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Nitrogen fluxes from marine sediments: quantification of the associated co-occurring bacterial processes

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Abstract Different models for calculate on of di-nitrogen fluxes using $^{15}NO_3$ tracers were tested for their congruence with experimental data obtained with marine sediment samples. The co-occurence of nitrification as source of substrate and the simultaneous N_2 production from denitrification and/or Anammox were taken into account as well as nitrous oxide production in the total denitrification rate. The results highlighted that isotope technique provides a powerful tool to evaluate, in the same experimental set up, the rates of total N_2 fluxes: denitrification and/or Anammox if it is carefully applied and its limitations, mainly the range of $^{15}NO_3$ inputs are adapted to the studied samples and the linearity of the kinetics of the products checked.

Keywords Anammox · Denitrification · Nitrification · Nitrous oxide · Isotope pairing method · Simulation

Introduction

The nitrogen cycle has received considerable attention in recent years because of its ecological importance. The potential importance of nutrient levels in the coastal zones is at the origin of the episode of eutrophication in the ecosystems. This eutrophication poses major ecological problems such as massive phytoplankton bloom and severe hypoxia. It was established since a long time that denitrification is the main microbial process through which N is transferred from aquatic system to the atmosphere. The main techniques that have been used to directly measure denitrification are the acetylene block methods for which the limitations are now well known (Bonin et al. 1998). During the last ten years with the appearance of important technological headways such as development of the use of isotope was led to revisit the nitrogen cycle. The isotope pairing method (IPM, Nielsen 1992) has generally been shown to be sensitive and robust (Cabrita and Brotas 2000; Dong et al. 2000; Glud et al. 1998; Ogilvie et al. 1997; Risgaard-Petersen et al. 1998). After the addition of known amount of $^{15}NO_3$ in the experimental system, denitrification is calculated from the formation of $^{29}N_2$ versus $^{30}N_2$. However, the use of this method is based on various assumptions (Nielsen 1992), such as the addition of $^{15}NO_3$ should not affect the production of N_2. Moreover, the estimate of the molar fraction of the various N_2 isotopes versus the molar fraction of $^{15}NO_3$ is possible only if the ratio $^{14}NO_3/^{15}NO_3$ remains constant in the zone where the activity takes place. The use of IPM in conjunction with other technique (i.e. dilution of $^{15}NO_3$) provides a powerful tool to

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evaluate, in the same experimental set up, the rates of not only denitrification as well as processes such as nitrification, mineralization or dissimilatory nitrate reduction to ammonium (Bonin and Michotey 2006). However, the original version of IPM focused only on N_2 production from denitrification without taking into account the other processes of nitrogen cycle as for example, nitrification. This transformation of ammonium into nitrate constitutes a continuous nitrate input in the medium and probably modifies the initial proportions of labelled nitrate leading to an error in the calculation of denitrification by IPM. During the last years, some authors have proposed to improve the original IPM in order to perform simultaneous measurements of denitrification and nitrogen fixation (An et al. 2001), and to gain an insight into the N_2O formation, or to assess inputs of nitrate-polluted water through the hyporheic zone (Master et al. 2005). Recently, it was discovered that Anammox (anaerobic oxidation of ammonium), can account up to 67% of N-loss in continental shelf sites (Thamdrup and Dalsgaard 2002). In the Benguela upwelling system, nutrient profiles, Anammox rates, abundances of Anammox cells, and specific biomarker lipids indicate that nitrate is not directly converted to N_2 by heterotrophic denitrification but Anammox bacteria are responsible for massive losses of fixed nitrogen (Kuypers et al. 2005). This process combines ammonia and nitrate or nitrite directly into dinitrogen gas. In consequences, the production of N_2 measured by IPM is not only formed by denitrification but is the resultant of both denitrification and Anammox processes, which may or may not act simultaneously in the sediment (Fig. 1). When Anammox and denitrification co-exist, the binomial law of the distribution of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ cannot be verified; since Anammox does not form of $^{30}N_2$ and thus the proportion of the various nitrogen isotopes is modified (Fig. 1). In a theoretical approach Risgaard-Petersen et al. (2003) have shown that Anammox activity causes overestimation of the N_2 production calculated by IPM and they proposed two indirect calculation procedures based on the determination of R14 (ratio between $^{14}NO_3$ and $^{15}NO_3$ undergoing reduction) in order to correct the overestimation generated by the calculation of IPM such as it was defined by Nielsen. More recently, the limits of these calculation procedures were investigated by Trimmer et al. (2005) who have proposed an alternative calculation procedure based on the direct estimation of R14 from the production of $^{45}N_2O$ and $^{46}N_2O$ assumed to be produced by denitrification only.

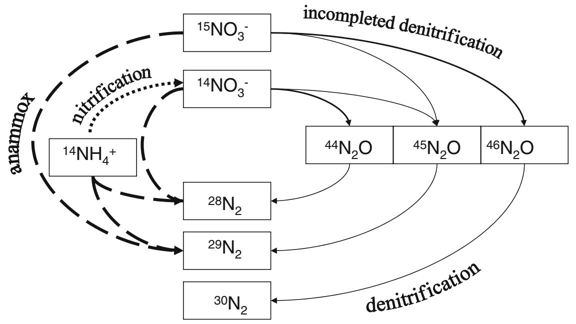

Fig. 1 Diagrammatic presentation of the various reactions implied in the production of the various N_2O and N_2 isotopes by the method of pairing isotopic

In spite of the common use of IPM, very few papers deal on the limitations of this method and they present mainly theoretical approaches. In this paper, the theoretical approaches are compared to experimental data: and we have investigated how the estimation of denitrification by IPM in coastal marine sediment is affected by the simultaneous expression of other processes associated with nitrogen cycle. Specifically, we ask three questions: (1) In which proportion, the nitrate production via nitrification affects the assumption of the original IPM? (2) How to give evidence for the expression of Anammox process in sediment? (3) How to take into account the nitrous oxide accumulation (from incomplete denitrification) in the determination of denitrification rates? Answering to these questions will allow improving the knowledge and the robustness of the Isotope Pairing Method. A systematic representation summarizing the different equations is proposed to help to decide which one should be used for the calculation of denitrification or nitrous oxide production rates when nitrification and or Anammox are simultaneously present in marine sediment.

Materials and methods

Sampling site

Sediments and waters used in this study were collected at three different stations. Two stations are located at Gulf of Fos (SE France), Mediterranean coast (Bonin et al. 1998). Station (B) is located inside Carteau Cove (Bonin et al. 1998) and station E outside this cove near the Rhone River mouth. The sediment samples were collected at different sampling times between October 2004 and October 2005. The third station is located in the Bassin d'Arcachon coastal lagoon. The Bassin d'Arcachon is a mesotidal lagoon on the SW coast of France (Atlantic Ocean). The total area of the lagoon is approximately 180 km^2, which includes the very dynamic tidal inlets and changing sand banks in the S.W. corner of the lagoon. Only 40 km^2 remains submerged at low tide (De Wit et al. 2005).

Undisturbed sediment cores were taken by hand in plexiglass tubes (inner diameter 18 cm) and experiments were carried out with the first 2 cm of the sediment. B and E sediment samples were collected in June 2005. The sediments were siltly and contained 45.11 and 40.26 g of organic carbon per kg of dried sediment. The percentage of interstitial sea water was about 18.7% and 18.3% (w/w). Nitrate and ammonium concentrations in interstitial water were 1.3 and 376.8 µM respectively for station B and 1.3 and 20.2 µM, respectively for station E. Sediment of Arcachon station was collected in emerged site at low tide in April 2005. The sediment was silty and contained 48.7 g of organic carbon per kg of dried sediment. The percentage of interstitial sea water was about 20.5% (w/w). Nitrate and ammonium concentrations in interstitial water were 22.3 and 2028 µM, respectively.

Sediment incubations experiments

From each sediment sample, 5 cm^2 sub-samples were placed into a 22 ml headspace vial containing 5 ml of filtered (<0.8 µm) seawater of the sampling site. The vials were sealed with a rubber stopper. All processes were measured under ambient oxygen level without nitrogen bubbling. At the beginning of the experiment, $^{15}NO_3^-$(97.4 atom%, Isotec Mathesson, USA) was added to each vial in order to obtain a final concentrations comprised between 5 and 250 µM. Direct measurement of Anammox rates were determined as recommended by Trimmer et al. (2003) in the same slurries enriched to approximatively 10% above the ambient level with concentrated stocks of labelled $^{15}NH_4$ (0.2 mM $^{15}NH_4Cl$, 99.1 atom%, Isotec Mathesson, USA) and 0.2 mM $^{14}NO_3^-$.

