Emissions de N2O par nitrification et dénitrification à l'échelle de la motte de sol: effet de la structure du sol, de l'aération et des activités microbiennes

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RESUME

La production de N_2O par les sols résulte des transformations microbiennes de l'azote: dénitrification et nitrification. L'objectif de ce travail était de caractériser les émissions de N_2O par dénitrification et nitrification dans un sol de limon en fonction de la structure du sol, à l'échelle de la motte. Nous avons étudié la dénitrification en combinant des mesures faites sur des boues de sol incubées en anaérobiose et un modèle de simulation des populations microbiennes réductrices de NO_3^- et N_2O. Les simulations démontrent que la capacité réductrice de N_2O est inductible, de sorte que la production nette de N_2O diminue fortement au cours du temps malgré une dénitrification active. Nous avons mis en évidence que la respiration et l'émission de N_2O par dénitrification par des mottes de sol incubées en aérobiose étaient fortement stimulées par une pré-incubation anaérobie. Ces résultats peuvent être expliqués à l'aide d'un modèle simplifié prenant en compte la structure des mottes caractérisée par analyse morphologique. La vitesse de nitrification et sa contribution à la production de N_2O ont été caractérisées sur des agrégats de sol en fonction de la pression partielle en O_2, en utilisant le traçage ^{15}N. Une réduction de la pression O_2 diminue la vitesse de nitrification et augmente fortement la production de N_2O par nitrification.

AVANT-PROPOS

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INTRODUCTION GENERALE

L'oxyde nitreux est un gaz à effet de serre impliqué dans la chimie de l'ozone, dans la haute troposphère et la basse stratosphère. Les estimations récentes font état d'émissions annuelles de N_2O vers l'atmosphère d'environ 14.7 Tg de N-N_2O, dont 9.5 Tg proviendraient des sols. La contribution des sols agricoles reste encore estimée de manière très imprécise. La production de N_2O dans les sols résulte des transformations microbiennes de l'azote, plus particulièrement de la dénitrification. La nitrification contribue aussi à ces émissions et pourrait en être une source importante.

Plusieurs motivations justifient notre travail. Les sources et puits des émissions de ce gaz sont mal évalués. Chacune des 2 étapes de la dénitrification (production et consommation) possède sa propre régulation. La production nette de N_2O dans les sols représente une part variable des produits de la dénitrification (N_2O et N_2) selon les conditions du milieu. Les émissions de N_2O varient donc aussi bien avec la vitesse de dénitrification qu'avec la vitesse de réduction du N_2O en N_2. Les facteurs qui contrôlent le ratio $N_2O/(N_2O+N_2)$ sont mal connus et peuvent différer de ceux qui régulent la dénitrification. Plusieurs modèles ont été proposés pour décrire la dénitrification. Ils prennent en compte plusieurs facteurs tels que la respiration, l'anaérobiose, … Néanmoins, les variations temporelles de ces facteurs ne sont pas considérées dans ces modèles. Par ailleurs, la structure du sol est un facteur important puisqu'elle conditionne l'anoxie et donc la dénitrification. Toutefois, ce paramètre n'est pas souvent pris en compte dans les modèles de prévision du N_2O. Enfin, la contribution de la nitrification aux émissions de N_2O reste encore mal estimée. Plusieurs facteurs pouvant influencer ces émissions ont été étudiés; toutefois, peu d'études ont analysé l'effet direct de la pression partielle en O_2 dans les sols.

Il est nécessaire de choisir une échelle expérimentale adéquate et compatible avec nos motivations environnementale et agronomique. Nous avons choisi une échelle millimétrique: boues, agrégats (2-3 mm) et mottes (2 à 3 cm de diamètre). Cette échelle d'étude se justifie par le fait que les conditions physiques (en particulier les teneurs en O_2) de la couche labourée sont en fait très variables à l'intérieur des mottes constituant cette couche, de telle sorte que la valeur moyenne ne suffit pas à prévoir l'activité des micro-organismes, en particulier la nitrification et la dénitrification.

Ce mémoire présente l'ensemble des résultats obtenus lors de cette étude et il est structuré en 6 parties.

Dans une première partie, nous avons commencé par une synthèse bibliographique décrivant l'état des connaissances actuelles sur les émissions de N_2O ainsi que les problématiques environnementales et agronomiques qui justifient notre travail (*Chapitre 1*).

Dans une seconde partie, nous avons décrit le fonctionnement de la production de N_2O en compétition avec sa réduction en N_2 sur des boues de sol avec différentes concentrations initiales en nitrate. A cette occasion, nous avons utilisé des modèles qui prennent en compte la dynamique microbienne ainsi que l'adaptation des microorganismes à réduire N_2O en N_2 (*Chapitre 2*).

Dans une troisième partie, nous avons étudié l'influence d'une pré-incubation anaérobie sur la respiration aérobie ainsi que sur les émissions de N_2O par dénitrification, sur deux populations de mottes de structure différente (*Chapitre 3*).

Au cours d'une quatrième partie, nous avons utilisé un modèle qui prend en compte l'activité dénitrifiante et l'aération en relation avec la structure des mottes décrite par des descripteurs synthétiques obtenus par analyse morphologique (*Chapitre 4*).

Dans une cinquième partie, nous avons étudié l'effet de la pression partielle en O_2 sur la nitrification et les émissions de N_2O par nitrification après un apport d'ammonium sur des agrégats de sol (*Chapitre 5*).

L'ensemble de ce travail de thèse est discuté dans une sixième partie dans laquelle nous avons positionné nos résultats dans un contexte d'étude plus large. Nous faisons quelques propositions de recherches ultérieures et d'application de ces résultats à la problématique environnementale.

