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EXPERIMENTAL DETERMINATION OF NITROGEN KINETIC ISOTOPE FRACTIONATION: SOME PRINCIPLES; ILLUSTRATION FOR THE DENITRIFICATION AND NITRIFICATION PROCESSES

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KEY WORDS

Denitrification Nitrification Nitrogen isotope fractionation Nitrogen-15 natural abundance

SUMMARY

A few principles relative to the presentation and use of nitrogen stable isotopic data are briefly reviewed. Some classical relationships between the isotope composition of a substrate undergoing a single-step unidirectional reaction, are introduced.

They are illustrated through controlled experiments on denitrification in a soil, and through nitrification by pure cultures of *Nitrosomonas europaea*. In the latter case, the isotope fractionation is calculated from the isotopic composition of the residual substrate, then of the product and the result is shown to be statistically the same for the two procedures.

The isotopic enrichment factor for denitrification is -29.4 ± 2.4‰ at 20°C, and -24.6 ± 0.9‰ at 30°C; for nitrification this factor is -34.7 ± 2.5‰ under the experimental conditions employed.

INTRODUCTION

Many agricultural and biochemical studies use isotopic tracers. Interpretations of variation in stable isotope abundance require a precise knowledge of the isotope fractionation occurring during physical, chemical or biochemical transformations undergone by the compound in question. We present here a method for the expression and calculation of kinetic isotope fractionation associated with transformations in the nitrogen cycle. Such methods have been standard in isotope geochemistry for H, O, C, S for several decades. The need for such a standard method comes from the variety of ways of presenting and discussing data published by agronomists and soil scientists using ^{15}N natural variations for the study of the nitrogen cycle in terrestrial ecosystems.

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TERMINOLOGY

Expression of isotope data. Standard

^{15}N abundance is expressed in atom per cent by the ratio:

$$A = \frac{^{15}N}{^{15}N + ^{14}N} \cdot 100$$

The value of A for atmospheric nitrogen is 0.3663 ± 0.0004 atom $^{15}N\%$[10].

If, as a convention, the abundance of the heavy isotope is referred to that of the lighter one, a dimensionless parameter may be defined, the isotope ratio R. For nitrogen: $R = {}^{15}N/{}^{14}N$. Due to the small abundance of ^{15}N in atmospheric nitrogen and the small variations of ^{15}N abundance in the nitrogen cycle, the following approximation holds:

$$R = \frac{^{15}N}{^{14}N} \simeq \frac{^{15}N}{^{15}N + ^{14}N}$$

Absolute abundance of ^{15}N cannot be measured with very great precision, but small differences in isotope ratio between two compounds are easily measured with high accuracy by using mass spectrometers fitted with double ion-collection, and double inlet systems equipped for rapid switching between standard and samples[17,18]. Technical reasons have led to the use of the so-called 'δ' notation to express results. For nitrogen, this relative unit is defined as:

$$\delta^{15}N = \frac{R_{sample} - R_{standard}}{R_{standard}}$$

As natural variations of ^{15}N are small, a common convention is to express δ in parts per thousand:

$$\delta^{15}N‰ = \delta^{15}N \times 1000$$

A $\delta^{15}N$ value of $+10$ ‰, for example, corresponds to a sample with an isotope ratio one per cent higher than that of the standard against which it is expressed. Some authors[11,20] use a slightly different expression:

$$\delta_a{}^{15}N = \left[\frac{\{^{15}N/(^{15}N + ^{14}N)\}_{sample}}{\{^{15}N/(^{15}N + ^{14}N)\}_{standard}} - 1 \right] \cdot 1000$$

It can be easily shown that the two modes of calculation differ by only 0.25‰ for delta values of 65‰ which are rarely met within natural settings. The two definitions can therefore be considered as equivalent. In order to be comparable from one laboratory to another, these measurements need to be done relative to a common standard. Such a standard should present several qualities; namely, perfect homogeneity and chemical and isotopic stability. Moreover, the isotopic composition of the standard must not be very different from the measured natural samples, in order to minimize systematic errors in the mass spectrometry, which increase with the 'isotopic distance' between standard and sample.

For these reasons, a standard which is abundant in nature and of simple chemical form (and coming from a medium where kinetic processes are rapid in order for the material to be well-mixed) is prefered.

We have carried out a series of measurements on atmospheric nitrogen from various origins in France and have assessed the constancy of its isotopic composition within mass spectrometer analytical precision: the standard deviation on 27 measurements is $\pm$ 0.026‰ (δ units). Therefore, the isotopic composition of atmospheric N_2 may be considered as practically constant and thus a reliable standard.

Expression of kinetic isotope fractionation. Isotope enrichment

Partition of isotopes between two compounds containing the same element (or between two phases) with different isotopic ratio is called isotope fractionation. This is due to different energy characteristics of the light and heavy molecule, from which comes a difference in the probability of reaction [2].