The kinetics of production of single-labelled ($^{14}N^{15}N$) and double-labelled ($^{15}N^{15}N$) di-nitrogen were followed during 10 h in incubation chamber maintained at in situ water temperature in triplicates. The rates of product accumulation were calculated from the changes in concentration, measured in triplicates, at each sampling time (0, 1, 3, 5, 7 and 10 h). For each point in the kinetic process, $HgCl_2$ (final concentration 10 mM) was added in order to block the activities, and the incubation flasks were frozen until analysis. After thawing and homogenization, gasses were directly sampled from the vial and injected into the mass spectrometer (Anagaz 100, MKS, England) equipped with direct inlet and $^{29}N_2$ and $^{30}N_2$ concentrations were then plotted against time and fitted to the linear model ($A(t) = A_0 \pm m * t$) using the least squares method, where t is the incubation time; A_0 is the concentration at $t = 0$; and m is the slope of the linear curve. The rates were calculated from the initial linear slope of the curve. Rate uncertainties were calculated from the errors in the linear regressions. The $^{29}N_2$ and $^{30}N_2$ productions were used to determine the total N_2 production and discriminate the part due to denitrification or to Anammox rates using equations developed in the results part of the manuscript. All abbreviations were reported in Table 1.

N_2 and N_2O isotope measurements with quadruple mass spectrometry

$^{29}N_2$ and $^{30}N_2$ in addition to $^{28}N_2$, Ar and O_2 were measured by mass spectrometry (Quadruple mass spectrometer Anagaz 100, MKS, England). Signals at 4 m/z values were collected every 0.5 s intervals and were stored by a desktop computer for later analysis. N_2 was measured at m/z = 28, 29 and 30 corresponding to $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ respectively, N_2O was measured at m/z = 45 and 46 corresponding to ^{45} or $^{46}N_2$ and O_2 or Ar were measured at m/z = 32 and 40 respectively. Ar was used as internal standard. The ratio between $^{29}N_2$/Ar and $^{30}N_2$/Ar is 0.00732 (0.0036 × 2) in natural sample since the natural abundance of ^{15}N is 0.366%. The ratio measured in mass spectrometry was very close to the theoretical value and calculated to account small variability between measurements. There is a possible interference of m/z = 30 signal by O_2, probably due to NO^+

Table 1 Abbreviation used in the text

Abbreviations	Definitions
2*n*	Total number of NO_3 ($^{15}NO_3$ plus $^{14}NO_3$)
A28	Production of $^{28}N_2$ coming from Anammox
A29	Production of $^{29}N_2$ coming from Anammox
D14	N_2 production from $^{14}NO_3^-$ as substrate of denitrification
D14*niel*	N_2 production from $^{14}NO_3^-$ as substrate of denitrification calculated using Nielsen method
D15	N_2 production from $^{15}NO_3^-$ as substrate of denitrification
D28	Production of $^{28}N_2$ coming from denitrification
D29	Production of $^{29}N_2$ coming from denitrification
D30	Production of $^{30}N_2$ coming from denitrification
Dtot	Production of N_2 coming from denitrification (sum of D14 and D15)
D#14	N_2 production from $^{14}NO_3^-$ as substrate of denitrification including N_2O production
D#15	N_2 production from $^{15}NO_3^-$ as substrate of denitrification including N_2O production
D#tot	N_2 production from NO_3^- as substrate of denitrification including N_2O production
E	Error in N_2 production due to Anammox presence calculated by the method of Nielsen
IPM	Isotope Pairing Method
p'	Probability that one $^{15}NO_3^-$ is introduced into the reaction
p	Number of $^{15}NO_3$
P14	N_2 production by reactions using $^{14}NO_3^-$
P14*niel*	N_2 production by all reactions using $^{14}NO_3^-$ as substrate calculated by the Nielsen IPM
P15	N_2 production by reactions using $^{15}NO_3^-$
P28	Production of $^{28}N_2$
P29	Production of $^{29}N_2$
P30	Production of $^{30}N_2$
P44	Production of $^{44}N_2O$
P45	Production of $^{45}N_2O$
P46	Production of $^{46}N_2O$
q	Number of reactions of nitrification
R14	Ratio between $^{14}NO_3^-$ and $^{15}NO_3^-$ undergoing reduction
$R14_{N_2O}$	Method for calculating P14 recommended by Trimmer et al. (2006)
$R14_V$	Method for calculating P14 recommended by Risgaard-Petersen et al. (2003)
Ra	Percentage of Anammox
S	Number of reactions of denitrification
V	Ratio between $^{15}NO_3$ added in different incubations

ions formations from N_2 and O^+ inside the MS. According to Jensen et al. (1996b), we might expect a correlations between the product at signals $m/z = 32$ and 30. We observed a linear relationship between m/z 30 and (m/z 28 × m/z 32) (Fig. 2) as suggested by Jensen et al. (1996b). This representation gave better regression than the square root relationship suggested by An et al. (2001). This relationship was used to determined $m/z = 30$ in excess by comparing the signal with those of the standard.

Modelling the behaviour of di-nitrogen production

Simple models were developed in W-maple V4 software to examine the behaviour (shape response)

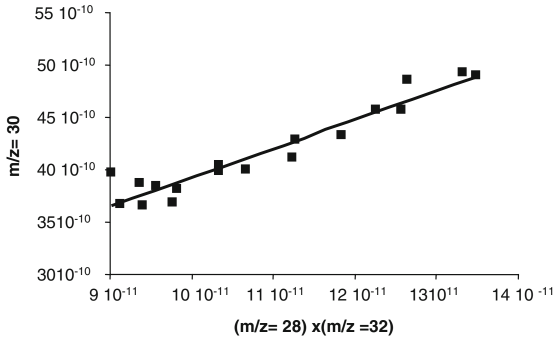

Fig. 2 Relationships between different m/z signals in gaseous air phase amended with various amount in N_2 measured in quadruple mass spectrometer in order to correct the quantification $^{30}N_2$. Indeed, interpreting the m/z 30 signal is complicated due to the reactions between N_2 and O_2 in the ion source of the mass spectrometer. The NO^+ ions (m/z = 30 signal) formed from N_2 and O^+ inside the mass spectrometer can cause a linear relationship between m/z 30 and (m/z 28 × m/z 32). The signal (m/z) represents the partial pressure of gases (in pascals 10^{-2}) inside the mass spectrometer

of $^{28}N_2$, $^{29}N_2$, $^{30}N_2$ productions and denitrification rates in the presence of nitrification and Anammox in slurry systems.

Nitrification rates

Nitrification rates were obtained by measuring the changes of NO_2 concentrations in the same sediment slurries containing sodium chlorate (10 mM NaClO) to inhibit oxidation of nitrite (Gilbert et al. 1997). Nitrification activity rate was calculated from data obtained during the linear NO_2 production phase in the first 10 h of incubation. The incubation flasks were first vigorously shaken before 10 ml samples were centrifuged at 5,000 rpm for 15 min. No filtration was performed in order to avoid the leaching of particulate matter retained by the filter. The supernatant was fixed with $HgCl_2$ and stored at 4°C for later analysis of nitrate and nitrite using the Technicon autoanalyser (Tréger and Le Corre 1975). Nitrification rate were linear in time, the accuracy was given by the standard error of the slope.

Statistical analyses

The paired Student *t*-test was used in this study in order to compare the quantitative variables obtained from 2 small independent groups. The statistical differences between: (1) experimental values and (2) the theoretical values obtained from modelling were tested.

Results

Determination of N_2 production in the presence of denitrification only: the original method of Nielsen

The IPM developed by Nielsen (1992) estimates denitrification by monitoring changes in di-nitrogen gas with different isotope compositions ($^{29}N_2 = {}^{14}N + {}^{15}N$, $^{30}N_2 = {}^{15}N + {}^{15}N$) after enrichment with $^{15}NO_3$. This method has been successfully used in numerous environmental studies. Nielsen has considered that only denitrification was implied in the production of N_2. After addition of $^{15}NO_3$ as tracer, Nielsen quantified the N_2 production from $^{15}NO_3$ as substrate (D15) and that from $^{14}NO_3$ (D14).