CHAPITRE 1

SYNTHESE BIBLIOGRAPHIQUE

I. Problématiques environnementales et agronomiques

I.1. Effet de serre et forçage radiatif

Une part importante du rayonnement infrarouge émis par le sol est absorbée par certains constituants atmosphériques mineurs (les gaz dits à effet de serre). Le rayonnement ainsi piégé réchauffe la basse atmosphère qui émet elle-même un rayonnement infrarouge. Une fraction de ce dernier rayonnement réchauffe la surface de la terre : c'est l'effet de serre. On parle d'effet de serre "naturel" pour caractériser le réchauffement de la planète dû à la présence de gaz absorbant dans l'infrarouge aux teneurs qu'ils avaient avant le début de l'ère industrielle, i.e. avant que l'homme ne commence à modifier significativement la composition de l'atmosphère. Cet effet de serre naturel a été estimé à 33°C : en absence de nuages et des gaz traces, la température moyenne de la terre serait ainsi d'environ -18°C, au lieu des 15°C environ vers 1800-1850. L'essentiel de l'effet de serre naturel est lié à la présence d'eau dans l'atmosphère (vapeur, nuages …) et à la présence de dioxyde de carbone (Figure 1a). Un changement dans l'intensité du rayonnement du soleil ou des capacités de l'atmosphère à absorber le rayonnement infrarouge constituent un forçage radiatif. Le forçage radiatif actuel serait à l'origine d'un accroissement de température d'environ 0.5-0.8°C depuis l'ère préindustrielle. Selon le rapport de l'IPCC (1996), entre 50 et 60 % du forçage radiatif résulte de l'accroissement de la concentration atmosphérique en dioxyde de carbone (Figure 1b). Le reste du forçage radiatif résulte de l'accroissement de concentrations d'autres gaz : méthane, CFC, ozone, protoxyde d'azote et vapeur d'eau stratosphérique. D'après l'évolution de la composition atmosphérique, l'accumulation atmosphérique du protoxyde d'azote (N_2O) s'élève à environ 4 Tg N an^{-1} (IPCC, 1995). Sa durée de vie est estimée entre 110 et 166 ans, avec un temps de résidence de 25 ans dans la troposphère (Jambert, 1995). Le N_2O possède un effet radiatif 200 à 300 fois supérieur à celui du CO_2. Les estimations actuelles indiquent que près de 65 % des émissions (soit 9.5 Tg N an^{-1}) sont issus du sol, dont 1/3 (3.5 Tg N an^{-1}) proviendrait des sols cultivés. Environ 20 % (3 Tg N an^{-1}) des émissions totales de N_2O proviennent des océans, 3 % (0.4 Tg N an^{-1}) serait produits par les animaux et l'élevage, 3 % (0.5 Tg N an^{-1}) seraient émis au cours du brûlage de la biomasse et environ 9 % (1.3 Tg N an^{-1}) proviendraient des rejets industriels lors de la fabrication de produits chimiques tels que l'acide nitrique ou les engrais.

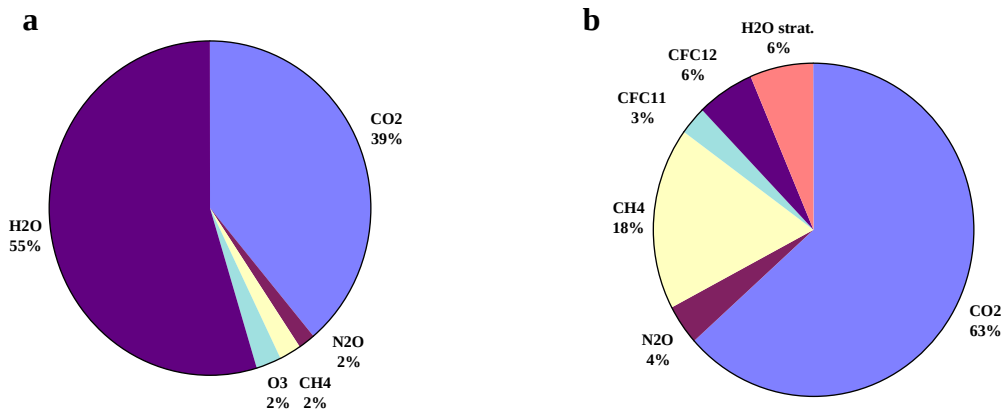

Figure 1: *Contribution relative (%) des gaz de l'atmosphère a) à l'effet de serre naturel et b) au forçage radiatif, d'après Mac Kay et Khalil (1991)*

I.2. Effets sur l'ozone de la haute troposphère et de la basse stratosphère

L'ozone (O_3) est le composant le plus paradoxal de l'atmosphère terrestre. Ce gaz est à la fois indispensable et néfaste pour le maintien de la vie sur terre. Situé dans la haute troposphère et la basse stratosphère, il filtre les rayonnements ultraviolets de longueur d'onde inférieure à 290 nm mortels pour la plupart des formes de vie, alors que l'ozone situé dans la troposphère est néfaste pour les espèces vivantes. Différents composés interviennent dans le devenir de l'ozone avec l'implication de réactions chimiques et photochimiques. Dans la basse troposphère, le N_2O est un composé très stable. Par contre, quand il atteint la haute troposphère et la basse stratosphère, il peut donner naissance à des radicaux libres NO• grâce à la présence d'atomes d'oxygène excités. Le monoxyde d'azote (NO) a alors un double rôle vis-à-vis de l'ozone :

- il est l'un des catalyseurs de la destruction de l'ozone ;
- il peut se combiner (sous la forme NO_2•) à d'autres radicaux libres qui sont plus nocifs pour l'ozone (notamment les radicaux libres chlore Cl•) et aboutit à inactiver temporairement ces différents catalyseurs. Ainsi en terme de bilan, le N_2O a probablement eu ces dernières années un effet plus protecteur que destructeur; ainsi au-dessus de l'Antarctique, le trou d'ozone se reforme chaque année au début du printemps austral lorsque les radicaux libres NO• sont piégés sous forme d'acide nitrique dans des nuages de glace et que les radicaux libres Cl• se reforment par photodissociation de la molécule Cl_2. Toutefois, dans certaines situations, les radicaux

libres NO• sont plus nocifs pour l'ozone : c'est notamment le cas lorsqu'ils se forment en très grande quantité suite à des bombardements solaires de protons mais, ils ne proviennent alors plus de l'oxyde nitreux. (Graedel et Crutzen, 1992). Les autres sources de NO dans la haute troposphère et la basse stratosphère sont les rejets directs à partir d'avions, d'explosions nucléaires, …

I.3. Pertes de fertilisants azotés en agriculture

L'augmentation de la teneur de l'atmosphère en protoxyde d'azote (N_2O) est à mettre en relation avec l'intensification de l'agriculture et l'utilisation de fertilisants azotés (Eichner, 1990; Beauchamp, 1997). Le N_2O émis par les sols constitue ainsi une perte d'azote pour les cultures, mais la perte totale d'azote est généralement beaucoup plus conséquente que la seule perte en N_2O. D'une part, le rapport $N_2O/(N_2O+N_2)$ des produits terminaux de la dénitrification peut prendre des valeurs très variables comprises entre 0 et 1 (Granli et Bockman, 1994) ; d'autre part, il semble que lorsque N_2O est émis par nitrification, du NO• est émis dans les mêmes proportions, voire en quantité plus importante (Williams *et al*., 1992 et Garrido, 2001). L'estimation moyenne actuellement retenue des émissions de N_2O à partir des sols est $Y = 1 + 0.0125X$, où X représente la quantité d'azote apporté par les produits fertilisants (X et Y en kg N/ha; Bouwman, 1996). La contribution directe ou indirecte des pratiques agricoles est estimée entre 1.8 et 5.3 Tg N_2O an^{-1}, et la production totale à 9.5 Tg N_2O an^{-1} (IPCC, 1995).

II. Processus impliqués dans la production et la consommation de N_2O dans les sols

Les émissions de N_2O sont principalement dues à l'activité des micro-organismes (dénitrification, nitrification, réduction de NO_3^- en NH_4^+), et parfois à la dénitrification chimique. Les émissions de N_2O par réduction microbienne dissimilative de NO_3^- en NH_4^+ semblent négligeables (Knowles, 1982). La dénitrification et la nitrification sont les principaux processus biologiques impliqués et sont réalisés chacun par des groupes fonctionnels spécifiques de la microflore du sol.

