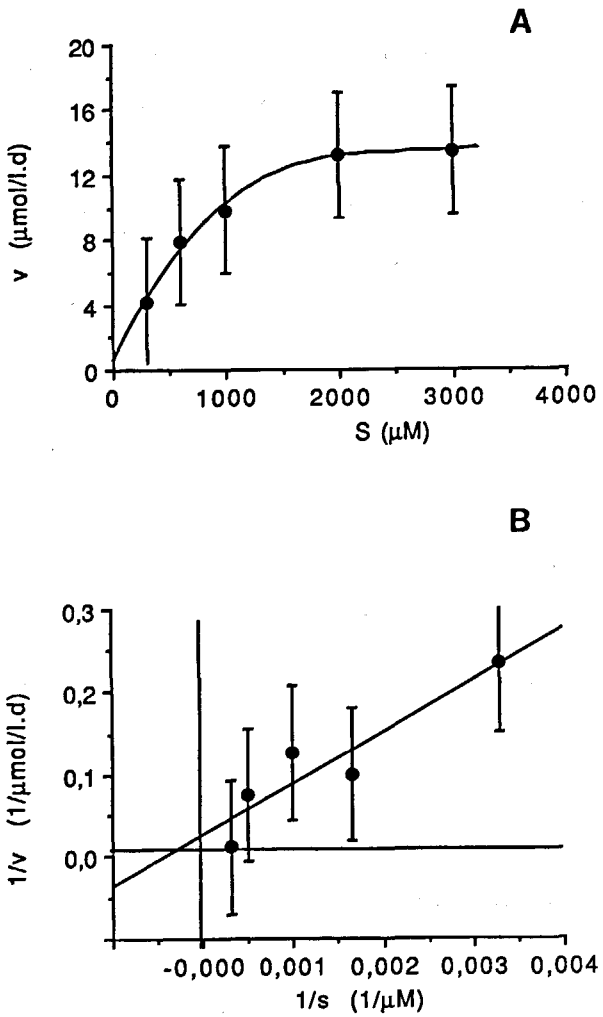

Figure 2

Denitrification rate versus *nitrate concentration in 0-2 cm depth sediment from Carteau bay (A); Lineweaver-Burk representation (B).*

Vitesse de dénitrification en fonction de la concentration en nitrate dans les deux premiers centimètres de sédiment de la baie de Carteau (A) ; représentation de Lineweaver-Burk (B).

The use of the whole core seemed to be better suited for estimating natural denitrifying activity, since this technique involves no alteration of the natural sediment structure or of the oxygen and nitrogen oxide gradient. However, our results clearly show that this method results in a decrease of the activity measured in the deep sediment. This could be due to incomplete inhibition of N_2O reduction by water-saturated acetylene, *i. e.* injected through the core. Koike and Sorensen (1988) have pointed out the difficulty of obtaining the complete inhibition if the C_2H_2 is not rapidly and homogeneously distributed during injection. Data obtained on intact core could be distorted by inadequate control of phenomena such as acetylene and nitrous oxide diffusion through sediment. Incubation in a sealed system means that the C_2H_2 concentration necessary to prevent further reduction of N_2O once this gas is produced can be determined. The latter method is therefore more appropriate for realizing denitrifying activity profiles.

Figure 2 shows the result of an attempt to determine the kinetic parameters Km and V_{max} by measuring denitrification rate versus nitrate concentration. Anaerobic sediment slurries were amended with NO_3^- (300-3 000 µM) and N_2O reduction was blocked by 15 KPa of acetylene. The relation between production of N_2O and NO_3^- concentration is depicted by a hyperbolic curve which corresponds to Michaelis-Menten kinetics. For the 0-2 cm layer, by the method of Lineweaver-Burk, a value of 1428 µM is found for Km and a value of 600 $\mu mol.l^{-1}.d^{-1}$ for V_{max}; which corresponds to the potential denitrifying activity. Data concerning all the layers are summarized in Table 2. Denitrifying activity decreased from 600 $\mu mol.l^{-1}.d^{-1}$ in the top layer to 20 $\mu mol.l^{-1}.d^{-1}$ in the 4-6 layer.