Total denitrification, Dtot, is the sum of D14 and D15.

$$\mathrm{Dtot} = \mathrm{D14} + \mathrm{D15} \tag{1}$$

D15 being the denitrifying activity that implies the participation of $^{15}NO_3$ in the production of $^{29}N_2$ and $^{30}N_2$ (one or two $^{15}NO_3$ for the production of $^{29}N_2$ or $^{30}N_2$ respectively). Thus:

$$\mathrm{D15} = \mathrm{P29} + 2\mathrm{P30} \tag{2}$$

P29 representing the production of $^{29}N_2$ and P30 the production of $^{30}N_2$.

In the same way, D14 being the denitrifying activity that implies the participation of at least one $^{14}NO_3$ leading to the production of $^{28}N_2$ and $^{29}N_2$. Thus:

$$\mathrm{D14} = \mathrm{P29} + 2\mathrm{P28} \tag{3}$$

P28 cannot be directly measured. In order to express D14 as function of $^{29}N_2$ and $^{30}N_2$, Nielsen (1992) uses the approximation of the binomial law. D14 depends on the probability of the presence of the two isotopes of N–NO_3 in the medium. Thus, denitrification is comparable with two independent drawings of one NO_3 for each reaction, p' and $(1 - p')$ being the probability of introducing one $^{15}NO_3$ or one $^{14}NO_3$ into the reaction respectively.

$$\mathrm{P30} = p'^2 \quad (4)$$

$$\mathrm{P29} = 2p' \times (1 - p). \quad (5)$$

This probability p' depends on the initial quantity of $^{15}NO_3$ and consequently on the percentage of $^{15}NO_3$. In consequence, in order to be able to use this formula, the percentage of $^{15}NO_3$ must remain constant during the kinetic of incubation in the zone where the activity takes place. Moreover, the use of the binomial distribution utilized by Nielsen (1992) implies an indefinite number of reactions of denitrification.

When denitrification is the sole process producing D14 and D15, the ratio of D14 versus percentage of $^{14}NO_3$ is equal to ratio of D15 versus percentage of $^{15}NO_3$

$$\frac{\mathrm{D14}}{\%^{14}\mathrm{NO}_3^-} = \frac{\mathrm{D15}}{\%^{15}\mathrm{NO}_3^-}.$$

Thus:

$$\mathrm{D14} = \mathrm{D15} \times \frac{(1-p')}{p'}. \quad (6)$$

Consequently, from Eqs. 4 to 6:

$$\mathrm{D14} = \frac{\mathrm{P29}}{2 \times \mathrm{P30}} \times \mathrm{D15}$$

$$\mathrm{D14} = \frac{\mathrm{P29}}{2\mathrm{P30}} \times (\mathrm{P29} + 2\mathrm{P30}). \quad (7)$$

Nielsen's theoretical curves were compared to the experimental results obtained from coastal marine sediment collected in station B at Carteau Cove (Fig. 3). We can note a similar shape between the original Nielsen's theoretical curves P29 and P30 experimentally obtained (Fig. 3a, c). According to the above equations, denitrifying activity (D14, D15 and Dtot) were calculated from P29 or P30 measurements and plotted as function of the $^{15}NO_3$ percentage (Fig. 3b). No significant statistical difference was found between the two variables (Student, $n = 18, p > 0.05$).

However, the obtained results showed that the use of the binomial law leads to an overestimation of denitrification rates for $^{15}NO_3$ inputs leading to low $^{15}NO_3$ percentage (Fig. 3d). This overestimation of denitrification rate estimated in the presence of low $^{15}NO_3$ input is confirmed for different sampling times all over the year (Table 2).

In consequences, it is strongly recommended to test incubation conditions for different time series and for different $^{15}NO_3$ concentrations in order to determine the best range of added $^{15}NO_3$. In this range of $^{15}NO_3$ concentrations, relationship between P30 and the concentration of added $^{15}NO_3$ must be obtained and P29 must be independent of the amount of $^{15}NO_3$ input. In the example shown in Fig. 4, the optimal incubation conditions were obtained for $^{15}NO_3$ concentrations above 50 μM.

In order to explain the overestimation of denitrification rate observed in the presence of low $^{15}NO_3$ amendments (Fig. 3d) we have developed combinatory approach that seems more suitable than the binomial law previously tested. Indeed, in contrast to binomial law the combinatorics introduces a number of reactions of denitrification and tends to take into account the evolution of the nitrate concentration for each reaction of denitrification (as a result of nitrate exhaustion or on the contrary of nitrate supply by nitrification).

In this case, P30 is the number of reactions of denitrification associating two molecules of $^{15}NO_3$ among the total amount of NO_3.

$$\mathrm{P30} = \frac{C_p^2}{C_{2n}^2} = \frac{p \times (p-1)}{2n(2n-1)} \quad (8)$$

where $2n$ is the total number of NO_3 ($^{15}NO_3$ plus $^{14}NO_3$), p the number of $^{15}NO_3$ and $2n - p$ is the number of $^{14}NO_3$.

In the same way, we can write P28 and P29 representing the production of $^{28}N_2$ and $^{29}N_2$.

$$\mathrm{P29} = C_p^1 \frac{C_{2n-p}^1}{C_{2n}^2} = \frac{p \times (2n-p)}{n(2n-1)} \quad (9)$$

$$\mathrm{P28} = \frac{C_{2n-p}^2}{C_{2n}^2} = \frac{(2n-p)(2n-p-1)}{2n(2n-1)} \quad (10)$$

These equations allow obtaining a theoretical chart of the production of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ (Fig. 3e). We can note that the both theoretical curves (Fig. 3a, e) and the experimental results presented the same shape (Fig. 3c). However, the theoretical D14, D15 and Dtot obtained by combinatorics (Fig. 3f), allow to mimic the high D14 and Dtot rates experimentally observed in the presence of low $^{15}NO_3$ amendment (Fig. 3d) and then is more representative in these conditions.

The combinatorics will be used for the simulation of the $^{28,\ 29}$ and $^{30}N_2$ production when nitrification and/or Anammox processes occurred simultaneously with denitrification.