II.1. La dénitrification

II.1.1. Le processus

La dénitrification est un processus respiratoire microbien au cours duquel des micro-organismes sont capables de substituer à l'oxygène des formes oxydées de l'azote comme accepteur terminal d'électrons dans leur chaîne respiratoire. Les oxydes d'azote solubles, nitrates (NO_3^-) ou nitrites (NO_2^-), sont transformés en composés gazeux : oxyde nitrique (NO), protoxyde d'azote (N_2O) et/ou diazote (N_2) selon la chaîne de réactions suivante:

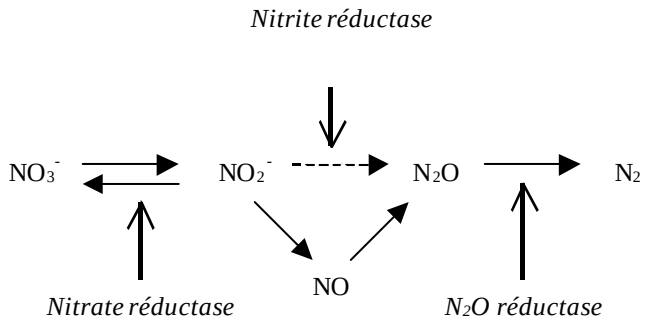

Figure 2: *Les étapes de la dénitrification (Hénault, 1995)*

Une chaîne respiratoire est un complexe membranaire associant transporteurs d'électrons, enzymes et transporteurs de protons. Elle permet notamment l'oxydation de composés réduits ($NADH+H^+$ en NAD^+ par exemple) avec la réduction concomitante d'accepteurs terminaux d'électrons (NO_3^- en NO_2^- par exemple) en combinant à certaines étapes du transfert des électrons le transport de protons de l'intérieur vers l'extérieur de la cellule. Le retour des protons dans la cellule peut être associé à la création d'énergie cellulaire sous forme d'ATP. Il faut distinguer respiration aérobie et respiration anaérobie. L'accepteur final d'électrons est l'oxygène pour la respiration aérobie. Différents accepteurs d'électrons peuvent être utilisés pour la respiration anaérobie, tels que NO_3^-, Mn, Fe, SO_4^{2-}, CO_2 (Paccard, 1995). Dans le cas des micro-organismes dénitrifiants, ces accepteurs sont : NO_3^-, NO_2^-, NO et N_2O.

II.1.2. Les micro-organismes dénitrifiants

Les bactéries dénitrifiantes au sens strict sont des bactéries capables de réduire NO_3^- ou NO_2^- en N_2O et/ou N_2 et qui peuvent utiliser l'énergie libérée au cours de cette réduction pour se développer (Mahne et Tiedje, 1995). Parmi les micro-organismes hétérotrophes

présents dans les sols cultivés, 1 à 5 % d'entre eux sont des dénitrifiants (Tiedje et al., 1982). Selon Lensi (1995), les organismes dénitrifiants représentent 10 % des bactéries totales en sol cultivé et 5 % en pâturage permanent. D'après une étude de Gamble *et al*. (1977) réalisée sur 19 sols provenant d'écosystèmes variés, les genres majoritaires sont représentés par *Pseudomonas* et *Alcaligenes*. Les bactéries dénitrifiantes sont en majorité hétérotrophes, elles tirent leur énergie de l'oxydation des matières organiques (organotrophes). Elles peuvent aussi être autotrophes et oxyder des composés minéraux comme Fe^{2+} et HS^- (chimiolithotrophes). Toutes les étapes de la dénitrification sont productrices d'énergie sous forme d'ATP. Toutes les bactéries dénitrifiantes sont des bactéries aérobies pouvant s'adapter à une respiration sur NO_3^-, NO_2^- ou sur N_2O quand le milieu devient pauvre en O_2 (on parle de bactéries aérobies facultatives). Les 2/3 de la microflore réduisent les NO_3^- en NO_2^-, mais seulement un faible pourcentage semble capable de réaliser toute la chaîne de réactions (Germon et Hénault, 1994). Si le nombre de bactéries dénitrifiantes ne reflète pas l'activité dénitrifiante (Parson *et al.*, 1991), la dynamique de ces populations constitue sans doute un élément essentiel pour expliquer les émissions de N_2O par les sols (Granli et Bockman, 1994). Les micro-organismes du sol s'adaptent aux conditions environnementales et les individus d'une même espèce qui se développent dans différents environnements peuvent différer en phénotype (ensemble des caractères morphologiques et physiologiques visibles), dû aux différences génétiques ou aux effets environnementaux sur les phénotypes (Bergwall, 1999). On parle alors de plasticité phénotypique, c'est-à-dire de capacité d'un organisme à produire différents phénotypes en conditions environnementales variables (Pigliucci, 1996). On peut citer l'adaptation à une concentration variable en NO_3^- du sol ; pour des concentrations élevées, la plasticité phénotypique est telle qu'elle élargit la capacité compétitive des dénitrifiants au-delà des conditions d'habitat rencontrées (Bergwall, 1999). L'adaptation à un milieu plus ou moins riche en NO_3^- se fait après un temps de latence plus ou moins long .

II.1.3. Les enzymes de la dénitrification

Chacune des étapes de la chaîne de dénitrification est catalysée par une enzyme spécifique. Certaines bactéries ne possèdent pas la totalité des enzymes et par conséquent, elles ne peuvent réaliser qu'une seule partie de la chaîne de dénitrification.

Deux principaux types de nitrate réductase ont été distinguées : la nitrate réductase assimilatrice et dissimilatrice. Plusieurs nitrate réductases dissimilatrices ont été mises en évidence: deux sont des enzymes membranaires et sont inhibées par l'oxygène, une autre est

périplasmique et peut fonctionner en présence d'oxygène dès qu'il y a du NO_3^-. La synthèse de NO_3^- réductase est régulée par des protéines qui ont pour rôle de réguler certaines fonctions bactériennes en fonction de la pression partielle en oxygène. Elles sont responsables en anaérobiose de répressions et d'inductions selon leur état d'oxydation (Unden et Schirawski, 1997).

La N_2O réductase (N_2OR) est une enzyme périplasmique soluble; sa complexité peut être due à la possibilité d'une compétition entre NO_3^- et N_2O comme accepteurs d'électrons (Cho et Sakdinan, 1978). Le Cu est nécessaire pour la N_2O réductase (Zumft, 1997) et le Fe pour les autres porteurs d'électrons. Les N_2OR sont inhibées par le dithionite, le CO et le C_2H_2 (Paccard, 1995). Otte *et al.* (1996) ont directement observé une soudaine et considérable augmentation de la concentration de N_2O réductase après environ 25h. Les NO_3^- et NO_2^- réductases sont synthétisées en conditions aérobies (Patureau *et al.*, 1996), alors que la synthèse de la N_2O réductase commence seulement 16 à 33 h après disparition du O_2 (Firestone et Tiedje, 1979; MacConnaughey *et al*., 1985; Dendooven et Anderson, 1994).