Consider a single step unidirectional reaction (*i.e.* the so-called 'kinetic reaction'):

substrate $\rightarrow$ product: $S \rightarrow P$

Without any hypothesis concerning the intimate mechanism and reaction order, we represent the kinetic fractionation factor $\alpha_{p/s}$, as $\alpha_{p/s} = R_{pi}/R_s$ where R_{pi} is the isotope ratio of the increment of product which appears in an infinitely short time at time t, and R_s the isotope ratio of the substrate at the same time.

(Some authors [4, 9, 19] use the inverse ratio, *i.e.* $\alpha_{s/p}$).

We have:

$$\alpha_{p/s} = \frac{d^{15}N_p/d^{14}N_p}{^{15}N_s/^{14}N_s} \qquad (1)$$

Since $d^{15}N_p = -d^{15}N_s$ and $d^{14}N_p = -d^{14}N_s$, one gets

$$\alpha_{p/s} = \frac{d^{15}N_s}{{}^{15}N_s} \Big/ \frac{d^{14}N_s}{{}^{14}N_s} \tag{2}$$

If $\alpha_{p/s}$ is considered *a priori* as constant during the progress of the reaction, integration of (2) gives:

$$(\alpha_{p/s}) \ln \left\{ \frac{{}^{14}N_s}{{}^{14}N_{s,0}} \right\} = \ln \left\{ \frac{{}^{15}N_s}{{}^{15}N_{s,0}} \right\}, \tag{3}$$

where ${}^{15}N_{s,0}$ and ${}^{14}N_{s,0}$ stand for the quantity of the two isotopes at time zero.

Let f be the unreacted fraction of substrate at time t, and N_s the total quantity of nitrogen at time t ($N_s = {}^{14}N_s + {}^{15}N_s$).

$$f = \frac{N_s}{N_{s,0}} = \frac{{}^{14}N_s + {}^{15}N_s}{{}^{14}N_{s,0} + {}^{15}N_{s,0}}$$

In natural systems, ${}^{15}N$ abundance varies little around the value 0.3663 atom per cent (0.3663 ± 0.0183) and the approximation ${}^{14}N \simeq {}^{15}N + {}^{14}N$ is valid, so: $f \simeq {}^{14}N_s/{}^{14}N_{s,0}$.

Equation (3) becomes:

$$(\alpha_{p/s} - 1) \ln f = \ln \frac{R_s}{R_{s,0}}$$

or

$$\frac{R_s}{R_{s,0}} = f^{(\alpha_{p/s} - 1)} \tag{4}$$

In isotope geochemistry, this equation is classically termed the 'Rayleigh' equation. It was derived by Lord Rayleigh for the case of fractional distillation of mixed liquid.

With the δ‰ notation,

$$(\alpha_{p/s} - 1) \ln f = \ln \frac{10^{-3}\,\delta_s + 1}{10^{-3}\,\delta_{s,0} + 1} \tag{5}$$

As the fractionation factor generally differs by less than 5% from unity, it can be

expressed, as an analogy with the delta notation, as:

$$\alpha_{p/s} = 1 + 10^{-3}\,\varepsilon_{p/s} \tag{6}$$

$\varepsilon_{p/s}$ is called the 'per mil enrichment factor' (either positive or negative) of the product relative to the substrate. This notation allows easy comparisons.

From (5) and (6), one gets:

$$\varepsilon_{p/s} = \frac{10^3 \cdot \ln \dfrac{10^{-3}\,\delta_s + 1}{10^{-3}\,\delta_{s,0} + 1}}{\ln f} \tag{7}$$

As $\ln(1 + u) \simeq u$ when u is small relative to 1 and $\ln \dfrac{1+u}{1+v} \simeq u - v$ when u and v are small relative to 1, for most δ values, the approximation:

$$\varepsilon_{p/s} = \frac{\delta_s - \delta_{s,0}}{\ln f} \text{ holds} \tag{8}$$

So in the hypothesis where α (and therefore ε) is constant during the reaction ($f \to 0$), the isotope composition of the substrate is a linear function of ln f and the enrichment factor ε is the slope of the straight line which passes through the origin ($\delta_s - \delta_{s,0} = 0$ for $\ln f = 0$).

A priori, the simplified relation (8) holds for $|\varepsilon|$ values $< 20‰$ and for $\delta_{s,0}$ values not too different from zero. Equation (7) is to be preferred in more extreme cases.