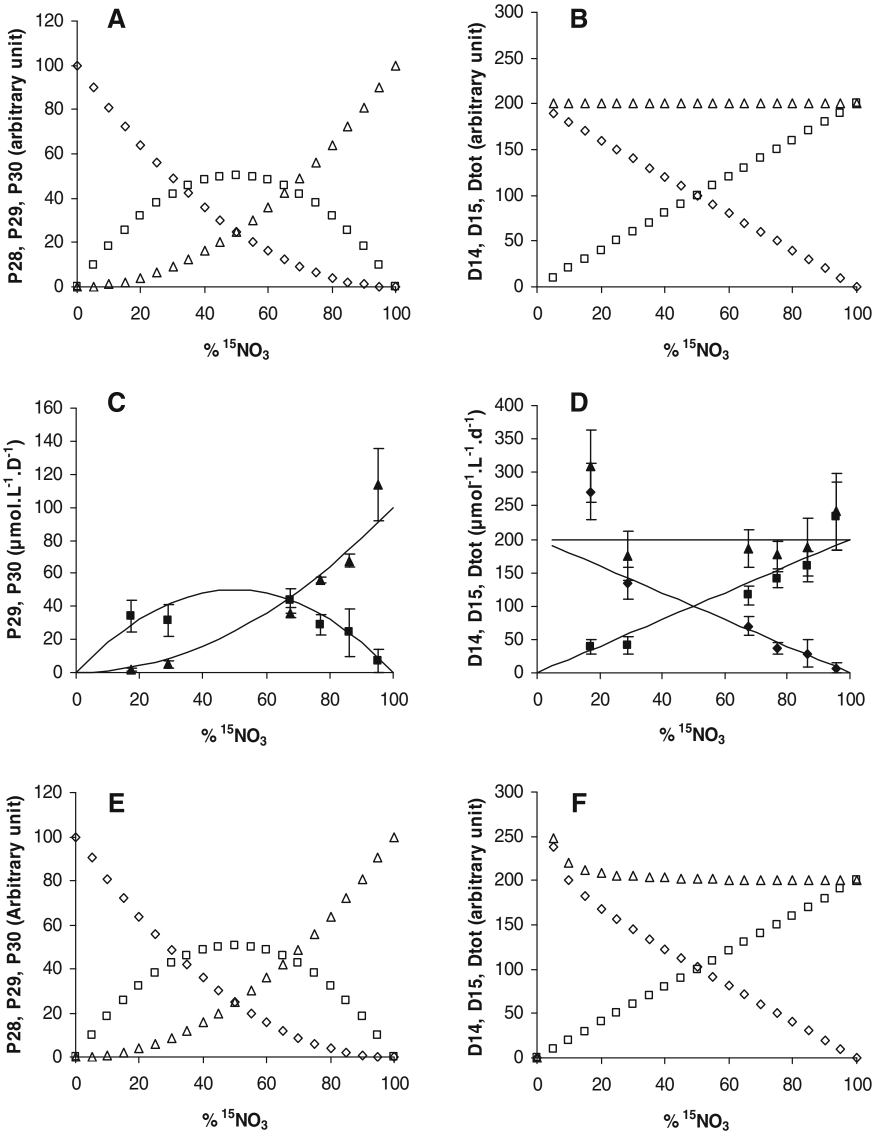

Fig. 3 Determination of denitrifying activity by isotope pairing method using binomial law or combinatorics approaches. (**a**, **b**) Utilisation of the binomial law for simulating the production of $^{28}N_2$ (P28, ◇), $^{29}N_2$ (P29, □) and $^{30}N_2$ (P30, △) (**a**) and simulation of the denitrifying activity (D15 (□), D14 (◇) and Dtot (△)) (**b**) as function of the percentage of $^{15}NO_3$ using the original IPM method. (**c**, **d**) Experimental determination of N_2 production: P29 (■) and P30 (▲) (**c**) and denitrifying activities: D15 (■), D14 (◆) and Dtot (▲) (**d**) as function of the percentage of $^{15}NO_3$ in sediment sample from Carteau cove (Station B, June 2005). Values are means ± 1 SD ($n = 18$). (**e**, **f**) Utilisation of combinatorics for simulating the production of $^{28}N_2$ (P28, ◇), $^{29}N_2$ (P29, □) and $^{30}N_2$ (P30 △) (**e**) and simulation of the denitrifying activity (D15 (□), D14 (◇) and Dtot (△)) (**f**) as function of the percentage of $^{15}NO_3$

Table 2 Illustration of the surestimation of Dtot for low $^{15}NO_3^-$ (<20%) compared with Dtot determined for higher $^{15}NO_3^-$ input for which the independence of Dtot towards $^{15}NO_3^-$ was verified

Sampling time	Dtot ($^{15}NO_3^- < 20\%$) (µmol L^{-1} D^{-1})	Dtot (µmol L^{-1} D^{-1})
October 2004	1160.8 ± 13.6	36.2 ± 17.8
December 2004	1595.3 ± 35.8	72.5 ± 35.9
February 2005	991.0 ± 102.6	169.2 ± 63.3
June 2005	459.2 ± 120.1	145.0 ± 29.8
August 2005	4350.8 ± 812.6	169.2 ± 50.8
October 2005	773.5 ± 38.0	241.7 ± 65.3

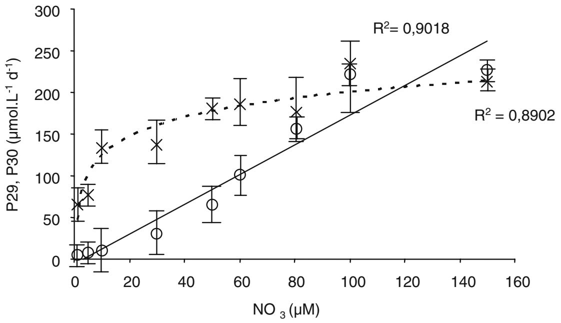

Fig. 4 P29 (×) and P30 (○) as function of $^{15}NO_3$ concentration in sediment sample from Carteau cove (Station B, June 2005)

Determination of N_2 production from denitrification in the presence of nitrification

Nitrification process leads to the NO_3 production from NH_4, and thus to permanent input of $^{14}NO_3$ in the zone of activity. This permanent flux of substrate must be taken into account in calculation.

If q is the number of reactions of nitrification, the number of initial molecules of NO_3 is not $2n$ but of $2n + q$, the number of labelled molecules ($^{15}NO_3$) being p. The number of reactions of denitrification is thus $n + q/2$.

The production of the various forms of N_2 ($^{29}N_2$, $^{30}N_2$) can be calculated according to the same method than that exposed previously (Eqs. 8–10).

$$\mathrm{P28} = \left(n + \frac{q}{2}\right) \times \frac{C^2_{2n+q-p}}{C^2_{2n+q}} = (2n + q - p) \times \frac{2n + q - p - 1}{2(2n + q - 1)} \tag{11}$$

$$\mathrm{P29} = \left(n + \frac{q}{2}\right) \times C^1_p \times \frac{C^1_{2n+q-p}}{C^2_{2n+q}} = p \times \frac{2n + q - p}{2n + q - 1} \tag{12}$$

$$\mathrm{P30} = \left(n + \frac{q}{2}\right) \times \frac{C^2_p}{C^2_{2n+q}} = \frac{p(p-1)}{2(2n + q - 1)} \tag{13}$$

The curves of P28, P29 and P30 production according to our simulations in the presence of nitrification (in the presented simulation, the additional source of NO_3 resulting from nitrification is considered to represent 26%, 42%, 60% or 80% of the nitrate available for reduction processes) are presented in Fig. 5. For comparison it was reported on the same figure P29 calculated without taking into account nitrification (Original Nielsen's calculation). The introduction of nitrification in the calculation modified the curve of P29 production. Indeed, for percentage of $^{15}NO_3$ close to 100%, in presence of denitrification alone, P29 decrease strongly and reach zero whereas in presence of nitrification and denitrification, P29 production decrease slightly and never reach zero (Fig. 5a). Indeed, nitrification involves the dilution of the initial labelling and this continuous input of $^{14}NO_3$ supports the formation of $^{28}N_2$ and $^{29}N_2$ isotopes.

According to Fig. 5b, it appears that the both profiles of D14 (D14*niel*, determined without permanent $^{14}NO_3$ input from nitrification, and D14) versus the percentage of $^{15}NO_3$, are superposed except for percentages of $^{15}NO_3$ lower than 20%. In consequences, the calculation of the denitrifying activities can be done according to the traditional calculation of isotopic pairing for percentages of labelling higher than 20%. Under this value, and when nitrification occurred D14*niel* is underestimated (Fig. 5b).

This simulation in the presence of nitrification was compared to experimental data obtained with sediment collected in station E showing nitrification activities (25 μM D^{-1}). As expected from theoretical approach, when the percentage of $^{15}NO_3$ added was higher than 90%, P29 as well as D14 never reached zero (Fig. 5c, d). Theoretical and experimental data showed similar profiles and were not significantly different (test Student, $n = 18$, $p > 0.05$).

Determination of N_2 production in the presence of denitrification and Anammox

Anammox is another process leading to the production of N_2. However Anammox can provide only $^{28}N_2$ and $^{29}N_2$. Thus, in the presence of $^{15}NO_3$, the production of N_2 from Anammox modifies the proportion of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ productions compared to the case where denitrification alone is implied. The total production of $^{28}N_2$ and $^{29}N_2$ depends on the ratio of intensities between the reactions of denitrification and Anammox. The net flux of $^{29}N_2$ corresponds to the number of reactions of denitrification (associating one $^{15}NO_3$ and one $^{14}NO_3$) plus the number of reactions of Anammox (implying one $^{15}NO_3$ and one $^{14}NH_4$).

The production of $^{29}N_2$ from Anammox process must be introduced in equation by applying the same combinatorics approach (previously described in Eqs. 8–10).