II.2. La nitrification

II.2.1. Le processus

La nitrification est due à des bactéries oxydant NH_4^+ en NO_2^- (nitritation) puis en NO_3^- (nitratation) en conditions aérobies. La nitrification peut être autotrophe ou hétérotrophe.

a. La nitrification hétérotrophe est réalisée par une microflore qui se développe sur des substrats carbonés organiques. Elle se limite principalement aux sols forestiers et aux milieux à faible pH et à température élevée. Elle est moins active que la nitrification autotrophe.

b. La nitrification autotrophe est le processus dominant en sol cultivé. En présence d'une source minérale de carbone, CO_2 ou bicarbonate, l'ammonium est oxydé successivement en hydroxylamine (NH_2OH), nitrite et nitrate. Ce processus fait intervenir des transporteurs d'électrons tels que NAD et FAD. Chaque étape est catalysée par un système enzymatique différent.

II.1.2. Les micro-organismes nitrifiants

Les nitrifiants hétérotrophes, largement représentés chez les champignons (ex: *Aspergillus flavus*, les bactéries et les actinomycètes, sont capables de réaliser la nitrification en culture pure à partir de sources d'azote organique ou ammoniacal (Killham, 1986). Toutefois, d'autres travaux sont nécessaires pour estimer l'importance de la nitrification hétérotrophe dans les sols.

Les nitrifiants autotrophes sont des bactéries nitrifiantes classées dans la famille des *Nitrobacteraceae* (Watson, 1971 ; Bock *et al*., 1986). Les bactéries oxydant l'ammonium en nitrite s'organisent en 5 genres : *Nitrosomonas, Nitrosospira, Nitrosococcus, Nitrosolobus et Nitrosovibrio*. Les bactéries oxydant les nitrites en nitrates s'organisent en 4 genres : *Nitrobacter, Nitrococcus, Nitrospira et Nitrospina.*

L'ammoniac mono-oxygénase (AMO) est une enzyme membranaire dont la structure n'est pas encore clairement identifiée. Des études ont montré une analogie de structure entre l'AMO de *N. europaea* et de *Paracoccus dénitrificans* (nitrifiant hétérotrophe). L'AMO présente la capacité d'hydroxyler une large gamme de substrats hydrocarbonés; elle est irréversiblement inhibée par l'acétylène (Hommes *et al*., 1998).

II.1.5. Les hypothèses de formation de N_2O liées à la nitrification

Plusieurs travaux ont mis en évidence la production de N_2O par nitrification (Blackmer *et al*., 1980 ; Stevens et Laughlin, 1998). Plusieurs hypothèses ont été émises pour expliquer l'origine de ces émissions :

H1) Une fraction du NH_4^+ oxydé est déviée vers la production de N_2O durant la nitrification, avec plusieurs réactions intermédiaires. Selon Conrad (1990) cette formation serait la conséquence de la transformation d'un composé intermédiaire formé au cours de l'oxydation de l'hydroxylamine en nitrite, identifié comme étant le nitroxyl (HNO). Selon Parton *et al*. (1996), l'oxydation du nitroxyl pourrait également conduire à la formation d'un autre composé inconnu qui serait ensuite oxydé en nitrite. Cette hypothèse est prise en compte dans le modèle NGAS de Parton *et al*. (1996) et des autres modèles (Linn et Doran, 1984; Davidson, 1993).

H2) La présence d'un système enzymatique sur *Nitrosomonas europaea* réduit NO_2^- en N_2O durant l'oxydation de carbone en anaérobiose (Ritchie et Nicholas, 1972). L'effet de la pression partielle de O_2 a été mis en évidence soit directement en faisant varier la

pression partielle en O_2 (Bollmann et Conrad, 1998), soit indirectement en faisant varier l'humidité du sol (Zanner et Bloom, 1995); il est pris en compte dans le modèle de Grant (1995).

H3) L'oxydation partielle de NH_4^+ en NO_2^- en aérobiose est suivie d'une diffusion du NO_2^- vers les sites anoxiques, puis d'une réduction en N_2O par dénitrification. Poth et Focht (1985) ont conclu que le processus de réduction de NO_2^- en N_2O par *N. europaea* correspond à la définition de la dénitrification. La production de N_2O par nitrification est non corrélée avec l'addition de nitrate mais corrélée avec l'addition d'ammonium, cela peut s'expliquer par l'utilisation de l'ammonium comme source d'électrons et par l'absence de l'activité du nitrate réductase dans *N. europaea*. Cette hypothèse est prise en compte dans quelques modèles (Poth et Focht, 1985).

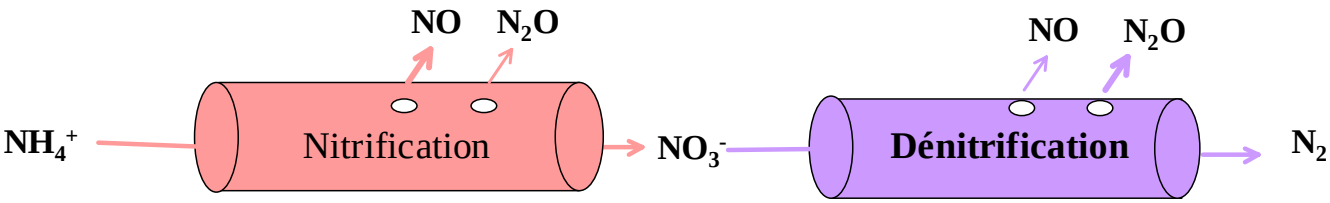

Figure 3: *Schéma conceptuel « hole in the pipe » (Firestone et Davidson, 1989)*

II. La régulation des émissions de N_2O dans les sols

La compréhension et la description des émissions de N_2O passe par la prise en compte des facteurs intervenant sur la nitrification et la dénitrification. Deux types de difficultés doivent être surmontées dans ce cadre :

- la prise en compte des interactions entre facteurs du milieu qui font que l'effet d'un facteur ne peut pas être décrit indépendamment de l'effet d'autres facteurs: citons les interactions entre les effets de la teneur en eau et de la température, ou entre la structure et la teneur en eau (Renault et Sierra, 1994) ;

- la contribution en proportion variable des deux processus et la consommation de N_2O par l'un des processus (dénitrification); celle-ci peut être du même ordre de grandeur que la production brute de N_2O.

L'échelle millimétrique adoptée par certains auteurs pour étudier et décrire les émissions de N_2O peut se justifier par le fait que les conditions expérimentées par les organismes du sol peuvent être très différentes des mesures globales faites sur des blocs de sol (Parkin, 1987). Ainsi la concentration en O_2 peut passer de la teneur atmosphérique à une valeur nulle sur une distance de quelques millimètres dans la motte de sol (Sextone *et al*., 1985; Sierra *et al*., 1995), et même sur une distance inférieure à 1 mm au voisinage de la matière organique particulaire (Parkin, 1987; Parry *et al*., 2000) ou dans la rhizosphère (Bidel *et al.*, 2000). Ce type d'échelle peut faciliter la prise en compte explicite des interactions entre facteurs du milieu.