In a closed system ($P + S = S_0$), the isotopic abundance of the *accumulated* or *instantaneous* product is easily described:

Accumulated product

One has

$$f \cdot R_s + (1 - f)\overline{R_p} = R_{s,0} \tag{9}$$

which is the isotope balance equation, where $\overline{R_p}$ is the isotopic ratio of the accumulated product. Combining equations (4) and (9) gives:

$$\overline{R_p} = R_{s,0} \cdot \frac{1 - f^{\alpha_{p/s}}}{1 - f} \tag{10}$$

As equation (9) may be written

$$f \cdot \delta_s + (1 - f)\overline{\delta_p} = \delta_{s,0}$$

combination with (8) gives:

$$\overline{\delta_p} = \delta_{s,0} - \varepsilon_{p/s} \frac{f \ln f}{1 - f} \tag{11}$$

The expression $\frac{f \ln f}{1 - f}$ tends towards -1 when f tends towards 1, thus the isotopic composition of the first product formed differs from the isotopic composition of the initial substrate by ε:

$$\overline{\delta_{p(0+dt)}} = \delta_{s,0} + \varepsilon_{p/s} \tag{12}$$

Instantaneous product

Equation:

$$\alpha_{p/s} = R_{pi}/R_s$$

gives:

$$\alpha_{p/s} = \frac{\delta_{pi} + 1000}{\delta_s + 1000}$$

or

$$\delta_{pi} = \delta_s \left(1 + \frac{\varepsilon_{p/s}}{1000}\right) + \varepsilon_{p/s}$$

If $\varepsilon_{p/s}$ is small relative to 1000, one gets the approximation

$$\delta_{pi} \simeq \delta_s + \varepsilon_{p/s} \tag{13}$$

So, the curve for δ_{pi} as a function of f is derived from the curve for δ_s by a translation of $\varepsilon_{p/s}$ on the δ coordinate.

Fig. 1 shows these variations for the theoretical case $\varepsilon_{p/s} = -20‰$ and $\delta_{s,0} = 0‰$, with the approximation of Equation (8)[12,16].

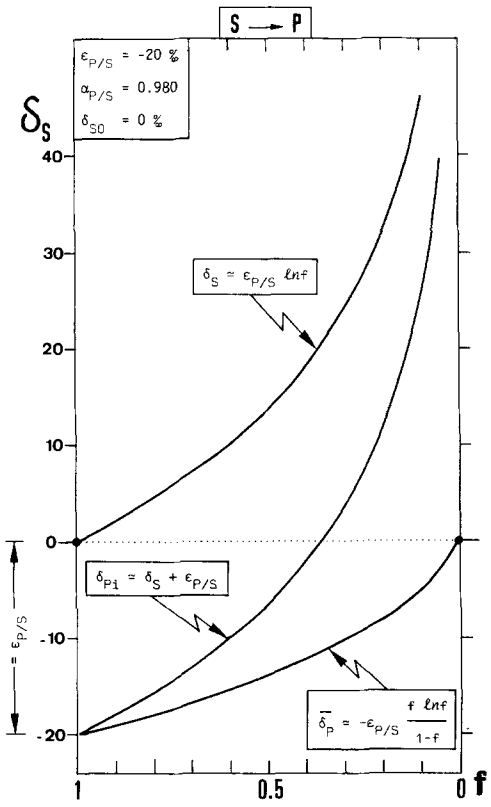

Fig. 1. Theoretical change in substrate and product isotopic composition in a single step unidirectional reaction. The approximation $\delta_s - \delta_{s,0} \simeq \varepsilon \ln f$ has been used with $\varepsilon_{p/s} = -20‰$ and $\delta_{s,0} = 0‰$. δ_s stands for the isotopic composition of the substrate, δ_p for the accumulated product and δ_{pi} for the instantaneous product.

In the case when the remaining substrate may be considered as an infinite reservoir with regards to the quantity of product (example: biological dinitrogen fixation by legumes, see ref. [15]), the ratio R_s of the substrate does not change, f is always $\simeq 1$ and one gets:

$$\delta_{pi} = \overline{\delta_p} = \delta_p \simeq \delta_{s,0} + \varepsilon_{p/s} = \delta_s + \varepsilon_{p/s}$$

More rigorously,

$$\alpha_{p/s} = R_p/R_s$$

whence,

$$\varepsilon_{p/s} = \frac{\delta_p - \delta_s}{\delta_s + 1000} \cdot 10^3$$

If δ_s is small with regard to 1000:

$$\varepsilon_{p/s} \simeq \delta_p - \delta_s$$

These relations are independent of the kinetics of the reaction. However, for a first order reaction, the following equations hold:

$$^{14}N_s = {}^{14}N_{s,0}\, e^{-kt}$$
$$^{15}N_s = {}^{15}N_{s,0}\, e^{-k't}$$

where k and k′ are respectively the rate constants for the reactions involving lighter and heavier isotopes, respectively.

Therefore, $f = e^{-kt}$, and

$$\frac{^{15}N_s}{^{14}N_s} = \frac{^{15}N_{s,0} \cdot e^{-k't}}{^{14}N_{s,0} \cdot e^{-kt}}$$

whence

$$R_s = R_{s,0}\, f^{(k'/k - 1)}$$

or:

$$\ln \frac{10^{-3}\delta_s + 1}{10^{-3}\delta_{s,0} + 1} = \left(\frac{k'}{k} - 1\right) \ln f \qquad (14)$$

Comparison of equations (5) and (14) shows that in the case of first order reactions, $\alpha_{p/s}$ is the ratio of the rate constants of the heavy to the light isotope. The same formulation has been given by Tong and Yankwich[22] which call r = k/k′, a factor named β by various authors[5,8,21]:

$$\beta = 1/\alpha_{p/s} = \alpha_{s/p}.$$

Equations (5) and (14) are identical with those of Tong and Yankwich[22] and are easier to use.