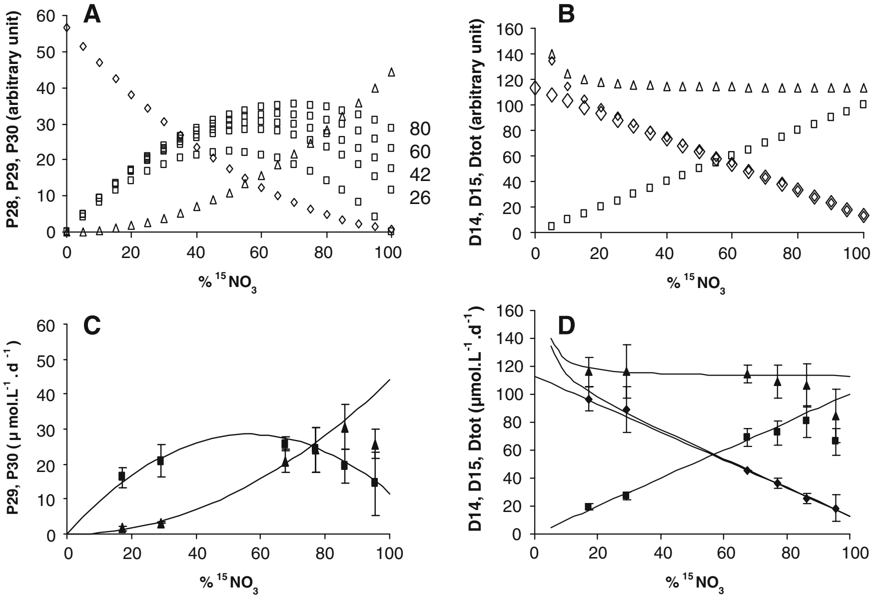

Fig. 5 Determination of denitrifying activity in the simultaneous presence of nitrification by isotope pairing method using combinatorics. (**a**, **b**) Simulation of the production of $^{28}N_2$ (P28, ◇), $^{29}N_2$ (P29, □) and $^{30}N_2$ (P30, △) as function the percentage of $^{15}NO_3$ in the system. Nitrification is considered to represent 0%, 28%, 44%, 55% or 62% of the nitrate input for reduction processes in these simulations. P29 profile (□) (**a**). Simulation of the denitrifying activity as function of the percentage of $^{15}NO_3$. D15 (□), D14 in presence of nitrification calculated by the binomial law (large ◇); D14 in presence of nitrification calculated by combinatorics (small ◇) and Dtot (△), (**b**); (**c**, **d**) Experimental determination of N_2 production : P29 (■) and P30 (▲) (**c**) and denitrifying activities (D15 (■), D14 (◆) and Dtot (▲) (**d**) as function of the percentage of $^{15}NO_3$ in sediment sample from Carteau cove (Station E, June 2005). Nitrifying activity experimentally determined was 25 µM D^{-1} in sediment sample used for this experiment. Values are means ± 1 SD ($n = 18$)

$$\mathrm{P29} = \frac{S\times C_p^1\times C_{2n-p}^1}{C_{2n}^2} + 2(n-S)\times\frac{C_p^1}{C_{2n}^1} = S\times\frac{p(2n-p)}{n(2n-1)} + 2(n-S)\times\frac{p}{2n} \qquad (14)$$

where S representing the number of reactions of denitrification, $2n$ the total number of nitrate molecules present in the system, p the number of labelled nitrate molecules, and, consequently, $2(n - S)$ the number of reactions of Anammox.

In the same way, it possible to calculate the production of $^{28}N_2$:

$$\mathrm{P28} = \frac{S\times C_{2n-p}^2}{C_{2n}^2} + 2(n-S)\times\frac{C_{2n-p}^1}{C_{2n}^1} = S\times\frac{(2n-p)\times(2n-p-1)}{2n(2n-1)} + 2(n-S)\times\frac{2n-p}{2n}. \qquad (15)$$

As $^{30}N_2$ can be produced only by denitrification, the production of this isotope can be calculated as follows:

$$\mathrm{P30} = \frac{S\times C_p^2}{C_{2n}^2} = S\times\frac{p(p-1)}{2n(2n-1)}. \qquad (16)$$

In the same way than that previously described for nitrification, when reactions of Anammox and denitrification occur simultaneously, we have simulated the production of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ and compared the curves with and without taking into account Anammox. Figure 6a presents an example of simulation when the reaction of Anammox accounts for 44% of the production of N_2. The production of N_2 and the proportion of the various isotopes were modified in the presence of Anammox. For the same percentage in $^{15}NO_3$ in the presence of both processes, P30 is lower than in the presence of denitrification alone, since $^{30}N_2$ is only produced by denitrification. On the other hand, P29 was higher because it is also produced by the reaction of Anammox and its maximum value was reached for higher percentages of $^{15}NO_3$.

According to the Nielsen's method (Eq. 7), the N_2-production from $^{14}NO_3$ (P14*niel*), can be written:

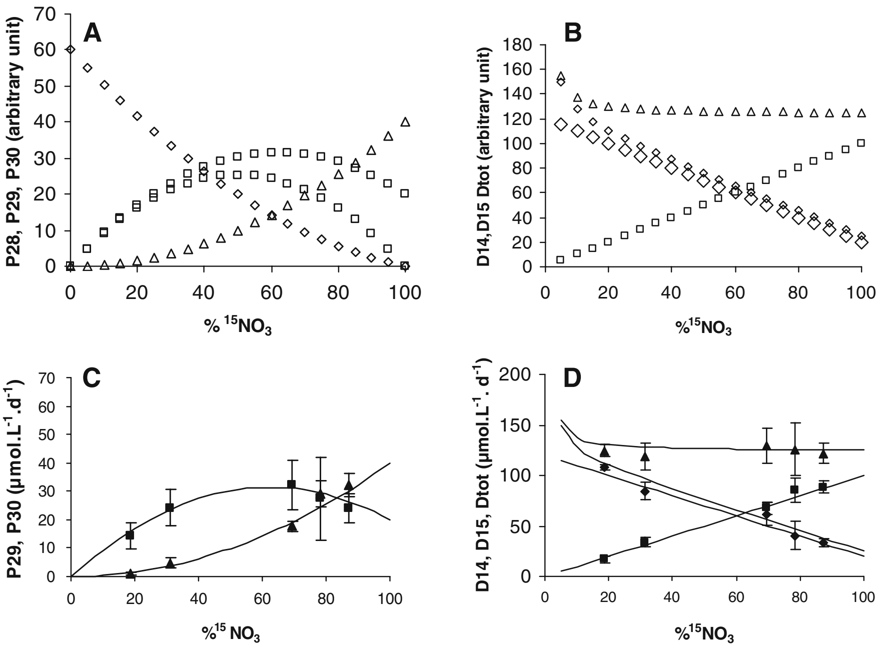

Fig. 6 Determination of denitrifying activity in the simultaneous presence of Anammox using combinatorics. (**a**, **b**) Simulation of the production of $^{28}N_2$ (P28, ◇), $^{29}N_2$ (P29, □) and $^{30}N_2$ (P30, △) as function the percentage of $^{15}NO_3$ in the system when Anammox accounts for 40% of the production of N_2. P29 profile (□) is compared with the P29 profile obtained in absence of Anammox (□) (**a**). Simulation of the N_2 production (denitrification plus Anammox) as function of the percentage of $^{15}NO_3$. P15 (□), P14 corrected by $R14_V$ method (large ◇) and Ptot (△), P14*niel* in presence of Anammox calculated by combinatorics (small ◇) (**b**); (**c, d**) Experimental determination of N_2 production as function of the percentage of $^{15}NO_3$ in sediment sample from Arcachon (April 2005). Values are means ± 1 SD ($n = 18$): P29 (■) and P30 (▲) (**c**) and the rates of N_2 production (denitrification plus Anammox) P15 (■), P14 corrected by $R14_V$ method (◆) and Ptot (▲) (**d**)

$$\mathrm{P14}\textit{niel} = \frac{(\mathrm{P29})}{(2\mathrm{P30})} \times (\mathrm{P29} + 2\mathrm{P30}).$$

In the simultaneous presence of denitrification and Anammox:

$$\mathrm{P14}\textit{niel} = \frac{(\mathrm{A29} + \mathrm{D29})}{(2\mathrm{D30})} \times (\mathrm{A29} + \mathrm{D29} + 2\mathrm{D30}). \tag{17}$$

D29 and D30 represent the productions of the $^{29}N_2$ and $^{30}N_2$ coming from denitrification, respectively and A29 the production of $^{29}N_2$ coming from Anammox.