Figure 4: *Les facteurs de contrôle de la dénitrification dans les sols (Robertson, 1989)*

Figure 5: *Les facteurs de contrôle de la nitrification dans les sols (Robertson, 1989)*

III.1. La teneur en eau

La teneur en eau affecte différemment la nitrification et la dénitrification, surtout au voisinage de la saturation en eau. De fortes corrélations ont été observées entre le taux de dénitrification et la teneur en eau du sol (Parson, 1991; Mosier *et al.*, 1986; Rolston *et al.*, 1984; Grundman et Rolston, 1987). Les auteurs qui ont tenté de décrire les effets de l'humidité indépendamment des effets d'autres facteurs ont établi un seuil minimum de taux de saturation en eau de la porosité (WFPS = water filled pore space) au delà duquel la dénitrification peut se produire. Ce seuil est égal à 0.6 selon Grundman et Rolston (1987) et Terry *et al.* (1981) ou 0.8 pour Rolston *et al.* (1984). La dénitrification croît exponentiellement au delà de ce seuil jusqu'à la saturation du sol (Terry *et al.*, 1981).

D'autres auteurs ayant une approche plus mécaniste des processus montrent que le WFPS minimum et la relation WFPS - dénitrification varient en fonction de plusieurs facteurs, tels que la structure du sol (densité apparente et granulométrie de motte) et la température (Renault et Sierra, 1994). Le rapport $N_2O/(N_2O+N_2)$ des produits terminaux de la dénitrification diminue avec une augmentation de la teneur en eau du sol (Terry *et al*, 1981; Weir *et al* , 1993). Linn et Doran (1984) supposent que la nitrification est active pour WFPS compris entre 10 et 80% avec un maximum d'activité à 60%. Ces auteurs représentent leurs résultats par la figure suivante :

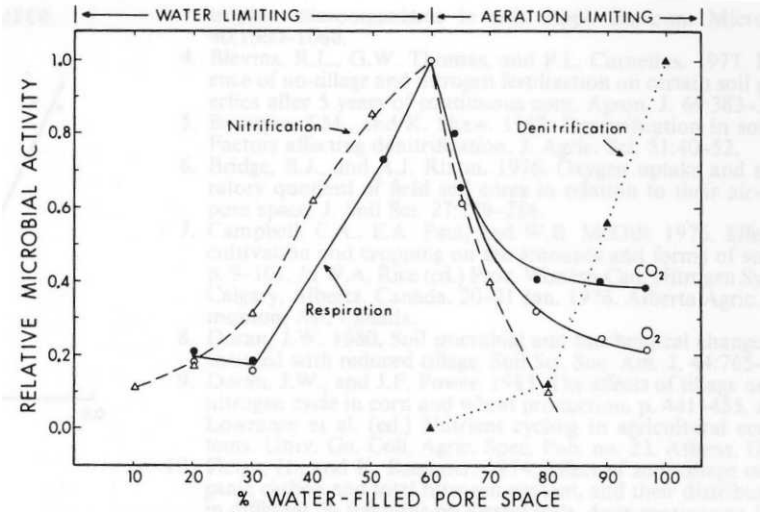

Figure 6 : Effet du taux de saturation sur l'intensité relative des processus (Linn et Doran, 1984)

La teneur en eau du sol influence les émissions de N_2O par son action sur l'activité des microflores impliquées, sur la dissolution des substrats des réactions et sur la diffusion des gaz dans les sols, en particulier sur la transport d'oxygène. Les événements pluvieux importants ou l'irrigation peuvent induire des pics de N_2O très importants (Sextone *et al*., 1985).

III.2. L'aération du sol

Le sol est un milieu hétérogène où peuvent exister des zones aérobies et anaérobies. La dénitrification apparaît à de faibles teneurs en O_2 (Jambert, 1995; Granli et Bockman, 1994). Le rapport $N_2O/(N_2O+N_2)$ tend à augmenter lorsque la concentration en O_2 diminue. La N_2O-réductase semble plus sensible à la teneur en O_2 que d'autres enzymes de la dénitrification (Betlach et Tiedje, 1981). Des sites anoxiques peuvent exister dans un sol présentant de bonnes conditions d'aération. Ils apparaissent au cœur des agrégats (Sextone *et al*, 1985, Sierra *et al*., 1995), au voisinage des matières organiques particulaires en décomposition (Parkin, 1987 ; Parry *et al*., 2000) et dans la rhizosphère (Bidel *et al*., 2000). La taille de ces sites joue sur l'importance de la dénitrification et sur le rapport $N_2O/(N_2O+N_2)$: plus le site est petit, plus le N_2O pourra atteindre rapidement les zones aérées sans être réduit (Lafolie *et al*., 2001).

La dénitrification a longtemps été considérée comme exclusivement anaérobie, en supposant que les enzymes de la dénitrification sont inhibées par les environnements oxiques. Il semble maintenant qu'elle puisse apparaître aussi en aérobiose. Cette activité est attribuée à l'existence d'une NO_3^- réductase distincte (appelée Nap), localisée dans l'espace périplasmique des dénitrifiants gram -, alors que le site actif de la membrane liée à la NO_3^- réductase (Nar) est située dans le cytoplasme (Bell, 1990).

Tiedje (1988) a établi un schéma représentant les effets de la concentration en O_2 sur les étapes de la dénitrification:

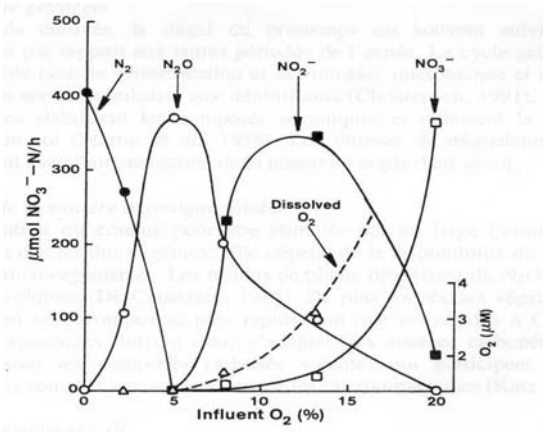

Figure 7 : *Effets de la concentration en O_2 sur les étapes de la dénitrification (Tiedje, 1988)*

Contrairement à la dénitrification, la nitrification est un processus aérobie qui requiert de l'oxygène pour l'activation de l'ammonium oxygénase (Wood, 1987). La vitesse de nitrification dépend de la pression partielle en O_2 (Bollmann et Conrad, 1998). Une diminution de l'aération du sol s'accompagne d'une augmentation de la pression partielle de CO_2, ce qui affecte directement ou indirectement (via les variations de pH) les nitrifiants autotrophes (Grant, 1993; Pennington et Ellis, 1993). Les émissions de N_2O par nitrification augmentent lorsque la pression partielle en O_2 diminue (Goreau *et al*., 1980). Le rendement de production en N_2O (rapport entre la production de N_2O et la quantité de N nitrifié) varie beaucoup selon les auteurs: 0.09 à 0.28% (Breitenbeck *et al*., 1980), 0.5 à 2% (Bolle, 1986), 0.02% (Tortoso et Hutchinson, 1990), 0.03 à 1% (Garrido, 2001).