EXPERIMENTAL RESULTS

They are related to the experimental determination of the isotopic enrichment factor during denitrification in soils samples and nitritation by pure *Nitrosomonas europaea* cultures.

1 *Experimental determination of the isotopic fractionation during denitrification in soils*

Selected soil samples were placed in experimental conditions favouring denitrification.

Materials and methods

100 ml of distilled water containing 0.1 ml N-serve (nitrification inhibitor) was added to 100 g of dried soil. After a few days of preincubation in order to eliminate the nitrates and nitrites (often present in the soil through nitrification), 10 ml of solution containing about 20 mg $N\text{–}NO_3^-$ was added to the soil. The flasks (600 ml) were placed under a helium atmosphere, of which 50 ml was then replaced by the same volume of acetylene in order to stop denitrification at the N_2O step [7]. N_2O is easily measured through gas chromatography and will give the extent of reaction (f). The unreacted nitrate was extracted with 1 *N* KCl and measured on a Technicon analyser. Isotope analyses follow the protocol described by Mariotti and Létolle [14].

At each step of NO_3^- reduction, nitrite was also measured. It represents an intermediary step between NO_3^- and N_2O:

$$NO_3^- \underset{1}{\rightarrow} NO_2^- \underset{2}{\rightarrow} N_2O \underset{3}{\rightarrow} N_2$$

NO_2^- was never detected during the experiment. It may be concluded that step 1 was rate controlling for the overall reaction. Any step in the further reduction of NO_2^- cannot lead to an isotope effect since all the NO_2^- was reduced as rapidly as it was formed.

Under these conditions, the measured isotope fractionation will correspond to the nitrate-nitrite step (step 1). Here, the two-step unidirectional reaction may be reduced to a single step unidirectional one, which would not be the case if nitrites were present [3].

Results

Changes in the concentration of NO_3^- and N_2O, as well as the isotope composition of remaining substrate (expressed as $10^3 \ln \{10^{-3}\,\delta_s + 1\}/\{10^{-3}\,\delta_{s,0} + 1\}$) are reported on Figures 2 and 3 respectively (two different temperatures: 20°C and 30°C).

The sum, $N\text{–}NO_3^- + N\text{–}N_2O$, is constant within analytical precision, which is the expected case for a closed system ($P + S = S_0$).

Correlation coefficients for the line $Y_1 = \delta_s - \delta_{s,0}$ and $Y_2 = 10^3 \ln \{10^{-3}\,\delta_s + 1/10^{-3}\,\delta_{s,0} + 1\}$ as a function of ln f have been calculated (Table 1).

Correlation factors are significant at the probability level 0.01. This indicates

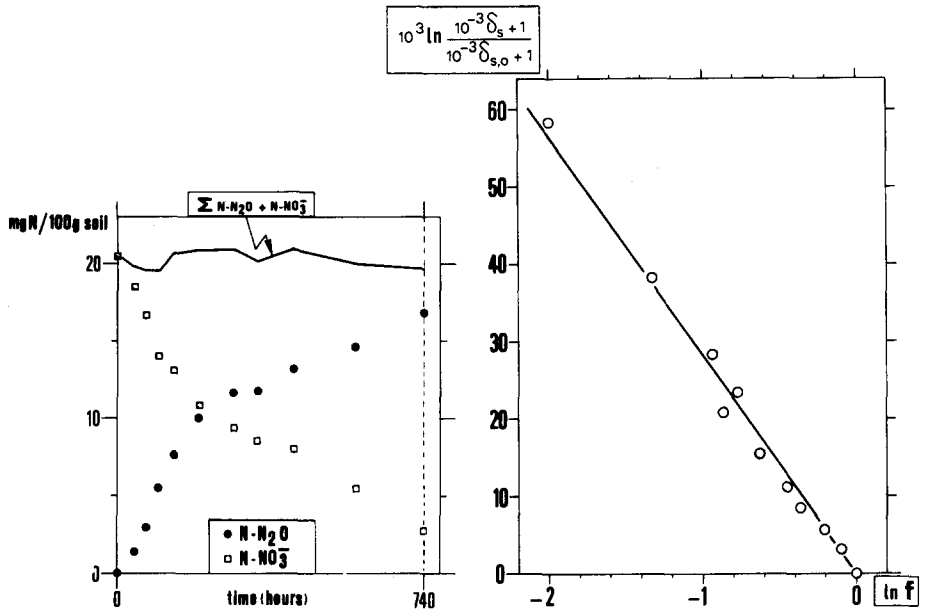

Fig. 2. Chemical and isotopic change in NO_3^- during denitrification; change in the concentration of the product N_2O and of the sum: $\Sigma(N\text{–}NO_3^- + N\text{–}N_2O)$. Incubation temperature was 20°C.