The relation between denitrification rate and NO_3^- concentration has been used to estimate the rate of denitrification (Vest). The activities estimated, using this method, are very low compared to those measured directly without addition of NO_3^-. The use of this relation to estimate denitrification does not seem suitable for Carteau cove coastal sediment. Results reported in Table 1 show no correlation between NO_3^- concentration *in situ* and the activity detected.

Given these results, it was particulary important, because of the high spatial variability of denitrification, to follow

Table 2

Potential and estimated denitrification in sediment collected from Carteau cove.

Activité dénitrifiante potentielle et estimée dans les sédiments prélevés dans l'anse de Carteau.

Sediment depth cm	Km µM	V_{max} activity $\mu mol.l^{-1}.d^{-1}$	NO_3^- µM	Estimated $\mu mol.l^{-1}.d^{-1}$
0-2	1428	600	4.67	1.95
2-4	1818	192	2.8	0.29
4-6	740	20	2.21	0.06
6-8	1666	37	1.62	0.036
8-10	1428	120	0.49	0.041

Table 3

Abiotic parameters in 0-2 cm depth sediment.

Paramètres physico-chimiques dans les deux premiers centimètres de sédiment.

	STUDY SITES					
	Carteau	Berre	Lavera	Carry-le-Rouet	Cassis	Port-Cros
Water depth (m)	7	1	0,5	30	20	10
Salinity	38	28	30	37	37	37
Redox (mVolt)	+ 150	- 75	- 170	+ 180	+ 140	+ 87
pH	7	7.5	7.8	7.3	6.9	7.5
Organic carbon (% dry weight)	2.8	5.7	8.2	0.29	0.42	0.02
NO_3^- (µM)	6.18	6.68	0.85	8.13	3.2	3.87
NO_2^- (µM)	1	4.46	0.5	0.91	0.73	0.39

this comparative study with experiments using various samples from the sediment surface. The investigation was carried out in six localities on the Western Mediterranean coast (France) during winter (December 1990 to February 1991). The sampling stations were chosen according to their carbon and nitrate contents, since the main factors that influence denitrification in the ecosystem include the supply of NO_3^- and organic matter.

During the sampling period, surface water temperature varied from 10 to 15° C. Table 3 characterizes the sediment by carbon and nitrogen contents. Port-Cros bay station presents lower organic content, whereas the Lavera, Carry-Le-Rouet and Cassis stations have particulary high organic carbon content. The latter stations showed NO_3^- contents ranging from 0.8 to 8 µM.

The rates of denitrification in sediment slurries prepared from the first two centimetres, measured by various procedures, are reported in Table 4. Values are means of triplicate experiments with a variability of generally less than 10 %.

Except for the Lavera site, denitrifying activity, measured without addition of nitrate in sealed tube, was always detected in these coastal sediments. The lack of activity at Lavera can be explained by the very low level of NO_3^- concentration in this sediment. For the other sites, activity ranged from 24 µmol.l^{-1}.d^{-1} to 58 µmol.l^{-1}.d^{-1}. Comparable values are reported in the literature (Tab. 5). There is no evident correlation between denitrifying activities and carbon contents in the study sites (Tab. 3).

Denitrification potentials, determined by Michaelis-Menten kinetics, ranged from 293 to 600 µmol.l^{-1}.d^{-1} for the whole set samples. The half saturation concentrations (Km) were higher than those generally reported (Tab. 5), but compa-

Table 4

Denitrifying activities in the top 2 cm in coastal marine sediments from the Western Mediterranean coast.

Activité dénitrifiante dans les deux premiers centimètres de sédiments marins prélevés sur la côte méditerranéenne occidentale.

	Study sites					
	Carteau	**Berre**	**Lavera**	**Carry**	**Cassis**	**Port-Cros**
Denitrifying activity (0-2 cm) (µmol.l^{-1}.d^{-1})	25.5	24.7	0	33	48	58
Potential activity (µmol.l^{-1}.d^{-1})	600	386	464.4	298	302	300
Km (µM)	1428	256	227.7	539	499	560
Estimated activity (µmol.l^{-1}.d^{-1})	2.58	9.81	1.73	4.33	1.92	2.05
Denitrifying Enzyme Activity (µmol.l^{-1}.d^{-1})	40	16.82	ND	29.32	40.5	15.9

Table 5

Denitrifying activities and half saturation constants (Km) in coastal marine sediments.