III.3. La disponibilité en azote minéral

La teneur et la forme de l'azote minéral contenu dans le sol exercent un effet important sur la nature et l'intensité des émissions de N_2O. Les bactéries dénitrifiantes ont la capacité d'utiliser NO_3^-, NO_2^- et/ou N_2O comme accepteurs d'électrons en absence de O_2. La dénitrification est généralement décrite par une cinétique de Michaelis-Menten, qui se caractérise par une dépendance de la vitesse aux faibles concentrations en NO_3^- et une indépendance aux plus fortes teneurs (Betlach et Tiedje, 1981; Myrold et Tiedje, 1985 ; Maag *et al*., 1997). Des constantes de Michaelis de dénitrification ont ainsi été déterminées sur

différents sols. Les principales incertitudes qui restent sur le processus de réduction du NO_3^- en N_2O sont les suivantes:

i) l'existence ou non d'un effet inhibiteur par les fortes concentrations en NO_3^- (Blackmer et Bremner 1978, Nakajima *et al*., 1984; Lalisse-Grundmann *et al*., 1988),

ii) la valeur de la constante de Michaelis décrivant l'affinité microbienne pour NO_3^-,

iii) l'existence ou non d'une compétition entre NO_3^- and N_2O pour les dénitrifiants pouvant réduire ces 2 formes.

Blackmer et Bremner (1979) considèrent que les faibles concentrations en NO_3^- augmentent la réduction de N_2O. Les fortes concentrations en NO_3^- ou NO_2^- diminuent généralement la réduction de N_2O et augmentent le rapport $N_2O/(N_2O+N_2)$ (Guthrie et Duxbury, 1978; Firestone *et al*., 1979; Terry et Tate, 1980; Letey *et al*., 1980; Gaskell *et al*., 1981; Nömmik *et al*., 1984; Weir *et al*., 1993; Luo *et al*., 1996). Plusieurs hypothèses sont proposées pour expliquer ces observations:

i) l'induction de la N_2O-réductase par le NO_3^- à faible concentration (Soohoo et Hollocher, 1990),

ii) l'inhibition par NO_2^- qui s'accumule durant la réduction de NO_3^- (Shimuzu *et al*., 1978; Firestone *et al*., 1979; McKenney *et al*., 1982),

iii) l'inhibition directe par NO_3^- (Gaskell *et al*., 1981),

iv) la compétition entre NO_3^- et N_2O comme accepteurs d'électrons (Cho et Sakdinan, 1978).

La compétition entre NO_3^- et N_2O comme accepteurs d'électrons pourrait varier dans le temps si les N_2O réducteurs s'adaptent aux fortes concentrations en NO_3^- (hypothèse suggérée par Terry et Tate, 1980; Guthrie et Duxbury, 1978). Il est donc important de prendre en compte la variation temporelle de la concentration en oxyde d'azote réductase et les dynamiques microbiennes, en particulier les populations dénitrifiantes et N_2O réductrices (Baumann *et al*., 1996; Otte *et al*., 1996).

L'apport d'engrais ammoniacaux peut induire une production de N_2O par nitrification. Peu d'études ont été publiées sur l'effet de la concentration en NH_4^+ sur la nitrification et sur les émissions associées de N_2O. Aux faibles concentrations, le taux de nitrification est proportionnel au taux de minéralisation (Bremner et Blackmer, 1981 ; Lensi *et al*., 1992), alors qu'il dépend de NH_4^+ aux fortes concentrations (Stevens *et al*., 1997). Aulakh *et al*. (1984) ont estimé que les pertes de fertilisants sous forme N_2O représentaient 0.1 à 0.5 % de l'apport de $N\text{-}NH_4^+$. Les émissions de N_2O par nitrification représentent souvent moins de 1%

du NH_4^+ nitrifié dans les sols (Bremner et Blackmer, 1978, Aulakh *et al*.,1984; Klemedtsson *et al*., 1988). Parton *et al*. (1996) ont suggéré que ces émissions ne sont influencées que par des fortes concentrations en NH_4^+, supérieures à 3 µg N g^{-1}.

III.4. La disponibilité en carbone et matière organique

Le métabolisme des bactéries hétérotrophes du sol est souvent limité par le carbone biodisponible dans le sol (Starr, 1993). La disponibilité en carbone dans le sol agit très différemment sur la dénitrification et la nitrification.

En ce qui concerne la dénitrification, le carbone organique joue un double rôle: un rôle indirect en stimulant la dégradation de la matière organique, donc la respiration microbienne et la création de sites anaérobies (Parkin, 1987); un rôle direct, en tant que donneur d'électrons pour les bactéries dénitrifiantes. La dénitrification peut être fortement corrélée à la quantité de carbone organique total ou soluble et surtout au carbone facilement minéralisable (Burford et Bremner, 1975; DeCatanzaro, 1985; Weier *et al.*, 1993). Le rapport $N_2O/(N_2O+N_2)$ diminue avec l'accroissement de la source carbonée. Dans les sols, Parkin (1987) décrit la présence de sites à fort potentiel de dénitrification, qui correspondent à des zones de forte décomposition de la matière organique (hot spots). La matière organique rapidement décomposable présente sans doute une distribution spatiale très hétérogène; ceci expliquerait la variabilité spatiale de la dénitrification.

En ce qui concerne la nitrification, le carbone assimilable exerce un effet indirect en jouant sur la consommation en O_2 et sur la concentration en NH_4^+ du sol par le biais des processus d'organisation ou de minéralisation d'azote. En effet, un apport de matière organique avec un rapport C/N élevé (type pailles de céréales) stimule l'organisation des ions NH_4^+ ce qui réduit la vitesse de nitrification, par compétition pour le substrat. Inversement, la minéralisation de la matière organique humifiée, à C/N faible, libère de l'ammonium et stimule la nitrification. Bremner et Blackmer (1981) ont montré, dans le cas d'un sol "aéré", que la vitesse de nitrification, le ratio N_2O/NO_3^- et l'intensité des émissions de N_2O augmentent lorsque le ratio C/N de la matière organique du sol diminue.

III.5. La texture et la structure du sol

La texture du sol influence aussi les activités microbiennes régulant le devenir de C et N dans le sol. La consommation de C et N par la biomasse microbienne est plus rapide dans les sols à texture grossière dont la capacité de protection physique de la matière organique est faible (Hassink, 1994; Sørensen, 1995). Les sols de texture grossière montrent des émissions de N_2O plus importantes que les sols à texture fine (Bouwman , 1996). En fait, il existe des différences de configuration enzymatique ou d'accessibilité du substrat sur les sites actifs entre les fractions du sol (Rojo, 1990). Si la biomasse microbienne globale est influencée par la texture du sol, il est probable que les micro-organismes dénitrifiants le soient également. C'est ce qu'a montré Lensi (1995) en caractérisant l'activité de différentes fractions granulométriques du sol: la capacité dénitrifiante varie selon la fraction granulométrique.