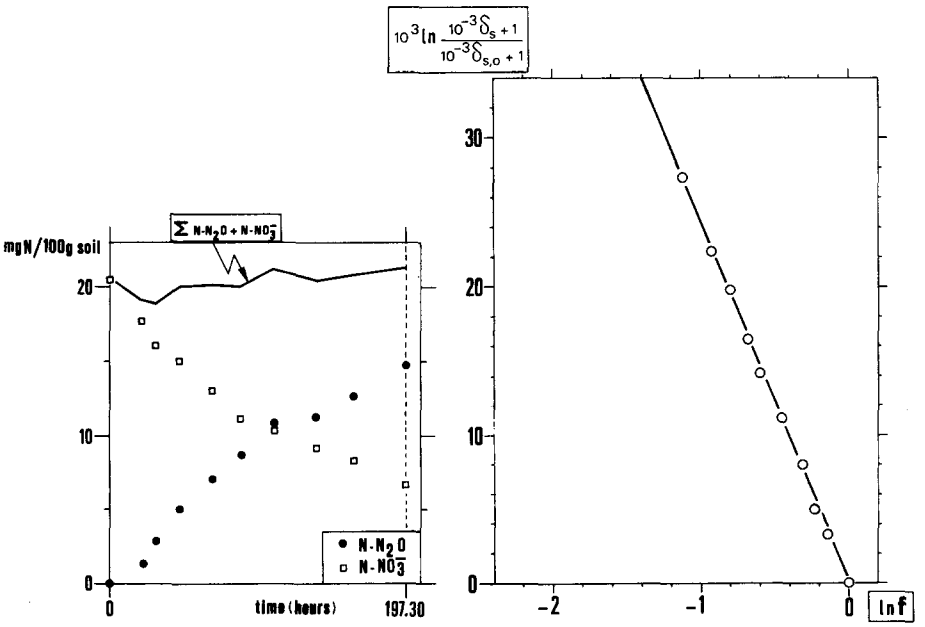

Fig. 3. Chemical and isotopic change in NO_3^- during denitrification; change in N_2O concentration and of the sum: $\Sigma(N\text{–}NO_3^- + N\text{–}N_2O)$. Incubation temperature is 30°C.

clearly that the assumption of the constancy of the isotope fractionation factor is valid, at least as far as the extent of reaction that has been studied, and therefore relations (7) and (8) are valid. The confidence limit for the slopes, ts_b, ($p = 0.05$) has been calculated. This gives the confidence limit for $\varepsilon_{p/s}$ experimentally measured. As these lines must pass at the origin, the slope was also calculated for the best fit of a line passing at this point. The slope is given by

$$m = \frac{\Sigma XY}{\Sigma X^2}$$

We have also tested the hypothesis that the intercept of the straight line calculated with experimental data is zero. Data are given in Table 1.

The difference of $\varepsilon_{p/s}$ values obtained either with equation (7) or approximation (8) is small and distinctly less than the confidence limit on $\varepsilon_{p/s}$. This confidence limit has been estimated for a series of 12 measurements of the denitrification fractionation factor, carried out under the same experimental conditions as described above (Mariotti *et al.*, to be published). Its mean value is 2.7‰ and may

Table 1. Regression parameters for experimental data of NO_3^- denitrification. (Step $NO_3^- \rightarrow N_2O$), reported on Figs. 2 and 3

Temperature	$Y = (\varepsilon \pm ts_b) \ln f + \beta$	Slope of the straight line passing through origin $\beta = 0$; $\varepsilon = \frac{\Sigma XY}{\Sigma X^2}$	Number of data	Correlation coefficient
20°C	$\delta_s - \delta_{s,0} = (-30.32 \pm 2.53) \ln f - 1.37$*	−29.12	11	−0.994**
	$10^3 \ln \frac{10^{-3}\delta_s + 1}{10^{-3}\delta_{s,0} + 1} = (-29.37 \pm 2.38) \ln f - 1.11$*	−28.39	11	−0.994**
30°C	$\delta_s - \delta_{s,0} = (-25.07 \pm 0.95) \ln f - 0.21$*	−24.79	10	−0.999**
	$10^3 \ln \frac{10^{-3}\delta_s + 1}{10^{-3}\delta_{s,0} + 1} = (-24.63 \pm 0.93) \ln f - 0.15$*	−24.43	10	−0.999**

* Intercept not statistically different from zero.
** Significant at $p = 0.01$.

reach 4.5‰. Due to analytical imprecisions, a relative precision of 10% of the value of $\varepsilon_{p/s}$ may be obtained at best.

One may conclude from the above experiments that the isotopic enrichment factor during denitrification, in the described conditions, is -29.4 ± 2.4‰ at 20°C and -24.6 ± 0.9‰ at 30°C.

In these experiments, the isotope composition of the gaseous product (N_2O) was not measured. However, in nitrification experiments it was possible to use the isotopic composition of the product (NO_2^-) in order to get two independant determinations of the fractionation factor: one based on the isotopic composition of the substrate, NH_4^+, and a second based on the isotopic composition of the product, NO_2^-.