Activité dénitrifiante et constante d'affinité (Km) dans des sédiments marins côtiers.

Location	**Km (µM)**	**Potential denitrification (µmol.l^{-1}.d^{-1})**	***In situ* denitrification (µmol.l^{-1}.d^{-1})**	**References**
Randers fjord (Denmark)	NR*	NR	99	Sorensen (1978 *a*)
Lymford (Denmark)	NR	NR	100-870	Sorensen (1978 *b*)
Randers fjord (Denmark)	NR	NR	14-100	Sorensen *et al.* (1979)
Lymford (Denmark)	NR	NR	30-510	Andersen *et al.* (1984)
Aarhus bight (Denmark)	NR	NR	50-100	Jorgensen and Sorensen (1988)
Japan	27-31	NR	NR	Koike and Hattori (1978 *a*; *b*)
Kysing fjord (Denmark)	344	422	NR	Oren and Blackburn (1979)
Deleware inlet (New Zealand)	NR	2 008	NR	Kaspar (1982)
Deleware inlet (New Zealand)	NR	971-13 878	NR	Kaspar (1983)
San Francisco bay (USA)	50	242-324	NR	Oremland *et al.* (1984)
Mediterranean coast	227-1 728	298-600	0-58	in this paper

* *not reported.*

rable to concentrations of 344 and 219 µM reported by Oren and Blackburn (1979) and Messer and Brezonik (1984) respectively. From these kinetic parameters, the denitrification rate has been estimated (Tab. 4). The values obtained were between 0.4 and 2.5 % of denitrification potential according to Kaspar observations (1982). The use of this relation to estimate denitrification is often considered as not straightforward (Seitzinger, 1988), and our results clearly show that there was no correlation between the vertical distribution of NO_3^- and denitrifying activity nor between denitrification rate and NO_3^- concentrations according to study sites. Similar observations have been reported by many other authors (Kaspar, 1983; Kaspar *et al.*, 1985 ; Sorensen, 1978 *b*). Potential activity is very high; it corresponds to the expression under optimal conditions of enzymes associated with the *in situ* population plus that of enzymes synthesized during the growth of denitrifying bacteria after a long term incubation (80 hours).

Tiedje (1988) recently suggested the use of the Denitrifying Enzyme Assay (DEA) to characterize the sediment. With this assay the concentration of functional denitrifying enzyme in the sample can be measured at the time of sample collection. Chloramphenicol (1 $g.l^{-1}$) is used to block protein synthesis. Electron donor and acceptor, which are limiting factors of denitrification, are added (1 mM NO_3^- and 1 mM glucose). The potential N_2O production rate is thus measured by making all the factors which affect the denitrifying rate, except enzyme quantity, non-limiting. After acetylene blockage, the rate of nitrous oxide production is assumed to be proportional to enzyme content. The incubation period recommended by Tiedje (1988) should not go beyond two hours, but using 10 mM NO_3^-, we have established the linearity of the kinetic of nitrous oxide accumulation during ten hours. The denitrification values obtained with DEA in our samples (Tab. 4) did not differ significantly from those obtained without NO_3^- after a short incubation period. In most sediments studied, the carbon and nitrate contents were not limiting factors.

The DEA method can be used to test when the substrate is limiting. For example, during the December cruise (1990), at the dyke sampling site of Carteau, the nitrate concentration was very low (0.48 µM). The *in situ* denitrifying activity was equal to 2.76 $\mu mol.l^{-1}.d^{-1}$. The DEA with addition of nitrate or glucose alone gave values of 18.06 and 3.24 $\mu mol.l^{-1}.d^{-1}$ respectively, a sixfold increase was obtained after addition of the limiting factor, namely nitrate.

A considerable amount of information is available on denitrification in coastal marine sediments. It is difficult to compare the results obtained by most of the authors because of the dissimilarity of the methods used. As we have shown in the present paper, the interpretation of the results is partly dependent on the method used. In conclusion, it should be stressed that each method attains different objectives, while potential activity, DEA and *in situ* denitrifying activity all provide complementary information. Refinements and intercalibration of the various techniques for measuring denitrification are thus desirable before *in situ* study of denitrification is undertaken.

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