La structure du sol est également un facteur très important, et elle est fortement modifiée par le travail du sol. Le travail du sol agit sur la structure qui régule le fonctionnement hydrique et le degré d'aération du sol et affecte la vitesse de dégradation de la matière organique (Aulakh *et al*., 1992). En modifiant la taille des agrégats et des mottes (Cambardella, 1993; Alvarez, 1998a), il modifie les conditions de transfert de l'oxygène. Le volume anoxique augmente avec la taille des mottes car l'oxygène a plus de difficulté à diffuser et donc à répondre à une demande respiratoire au sein d'un agrégat volumineux. Un gros agrégat a plus de chance d'avoir un centre anoxique important qu'un petit agrégat. Pour les mêmes raisons, le N_2O produit dans le cœur anoxique aura quant à lui, plus de probabilité d'être réduit en N_2 avant d'atteindre les régions oxygénées de l'agrégat. Plus le sol est compacté, plus la taille de ses agrégats augmente, plus sa porosité est faible, ce qui favorise la dénitrification (Bakken *et al.*, 1987).

III.5. La température

Lorsque la température augmente, les émissions globales de N_2O augmentent (Smith et Arah, 1990), tandis que le rapport $N_2O/(N_2O+N_2)$ tend à diminuer (Granli et Bockman, 1994). L'augmentation de température stimule la croissance bactérienne et son activité (Smith et Arah, 1990), d'où une respiration accrue pouvant entraîner la formation de zones anoxiques. Elle provoque aussi un accroissement de la diffusion et une diminution de la solubilité des gaz. La dénitrification se produit dans une gamme de température allant de 10 à 75-85°C,

avec un optimum situé entre 30 et 67°C (Smith et Arah, 1990; Granli et Bockman, 1994). Les températures optimales pour la nitrification se situent entre 25 et 35°C (Haynes, 1986). Le N_2O produit par nitrification augmente avec la température (Yoshida et Alexander, 1970 ; Bremner et Blackmer, 1981).

III.6. Le pH

Le pH du sol varie sous l'effet de plusieurs processus. La baisse de pH est en partie due à la production de CO_2 et d'acides gras volatils (Stumm et Morgan, 1996, Dassonville et Renault, 2002). Le pH peut augmenter sous l'effet de certains processus biologiques consommateurs de H^+ tels que la dénitrification (Mc Inerney, 1988). L'accroissement de pH observé ultérieurement résulte partiellement de la consommation des acides gras volatils et de la disparition du CO_2 avec la méthanogénèse (Stams, 1994). L'acidification du milieu peut modifier les équilibres chimiques en solution concernant les hydroxydes, les carbonates, les sulfures, les phosphates et les silicates et ainsi aboutir à la dissolution ou la précipitation des espèces solides et à la sorption ou la désorption des composés comme l'aluminium, le fer, le manganèse, et la calcite (Stumm et Morgan, 1996). Ces mêmes phénomènes sont impliqués dans le pouvoir tampon du sol concernant les variations de pH. Dans le cas des milieux calcaires, l'accroissement de la concentration en CO_2 aboutit à la solubilisation de calcite avec la formation de bicarbonate (Stumm et Morgan, 1996).

Les apports d'engrais peuvent modifier le pH; ainsi les fertilisants à base de NH_4^+ acidifient le sol. Le pH du sol peut s'acidifier sous l'effet des eaux de pluie qui se chargent sur leur trajet en divers gaz de l'atmosphère (CO_2, acide sulfurique, acide nitrique et acides organiques, notamment acide formique et acide acétique).

Un pH neutre ou supérieur à 7 semble favorable aux activités microbiennes nitrifiantes et dénitrifiantes. Le pH optimal d'une culture bactérienne dénitrifiante se situe à la neutralité. La N_2O réductase semble particulièrement sensible aux faibles pH. Elle est inhibée pour un pH inférieur à 5 (Knowles, 1982). Cela conduit à une augmentation des émissions de N_2O par dénitrification aux faibles pH, même si l'activité dénitrifiante est ralentie. En règle générale, les vitesses de dénitrification diminuent dans les sols acides et le rapport $N_2O/(N_2O+N_2)$ augmente simultanément avec la diminution du pH (Koskinen et Keeney, 1982).

La vitesse de nitrification est optimale à un pH de 7.5 à 8 (Bock *et al*., 1986). Toutefois, la nitrification peut se produire dans les milieux plus acides. Les émissions de N_2O par les sols en conditions aérées augmentent lorsque le pH passe de 5.9 à 8.3 (Bremner et Blackmer, 1981).

Peu d'études ont été réalisées expliquant le mécanisme d'action du pH sur les émissions de N_2O mais beaucoup d'hypothèses ont été émises. La diminution de l'activité dénitrifiante serait due à un effet direct des ions H^+ ou à un effet indirect de l'acidité provoquant des carences en nutriments (Koskinen et Keeney, 1982; Aulakh et al, 1992) ou des toxicités Al, Mn, NO_2^- (Firestone *et al*., 1982).

III. Modélisation des émissions de N_2O dans les sols

Différents modèles de simulation des émissions de N_2O ont été proposés. Ces modèles permettent notamment :

- de tester des hypothèses sur les processus, en analysant les écarts entre données expérimentales et données simulées ;
- de disposer d'outils d'interpolation plus élaborés que des outils purement statistiques pour passer de données acquises ponctuellement à des données distribuées dans l'espace et le temps;
- de réaliser des études de scénarios pour évaluer le poids de variables ou de leurs interactions dans les émissions de N_2O.

Les modèles de simulation de la dénitrification et des émissions de N_2O peuvent se classer en différents types : stochastiques, empiriques et mécanistes.

IV.1. Les modèles stochastiques

Un modèle stochastique tient compte de la variabilité spatiale des processus étudiés. Ainsi Parkin (1987) considère l'hétérogénéité spatiale de la matière organique pour simuler la variabilité spatiale de la dénitrification et notamment le phénomène de "hot spots". Son modèle suppose une loi de distribution log-normale pour l'activité dénitrifiante. Il obtient une bonne corrélation entre expérience et modèle. Par ailleurs, Parkin *et al*. (1987) ont étudié l'influence de la taille de l'échantillon sur la variabilité du taux de dénitrification. Récemment,

Parry *et al*. (2000) ont utilisé un modèle stochastique pour simuler la variabilité de la contribution des MOP au taux de dénitrification; cette contribution dépend en effet des activités dénitrifiantes et respiratoires et de la structure des mottes.