2 *Determination of the isotope fractionation factor during nitrification with* Nitrosomonas europaea

Material and methods

The study has been carried on a pure culture of *N. europaea* (Rothamsted collection). The growing medium has been described by Lewis and Pramer[13], the only difference being the ammonium sulphate concentration. A 1.25 fold concentration of the parent medium was added to four series of six flasks each, as follows: 100 ml in 500 ml Erlenmeyers flasks for series A, 200 ml in 1000 ml Erlenmeyers for series B and C, 400 ml in 2000 ml Fernbach flasks for series D. Before autoclave sterilization, water was added such that the four series would attain a final volume of: 125 ml (A), 250 ml (B and C) and 500 ml (D) respectively, when ammonium sulphate was added later.

The ammonium sulphate solution was prepared and sterilized separately and aseptically added to series A to D in order to get a decreasing concentration scale as shown on Table 2.

In each series, one control flasks was not inoculated. The other flasks were inoculated at 1% volume with a culture grown for 72 hours on the Lewis and Pramer[13] medium with a 10^{-2} *M* concentration of $(NH_4)_2SO_4$. All flasks, including the control flasks, were incubated and stirred at 28°C.

Staggered samples were taken out of each series in order to follow the nitrification process, nitrites being measured through the modified Griess-Islovay method[1]. The incubation was concluded at different times for each series, according to the extent of the reaction: from 39 h to 208 h for series C and D, from 39 h to 264 h for series B and from 48 h to 471 h for series A. After incubation the cultures were cooled, and $HgCl_2$ added. The samples were then frozen until the time of analysis. The chemical analyses were performed after distillation with MgO (NH_4^+), then with MgO + Devarda alloy (for NO_2^-), the NH_4^+ nitrogen from the distillate being measured with an Orion model 95-10 specific electrode.

Results

The determination of the isotopic enrichment factors was carried out separately on the four experiments with a different ammonium substrate concentration. The four values are not statistically different one from another. Therefore a single value can be proposed based on the 19 pairs of experimental values.

In these experiments, the bacterial biomass was growing. However, the quan-

Table 2. Data for nitrification experiment with *Nitrosomonas europaea*. Four experiments with different initial substrate concentrations are shown. The values $N\text{–}NH_4^+$ and $N\text{–}NO_2^-$ mg represents the quantity of nitrogen for these two ions present in the volume chosen for incubation

Experiment	$N\text{–}NH_4^+$ concentration mg/l	Volume of medium ml	Time hours	$N\text{–}NH_4^+$ mg	$\delta^{15}N$ NH_4^+	f	$N\text{–}NO_2^-$ mg	$\delta^{15}N$ NO_2^-	$\Sigma(N\text{–}NH_4^+ + N\text{–}NO_2^-)$ mg	$10^3 \ln \frac{10^{-3}\delta_s + 1}{10^{-3}\delta_{s,0} + 1}$	$\delta_s - \delta_{s,0}$	$\delta_p - \delta_{s,0}$	$-\frac{f \ln f}{1-f}$
A	350.0	125	0	43.8	5.1	1.000	0.4	—	44.2	0.00	0.0	—	—
			48	41.7	5.5	0.952	2.7	−24.7	44.4	0.40	0.4	−29.8	0.976
			87	30.8	15.4	0.703	7.9	−18.8	38.7	10.20	10.3	−23.9	0.834
			162	20.8	32.1	0.475	19.2	−17.3	40.0	26.51	27.0	−22.4	0.673
			246	20.8	33.1	0.475	19.2	−15.1	40.0	27.48	28.0	−20.2	0.673
			471	24.0	35.1	0.548	20.7	−22.5	44.7	29.41	30.0	−27.6	0.729
B	226.4	250	0	56.6	4.0	1.000	0.0	—	56.6	0.00	0.0	—	—
			39	55.7	5.4	0.984	4.0	−27.8	60.6	1.39	1.4	−31.8	0.992
			70	36.4	21.9	0.643	7.2	−10.3	43.6	17.67	17.9	−14.3	0.795
			117	13.4	56.1	0.237	26.4	−10.7	39.8	50.59	52.1	−14.7	0.447
			162	11.0	66.2	0.194	32.2	−10.8	43.2	60.11	62.2	−14.8	0.395
			264	7.0	79.1	0.124	37.5	− 7.1	44.5	72.14	75.1	−11.1	0.295
C	100	250	0	25.0	4.7	1.000	0.0	—	25.0	0.00	0.0	—	—
			39	22.9	10.6	0.916	3.1	−23.3	26.0	5.86	5.9	−28.0	0.957
			48	18.4	17.0	0.736	3.9	−17.2	22.3	12.17	12.3	−21.9	0.855
			70	6.8	49.2	0.272	9.2	−12.8	16.0	43.34	44.5	−17.5	0.486
			96	4.4	61.1	0.176	15.2	−13.5	19.6	54.62	56.4	−18.2	0.371
			208	1.3	—	0.052	20.7	4.7	22.0	—	—	0.0	0.162
D	66.2	500	0	33.1	1.3	1.000	0.0	—	33.1	0.00	0.0	—	—
			39	22.0	19.7	0.665	8.4	−23.2	30.4	18.21	18.4	−24.5	0.810
			48	13.8	41.9	0.417	16.1	−18.8	29.9	39.75	40.6	−20.1	0.626
			63	4.9	77.3	0.148	21.3	− 8.4	26.2	73.16	76.0	− 9.7	0.332
			72	3.4	87.0	0.103	21.6	− 5.6	25.0	82.12	85.7	− 6.9	0.261
			208	2.9	—	0.088	23.8	2.1	26.7	—	—	0.8	0.234