P14*niel* is an approximation allowing calculating P14 from the measurements of P29 and P30. The true N_2 production from $^{15}NO_3^-$ by Anammox and denitrification processes corresponds to (see Eq. 3):

$$\mathrm{P14} = \mathrm{A28} + 2\mathrm{D28} + \mathrm{D29}. \tag{18}$$

In accordance with Eqs. 8–10:

$$\mathrm{D30} = n\frac{C_P^2}{C_{2n}^2} = \frac{p \times (p-1)}{2(2n-1)} \tag{19}$$

$$\mathrm{D29} = nC_p^1\frac{C_{2n-p}^1}{C_{2n}^2} = \frac{p \times (2n-p)}{(2n-1)} \tag{20}$$

$$\mathrm{D28} = n\frac{C_{2n-p}^2}{C_{2n}^2} = \frac{(2n-p) \times (2n-p-1)}{2(2n-1)} \tag{21}$$

$$\mathrm{A28} = n\frac{C_{2n-P}^1}{C_{2n}^1} = \frac{2n-p}{2} \tag{22}$$

$$\mathrm{A29} = n\frac{C_P^1}{C_{2n}^1} = \frac{p}{2}. \tag{23}$$

These equations allow to express and represent graphically P14 and P14*niel* (Eqs. 17 and 18) in the presence of Anammox according to the percentage of $^{15}NO_3$ (Fig. 6b). In the presence of high percentage in $^{15}NO_3$, P14*niel* does not reach the zero value since P14*niel* is calculated from the production of $^{28}N_2$ et de $^{29}N_2$ (Eq. 17) and $^{29}N_2$ produced by Anammox doesn't come from $^{14}NO_3$. The presence of Anammox induces an Error in the method of Nielsen that can be calculated from the difference between P14 and P14*niel*.

$$E = \mathrm{P14}niel - \mathrm{P14} \tag{24}$$

$$E = \frac{\mathrm{D29} + \mathrm{A29}}{\mathrm{2D30}} \times (\mathrm{A29} + \mathrm{D29} + \mathrm{2D30}) - (\mathrm{A28} + \mathrm{2D28} + \mathrm{D29}) \tag{25}$$

The percentage of Anammox is represented by Ra.

$$Ra = \frac{\mathrm{A28}}{\mathrm{P14}} \tag{26}$$

R14 is the ratio between the quantities $^{14}NO_3$ and $^{15}NO_3$ in the zone of expression of the activities, this ratio is different from the initial percentage of labelling

$$\mathrm{R14} = \frac{2n - p}{p} = \frac{\mathrm{2D28}}{\mathrm{D29}} = \frac{\mathrm{A28}}{\mathrm{A29}} = \frac{\mathrm{D29}}{\mathrm{2D30}} \tag{27}$$

$$\begin{aligned} E &= \mathrm{P14}\frac{Ra \times (1 + \mathrm{R14})}{\mathrm{R14} \times (1 - Ra)} \\ &= (\mathrm{A28} + \mathrm{D29} + \mathrm{2D28}) \times \frac{(\mathrm{R14} + 1)}{\mathrm{R14}} \times \frac{Ra}{(1 - Ra)}. \end{aligned} \tag{28}$$

From Eqs. 20 to 26 and after simplification, we can write:

$$E = 3n \times \frac{Ra}{1 - Ra}. \tag{29}$$

The Error (E) depends on P14 (Eq. 28), that is not constant but dependent itself on R14, and must be taken into account in calculations. After simplification, we demonstrate that this Error depends only on the proportion of Anammox (Ra) but not on the percentage of labelling (R14) when the initial nitrate concentration is sufficient to obtain rates independent of the nitrate concentration.

P29 and P30 production rates as function of $^{15}NO_3$ amendment determined in sediment collected at Arcachon in March 2005 were reported in Fig. 6c. The obtained profile showed that P29 never reached values close to zero for $^{15}NO_3$ amendment higher than 90%. This profile was comparable of that obtained in simulations when denitrification occurred in the presence of nitrification (Fig. 5a) and/or Anammox (Fig. 6a).

If the presence of Anammox is suspected, the traditional IPM (Nielsen 1992) is erroneous and must be modified. P14 can be expressed as function of P15 and R14.

$$\mathrm{P14} = \mathrm{R14} \times \mathrm{P15} = \mathrm{R14} \times (\mathrm{P29} + \mathrm{P30}) \tag{30}$$

The calculation of R14 is thus essential for the calculation of P14.

According to Risgaard-Petersen et al. (2003) R14 can be experimentally determined from measurements of P29 and P30 in the presence of different $^{15}NO_3$ concentrations.

$$\mathrm{R14}_V = \frac{\mathrm{P29}_1 - V \times \mathrm{P29}_2}{2 \times (\mathrm{P30}_1 - V^2 \times \mathrm{P30}_2)}. \tag{31}$$

Measurements of the P29 and P30 with different concentrations of $^{15}NO_3$ allow the calculation of R14 and consequently the determination of the production of N_2 formed from $^{14}NO_3$.

The rates of P14 (Fig. 6d), obtained using this latter method ($\mathrm{R14}_\mathrm{V}$) to correct the P14*niel*, were no significantly different (test student, $n = 18, p > 0.05$) to the theoretical profile (Fig. 6b), in the presence of high $^{15}NO_3$ level, the P14 never reached zero.

More recently Trimmer et al. (2006) proposed another method for the determination of R14 from the ^{15}N labelling of the N_2O produced during incubation and R14 can be expressed as

$$\mathrm{R14}_{N_2o} = \frac{\mathrm{P45}}{\mathrm{2P46}}. \tag{32}$$

According to our simulation P14 estimated with the original Nielsen IPM was overestimated (Fig. 7). The both methods ($\mathrm{R14}_\mathrm{V}$ and $\mathrm{R14}_{\mathrm{N_2O}}$) used to correct the error gave similar results for high $^{15}NO_3$

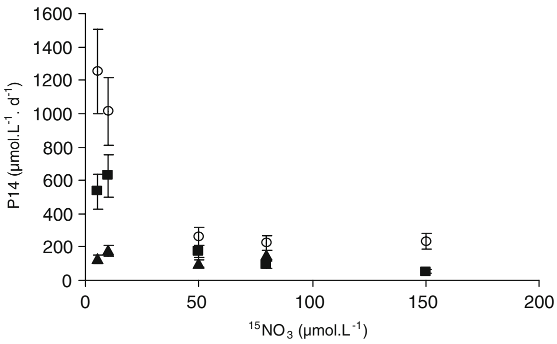

Fig. 7 Comparison of the N_2 production (P14) from marine sediment when denitrification and Anammox processes co-occured (means ± 1 SD) using the Nielsen's original IPM (○) and corrected values using R14 derived from the V method ($\mathrm{R14}_\mathrm{V}$) (Risgaard-Petersen et al. 2004) (▲) or $\mathrm{R14}_{\mathrm{N_2O}}$ method (Trimmer et al. 2006) (■) for as function of the $^{15}NO_3$ input in the system

concentrations (higher than 50 µM in this study) in contrast to that observed for lower $^{15}NO_3$ inputs. We can also notice that the range in which corrected P14 was independent of the $^{15}NO_3$ input was larger when the $R14_V$ method was used.

Finally, the balance between Anammox and denitrification in this total N_2 production can be calculated according to Eq. 26 allowing the Ra calculation:

$$\begin{aligned} Ra &= \frac{A28}{P14} = \frac{R14(P29 - 2R14 \times P30)}{R14(P29 + 2P30)} \\ &= \frac{P29 - (2R14 \times P30)}{P29 + 2P30}. \end{aligned} \tag{33}$$

Thus the use of $R14_V$ as input in Eq. 33 permits to evaluate that Anammox process is responsible of 54% of the N_2 production in the sampling site.

To confirm the occurrence of Anammox in these sediments and to compare rates obtained by direct measurement after $^{15}NH_4$ amendment and those calculated from the modified IPM slurries, sediment collected at Arcachon in March 2005, were spiked in parallel with $^{15}NO_3^-$ or $^{15}NH_4^+$ plus $^{15}NO_3^-$ and the production of $^{29}N_2$ was determined (Fig. 8). The Anammox rate reached 38.7 ± 1.9 vs. 42.2 ± 19.2 µmol L^{-1} D^{-1} using $^{15}NH_4^+$ or $^{15}NO_3^-$ modified IPM methods, respectively. Overall, there was a good agreement between the rates of anaerobic ammonium oxidation measured with labelling of either the NH_4^+ or the NO_3^- pool.