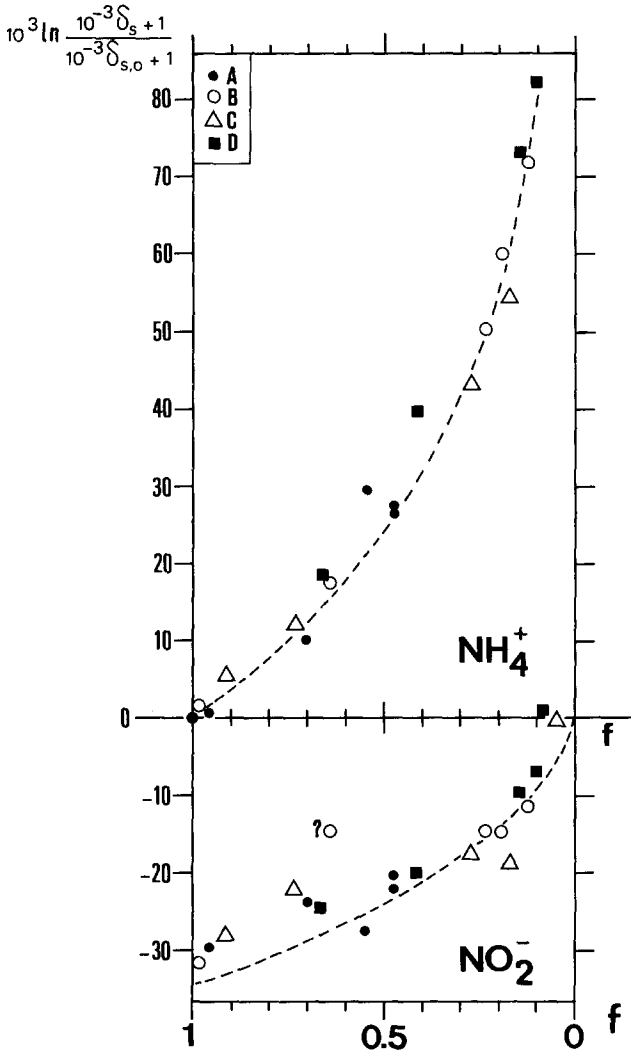

Fig. 4. Change in isotopic composition of substrate NH_4^+ and product NO_2^- as a function of unreacted fraction of substrate (f), during nitritation with *Nitrosomonas europaea*.

tity of nitrogen assimilated by the biomass was always negligible in comparison with the mineral species (NH_4^+ or NO_2^-). Data are presented on Table 2 and Figs. 4 and 5. Parameters of the regression line, using equation (7) are $\varepsilon_{p/s} = -34.7 \pm 2.5‰$ with the intercept value 1.8 which is not statistically different from zero. The correlation coefficient is 0.99. The same parameters calculated

with equation (8) are $\varepsilon_{p/s} = -36.2 \pm 2.6‰$; the intercept is 1.5 (not statistically different from zero) and the correlation coefficient, 0.99.

The isotopic enrichment factor and consequently the fractionation factor is therefore constant with the extent of reaction in the nitrogen concentration range from $2.5 \cdot 10^{-2} M$ to $5 \cdot 10^{-4} M$. The isotope enrichment factor may also be calculated using the isotopic composition of the product: NO_2^-. From the equation (11), the isotope enrichment factor is deduced from the slope of the