Determination of total denitrification taking into account N_2O accumulation

The original version of the IPM focussed on N_2 formation and neglected the formation of N_2O. In these conditions, the total denitrification capability is underestimated and important ecological information on N_2O fluxes disregarded. In a theoretical approach, Master et al. (2005) suggested to include the isotopic composition and the rates of N_2O production in the estimation of total denitrification rate. We have then decided to introduce in Dtot the production of N_2O via denitrification i.e. $^{44}N_2O$, $^{45}N_2O$ and $^{46}N_2O$ molecules corresponding to P44, P45 and P46, respectively.

According to Master et al. (2005), $^{45}N_2O$ and $^{46}N_2O$ molecules will be produced with frequencies similar to those of $^{29}N_2$ and $^{30}N_2$.

$$\frac{P29}{2 \times P30} = \frac{P45}{2 \times P46} \tag{34}$$

Including N_2O production, D#14 (from Eq. 7) can be now written:

$$D^{\#}14 = D15 \times \frac{P45 + P29}{2 \times (P46 + P30)} \tag{35}$$

with

$$D^{\#}15 = P29 + 2P30 + P45 + 2P46. \tag{36}$$

From these equations, we can calculate the $D^{\#}$tot which represents a more accurate estimation of denitrification capacity by including the N_2O production. The ratio between Dtot/$D^{\#}$tot expressed the percentage of complete denitrification. This ratio has been determined in Arcachon bassin in March 2005 for various $^{15}NO_3$ amendments. The results presented in Fig. 9 clearly showed a strong effect of the $^{15}NO_3$ concentration on the percentage of observed complete denitrification.

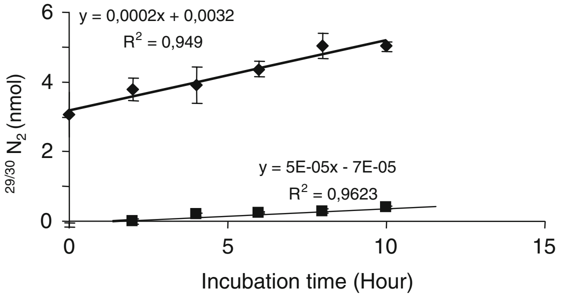

Fig. 8 Kinetics of $^{29}N_2$ (◆) and $^{30}N_2$ (■) production in marine sediment amended with 15-ammonium and unlabelled nitrate

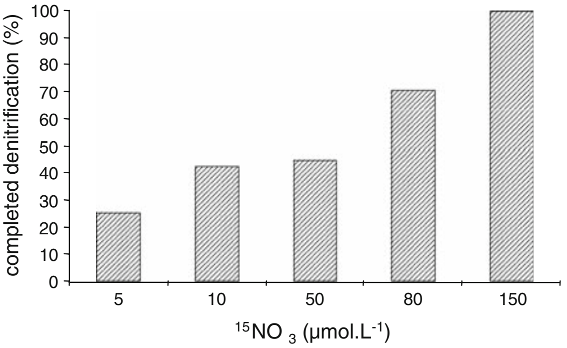

Fig. 9 Percentage of complete denitrification as function of $^{15}NO_3$ in sediment sample from Arcachon (April 2005)

Discussion and conclusions

The conventional sequence of organic nitrogen to N_2 in marine sediment involves ammonification, nitrification, DNRA and denitrification. The quantification of denitrification in these ecosystems is of major importance since until today denitrification was considered as one of the largest sinks for fixed nitrogen in marine budget (Codispoti et al. 2001; Herbert 1999). The Anammox process, was initially discovered in wastewater bioreactors (Mulder et al. 1995; Vandegraaf et al. 1995) constitute a shunt in the conventional sequence, since nitrogen originating from ammonium is paired with nitrogen originating from nitrate (or nitrite) which lead to anaerobic oxidation of ammonium into N_2 (Strous et al. 1999). Recently, Anammox was reported to account for 8% in estuarine and coastal marine sediments (Trimmer et al. 2003) and until as much as 24–67% of N_2 production in continental shelf sediment (Risgaard-Petersen et al. 2004; Thamdrup and Dalsgaard 2002). One of the basic assumptions of the original IPM is that N_2 is solely produced by denitrification and the co-existence of denitrification and Anammox activities thus constitutes a violation of one of the assumption on which IPM is based. The IPM has been largely used in numerous in situ studies during the last decade. A review of this method, its applications and its limitations has been recently published (Steingruber et al. 2001).

Three major limitations in the use of IPM deserve our attention in the present work.

Firstly, the requirement of sufficient $^{15}NO_3$ amendment in order to obtain denitrification rates independent of the amount of input of tracer. This point was explained by the limitation of the approximation of the binomial law for low $^{15}NO_3$ concentrations. For Nielsen (1992) the presence of low amount of $^{15}NO_3$ in the incubation system favours the probability of meeting between two $^{14}NO_3$ and thus could allow a $^{28}N_2$ production higher than that of $^{30}N_2$ and in consequence could lead to underestimation of denitrification rates calculated with IPM. However, from our experience, we have shown that the use of the binomial law induces an error since this approximation doesn't take into account the changes in the initial stocks of nitrate leading to an overestimation of the denitrification rates when insufficient amount of $^{15}NO_3$ were added in the incubation systems.

Furthermore, IPM considers that the reaction of denitrification is always complete and thus the entire nitrate stock is reduced to N_2. However, we have observed that the completion of denitrification reaction depends on the amount of nitrate amendment in the experimental system. In the presence of low nitrate input the percentage of incomplete denitrification is maximal and thus the rate of denitrification was underestimated. Whatever the reason, it is clear that the insufficient $^{15}NO_3$ input lead to erroneous denitrification measurements. In most of the studies of the literature, denitrification rates were determined in the presence of only one $^{15}NO_3$ concentration, depending of the paper the $^{15}NO_3$ was added from a minimum of 10 μM to a maximum of 400 μM without explanation, the final $^{15}NO_3$ concentration ranging between 5 and almost 100%. The biases induced in the presence of lower $^{15}NO_3$ spikes could be easily avoided by testing various different amounts of $^{15}NO_3$ in sediment overlying water. In literature, the production of $^{29}N_2$ and $^{30}N_2$ was determined in two ways: (i) by following these productions over the time (Bonin and Michotey 2006; Jensen et al. 1996a; Lohse et al. 1996; Nielsen 1992) or (ii) by determining the production at the end of the incubation (Cabrita and Brotas 2000; Christensen et al. 2000; Welsh et al. 2000). Both approaches presented advantages and limitations. The former, is generally performed in slurry incubation systems, and presents the advantages to control whether these productions occurred linearly with time and to take into account the heterogeneity of the sediment since experimental systems were sacrificed at the time of kinetic interval. The second was performed in intact sediment cores and present the advantage to maintain the microbial stratification; however no information is obtained about the linearity of $^{29}N_2$ and $^{30}N_2$ productions since the incubations were stopped after an arbitrary chosen incubation time by mixing the whole sediment with the overlaying water after $ZnCl_2$ addition. Furthermore from our experience, we underline the absolute necessity to test different concentrations of $^{15}NO_3$ before each experiment. As recommended in the original paper of Nielsen, incubations for both different times and for different concentrations of $^{15}NO_3$ must be employed, Nielsen suggests that

$^{15}NO_3$ concentrations are high enough when D14 becomes independent of the $^{15}NO_3$ concentrations.

Master et al. (2005) have modified the original IPM in order to include N_2O production. This approach leads additional valuable information on denitrification and on the gaseous nitrogen cycle. However our results show that the N_2O production via denitrification is dependent on the amount of nitrate amendment. At this stage it is still not clear whether these results reflected (1) a true partial denitrification in this ecosystem that we are unable to detect with partial inhibition methods (Bonin and Michotey 2006) in the presence of various partial pressures in acetylene or (2) a lack of sensitivity of the analytical procedure of the isotopic composition of the N_2O in the presence of low nitrate concentrations, more researches are needed in order to clarify this issue.