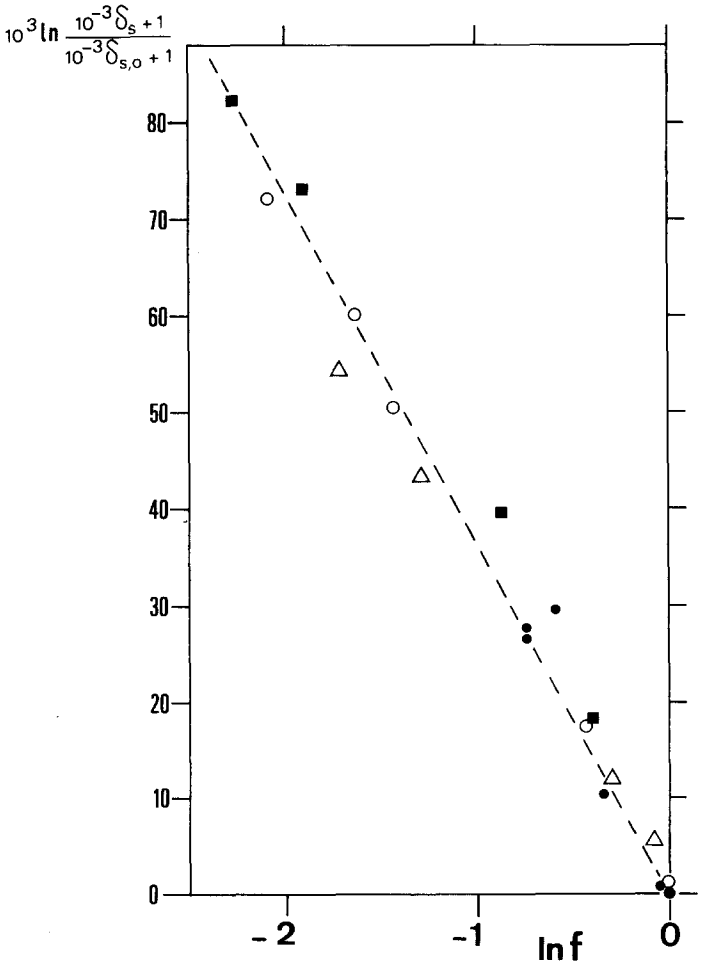

Fig. 5. Change in substrate NH_4^+ isotopic composition as a function of ln f during nitrification. The slope of the straight line is the isotopic enrichment factor $\varepsilon_{p/s}$.

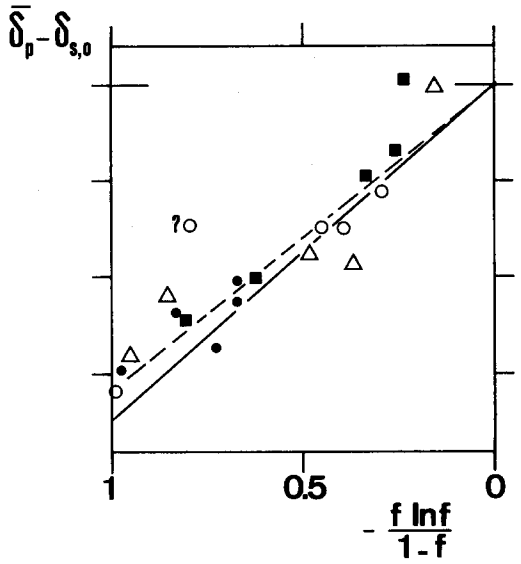

Fig. 6. Nitrification experiment. Isotopic composition of product (NO_2^-) as a function of $-f \ln f/1 - f$) (cf. equation 11). The slope of the solid straight line represents the isotope enrichment factor calculated from the change in the substrate (−34.7‰, see Fig. 5); the dotted line gives the isotopic enrichment factor calculated from the change in the product (−31.9, see text).

straight line $Y = \overline{\delta_p} - \delta_{s,0}$, $X = -f \ln f/(1 - f)$. f will be taken as $[NH_4^+]_t/[NH_4^+]_0$ (Fig. 6). One of the experimental values obtained for the isotopic composition of NO_2^- (experiment B, 70 h, Table 2) seems incorrect (see Fig. 4 and 6) and has not been taken into account in the calculation.

One gets:

$$\overline{\delta_p} - \delta_{s,0} = 0.64 - (31.91 \pm 6.39) \cdot \left(-\frac{f \ln f}{1 - f} \right); \quad r = -0.931$$

The intercept is not statistically different from zero.

It may be concluded that the estimation of the isotopic enrichment factors, calculated from changes in isotopic composition of either the substrate or the product, are not significantly different. Nevertheless, the first estimation is more precise. A second conclusion is that the system under study could be considered, within analytical errors, as a closed system. The isotope enrichment factor during nitrification in a pure culture of *N. europaea* is therefore about −35‰ in the given conditions, very close to the value published by Domenach and Chalamet[6].

CONCLUSION

Isotopic kinetic reaction studies, such as the one presented here, are needed in order to ascertain the magnitude of isotope fractionation associated with important N transformations. This knowledge is essential for balance estimations of the nitrogen budget.

The present study shows that under the experimental conditions we used, the isotope effects associated with nitrification and denitrification are constant with extent of reaction and with substrate concentration and that the substrate-product relation fits the model of a simple and unidirectional reaction.

The isotope fractionation factor for a reaction, the mechanism of which is not well known, cannot be estimated through a single determination of the isotopic enrichment of the product *versus* substrate, at any step in the progress of the reaction, since there are no *a priori* reasons for this fractionation factor to be constant.

The present data also show that the kinetic isotope fractionation factor cannot be estimated presently with a precision better than 10% of the measured value.

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