The second point concerns the dilution of the initial $^{15}NO_3$ labelling during the incubation time when $^{14}NO_3$ is produced via nitrification of $^{14}NH_4$. Indeed, it was not clear whether an underestimation of denitrification in the case of coupled nitrification–denitrification can be excluded (Steingruber et al. 2001). In the simulations presented in the present study, the ^{15}N percentage in nitrate decreased during the simulated incubation due to the dilution with ^{14}N from nitrification, but in the presence of recommended $^{15}NO_3$ concentrations, during most of the reaction the random pairing could have occurred and $^{15}NO_3$ would have been combined to $^{14}NO_3$ to form $^{29}N_2$. Our results clearly show that the occurrence of nitrification does not question the original method of IPM of Nielsen based on the binomial law of the N_2 species. Only when the percentage of $^{15}NO_3$ input reached values close to 100%, P29 as well as D14 were never equal to zero. Under these conditions, one of the advantages of the IPM is that both nitrate diffusing from the overlaying water and coming from nitrification within the sediment can be quantified. In conjunction with quantification of ^{15}N dilution of NO_3, nitrification can be estimated in the same experimental system (Bonin and Michotey 2006; Glibert et al. 1982) in order to be sure that the rate of coupled nitrification-denitrification is in accordance with the rate of nitrification.

The last point concerns the co-existence of denitrification and Anammox activities. In a theoretical approach Risgaard-Petersen et al. (2003) have shown that the co-existence of denitrification and Anammox activities causes an overestimation of the N_2 production calculated by IPM. They calculated that the overestimation of the total N_2 production rates were from 2.5% to 82% relative to the revised estimates when Anammox accounted for 6% and 70% of the N_2 production, respectively. For Risgaard-Petersen et al. (2003), this Error depends on the proportion of Anammox (Ra) but also on the percentage of labelling (R14), whereas from our calculations, the error seems only to depend on the proportion of Anammox (Ra). Risgaard-Petersen et al. suggest that IPM does not seriously overestimate N_2 production, because Anammox accounted for less than 6% of N_2 production in most of sediment. However, the error calculated with the method developed in the present paper showed that the true error in the total N_2 production due to the presence of Anammox is higher than that estimated by Risgaard-Petersen (2003). This fact reinforced the necessity to determine the part of Anammox in the total production of N_2 for more accurate estimate of denitrification rates. Experiments with different amounts of $^{15}NO_3$ and mainly in the presence of high amendment in order to reach $^{15}NO_3$ percentage close to 100% revealed the potentiality of Anammox activity in the sample and allowed the determination of $R14_V$ value necessaries to correct the total N_2 production rates when denitrification and Anammox processes co-occurred. The direct technique ($R14_{N_2O}$) developed by Trimmer et al. (2006) is based on the assumption that the isotopic composition of $^{15}N–N_2O$ directly reflects the ratio of $^{14}NO_3$ to $^{15}NO_3$, i.e. R14 (Trimmer et al. 2006). The robustness of the $R14_{N_2O}$ method hinges on denitrification being the only significant source of $^{15}N–N_2O$ from $^{15}NO_3$. We have compared the two latter approaches, and showed that both were not significantly different when sufficient amount of $^{15}NO_3$ were added. Trimmer et al. have reported that the $R14_V$ method was ineffective in natural intact sediment and they recommended using the $R14_{N_2O}$, moreover they showed that the contribution of Anammox to N_2 production is greater than that measured using slurries. In our study, for low $^{15}NO_3$ input, the ratio P29 on 2P30 was not equal to the ratio P45 on 2P46 showing that denitrification was not the only source of N_2O under these incubation conditions. At this stage, it is difficult to attribute the production of N_2O to one of the potential productive processes: nitrification, nitrifier denitrification, heterotrophic nitrification (Schmidt et al. 2002; Shaw et al. 2006; Wrage et al. 2001) or DNRA (Bonin 1996; Senga et al. 2006; Smith and Zimmerman 1981). Recently it has been reported that Anammox bacteria can also produce N_2O during nitrate reduction (Kartal et al. 2007). These latter authors have also shown the role of RDNA by Anammox bacteria which would convert a significant part of $^{15}NO_3$ to $^{15}NH_4$, this would lead to the production of $^{30}N_2$. In consequences, the contribution of Anammox bacteria to N-losses would be thus underestimated.

According to the present knowledge, and the low number of publications reporting Anammox rates in marine sediment, it is essential to quantify systematically this process in all studies using IPM. In order to ensure a better quantification of the denitrifying activities but also to increase the knowledge of the ecological importance of the Anammox process actually studied in not enough ecosystems. IPM, in spite of its limitations, is a powerful technique for quantifying denitrification rates if it is carefully applied and if the results are critically evaluated. In this paper, we purpose a decisional flow chart (Fig. 10) allowing to easily interpret the P29 and P30 profiles according to the amount of added $^{15}NO_3$ in order to choose the most suitable method for P14 calculation. The profiles of production of $^{29}N_2$ and $^{30}N_2$ in the presence of Anammox or nitrification alone are rather similar. Indeed, in both cases, the

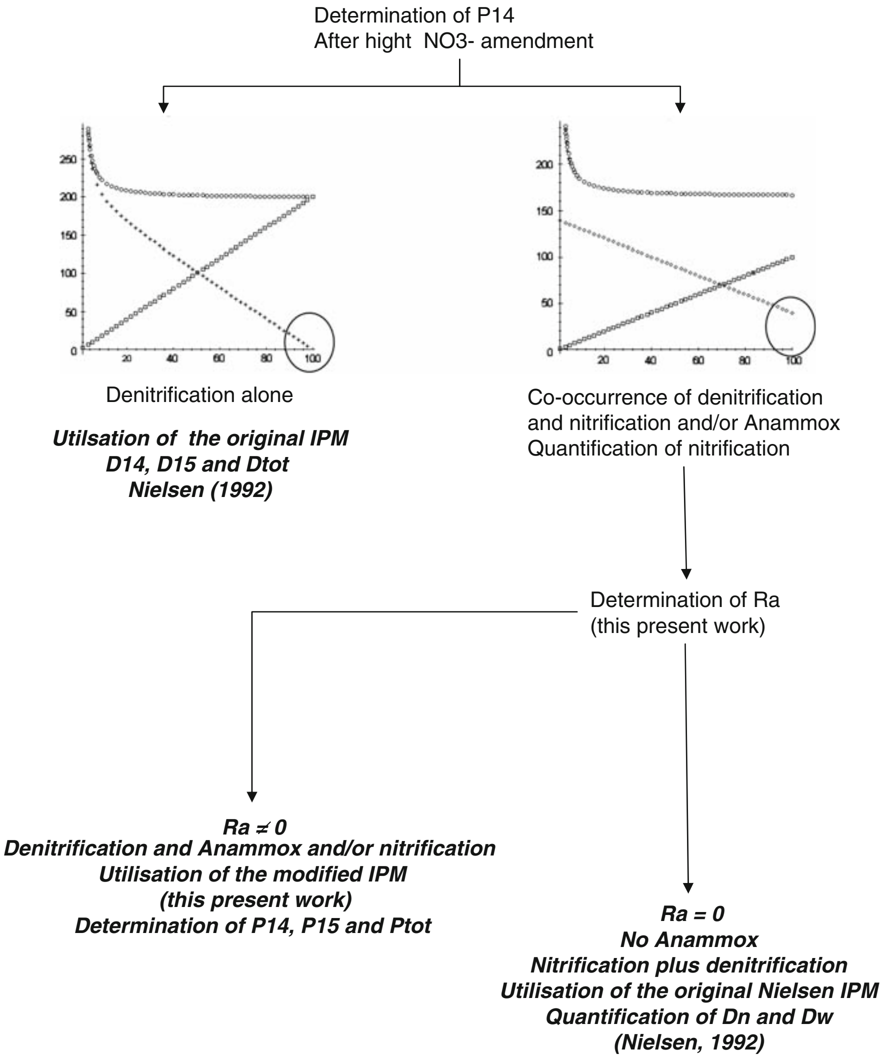

Fig. 10 Summarize of the different calculations methods and the conditions for their utilisations in the determination of denitrification and Anammox activities

formation of $^{29}N_2$ is not null for a labelling percentage close to 100%. In conclusion, the profiles are different when denitrification activity alone is present in the samples and when this activity is accompanied by a nitrifying activity or Anammox or both. The Ra determination (different to zero or not) allows to determine the presence of Anammox or not. The presence of nitrification activity in the sample could be detected by complementary methods of measurement such as isotopic dilution of the initial $^{15}NO_3^-$ labelling or inhibition technique with chlorate as it was done in this study. In the presence of Anammox, the use of modified IPM method proposed in this work permits to estimate the total production of N_2, the denitrifying activity and the Anammox activity.

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