IV.2. Les modèles empiriques

Ces modèles utilisent des fonctions empiriques établies à de grandes échelles d'espace et de temps (parcelle agricole, année culturale), dans des conditions d'expérimentation forcément restreintes (compte tenu de la lourdeur des expérimentations au champ). Ces modèles ne cherchent pas à décrire les processus élémentaires de la dénitrification mais à rendre compte de cette dernière en considérant les principaux facteurs (directs ou indirects) qui l'affectent. La dénitrification effective est supposée être le produit de *n* fonctions décrivant l'effet individuel de chacun des *n* facteurs (teneur en eau, NO_3^-, matière organique, température, pH, ...). Ces modèles prennent en compte les effets moyens de ces paramètres mais négligent de ce fait leurs distributions spatiales. Ils peuvent être appliqués à de grandes échelles d'espace et de temps (parcelle agricole, année culturale), mais risquent de ne pas être généralisables à d'autres situations que celles sur lesquelles ils ont été calibrés. Le modèle de Rolston *et al.* (1984) décrit le taux de dénitrification comme une multiplication d'une fonction teneur en eau, d'une fonction température et d'une fonction concentration en NO_3^-. Cette dernière est représentée par une cinétique d'ordre 0 ou 1. Hénault et Germon (2000) ont amélioré ce modèle en y ajoutant une fonction liée à la vitesse de dénitrification décrite par une cinétique de Michaelis-Menten. Cette fonction prend en compte la disponibilité du carbone. Parton *et al*. (1988, 1996) ont développé un modèle prédictif des émissions de N_2O par les sols (NGAS). Ce modèle tient compte de la texture du sol, de la température, de la teneur en eau du sol, de la disponibilité en azote et de la respiration du sol. Un autre modèle (CASA) a été conçu pour simuler la distribution mondiale des émissions de N_2O (Potter *et al*., 1996). Il associe un modèle de production végétale à un modèle de fonctionnement du sol (physique et biologique) et tient compte des conditions climatiques. Concernant les émissions de N_2O, le modèle ne fait pas la distinction entre nitrification et dénitrification. Il suppose que la somme des composés azotés gazeux produits (N_2+N_2O+NO) correspond à 2 % de l'azote minéralisé, quelles que soient les conditions du milieu. Le modèle NLEAP-N_2O (Xu *et al*., 1998) a été conçu pour étudier l'effet des pratiques agricoles (travail du sol, irrigation et fertilisation) sur les émissions de N_2O. Il est basé sur une description empirique des effets des

facteurs du milieu. Les émissions sont la somme des émissions associées à la nitrification et des émissions associées à la dénitrification. Les émissions de N_2O par nitrification sont supposées dépendre de l'importance de la nitrification et des conditions du milieu incluant les effets de la température et la teneur en eau.

IV.3. Les modèles mécanistes

Les modèles mécanistes reposent sur une description fine dans l'espace et le temps de tous les phénomènes impliqués directement ou indirectement dans les émissions de N_2O. Un modèle ne pouvant être qu'une représentation simplifiée de la réalité, plusieurs hypothèses caractérisant le milieu et les mécanismes physiques, chimiques et biologiques sont posées. Ces modèles mécanistes donnent des résultats pertinents sur des échantillons de sol de faible taille mais nécessitent un travail très important d'acquisition des paramètres. Plusieurs hypothèses caractérisant le milieu physique du sol ont été proposées. Les premiers modèles supposaient le sol comme un milieu homogène pour les transferts. Le transport de gaz était simulé sur la seule dimension verticale (Van Bavel, 1951). Plus récemment, le milieu a été considéré comme agrégé et hétérogène pour les transferts de O_2 (Currie, 1961; Leffelaar, 1979; Arah et Smith, 1989; Renault et Sierra, 1994; Renault et Stengel, 1994). Ce type de milieu permet de décrire 2 modes de transferts : un transfert rapide dans la porosité inter-agrégats et un transfert lent au sein des agrégats. Dans l'espace poral inter-agrégats, l'eau apparaît d'abord en ménisques autour des agrégats, ce qui réduit la surface où les transferts de O_2 sont possibles. Les autres modèles se différencient par des hypothèses sur la forme des agrégats: type parallélépipède (Mc Connaughey et Bouldin, 1985) ou cylindrique (Leffelaar et Wessel, 1988). Il existe un effet de la structure du sol qui s'exerce principalement par l'intermédiaire de la distribution granulométrique des agrégats (Renault et Stengel, 1994). Un milieu agrégé pose des problèmes pour expliquer l'anoxie en raison de la distribution des pores. C'est pourquoi quelques modèles considèrent le sol comme un milieu mal structuré et hétérogène pour les transferts de O_2 à l'aide d'une distribution aléatoire de pores (Arah, 1988). La loi de Fick est généralement utilisée pour caractériser les transferts par diffusion, la convection n'étant généralement pas prise en compte. La respiration est décrite par une cinétique de Michaelis Menten (Greenwood et Berry, 1962; Leffelaar et Wessel, 1988; Grant, 1991). Sierra et Renault (1995) a essayé de caractériser l'effet inhibiteur de CO_2 sur la respiration. Il trouve une inhibition de type compétitive sur des agrégats de sol remaniés. Sur

le même sol non remanié, il trouve une activation aux faibles teneurs en CO_2 et une inhibition aux fortes teneurs. La dénitrification peut également être décrite par une loi de Michaelis Menten faisant intervenir une constante traduisant l'affinité du milieu pour NO_3^- (Maag *et al*., 1997). Cette constante tend à croître avec l'échelle d'observation, ce qui indique qu'on passe d'une concentration réelle au niveau des micro-organismes dénitrifiants à une concentration moyenne généralement supérieure au niveau d'un volume de sol (Murray *et al*., 1989). Leffelaar et Wessel (1988) prennent en considération la croissance bactérienne et la dépendance à la concentration en substrat disponible. Arah et Smith (1989) ont utilisé une fonction log-normale pour décrire la respiration maximale des agrégats pour tenir compte de la variabilité de la respiration entre agrégats. Li *et al*. (1996) ont proposé le modèle DNDC pour simuler la distribution régionale des émissions de N_2O et pour étudier les rôles respectifs de différents facteurs du milieu sur ces émissions. Dans ce modèle, un module d'estimation des émissions de N_2O par nitrification est couplé à la fois à un module prédictif de la décomposition de la matière organique du sol et à un module d'estimation des émissions de N_2O par dénitrification. Le modèle DNDC et le modèle ECOSYS (Grant, 1991, 1993a, 1995), décrivent très finement les processus microbiens du sol. Chaque transformation associée à la dénitrification dépend (i) des disponibilités en accepteurs et en donneurs d'électrons, (ii) de la taille des communautés microbiennes réalisant les différentes transformations et (iii) des conditions du milieu (température, pH, …). La taille des communautés microbiennes (NO_3^- réducteurs, NO_2^- réducteurs et N_2O réducteurs) résulte des processus simultanés de croissance, de maintenance et de mortalité. La mortalité est supposée proportionnelle à la taille de la population. La croissance microbienne tient compte de la disponibilité en NO_3^-, NO_2^- ou N_2O, et de la matière organique soluble.

V- Conclusion

Au travers de cette synthèse bibliographique, nous avons rappelé les enjeux agronomiques et environnementaux associés aux émissions de N_2O. Jusqu'à présent, les études sur les émissions de N_2O se sont surtout concentré sur le processus de dénitrification considéré comme le principal responsable de ces émissions. Nous avons vu que peu de travaux relatifs à la dénitrification décrivaient simultanément les processus de production et de réduction de N_2O, alors que ces processus risquent de répondre différemment aux conditions du milieu. L'objectif de la première partie de notre travail sera de caractériser et de

modéliser la production de N_2O, sa consommation et leurs interactions. D'autre part, plusieurs modèles ont été proposés pour décrire la respiration, l'anaérobiose et la dénitrification. Toutefois, aucun de ces modèles ne prend en compte les variations des activités respiratoire et dénitrifiante, alors que l'on sait que ces activités puissent varier au cours du temps, en conséquence des dynamiques microbiennes, des inductions et activations des enzymes. La prise en compte de ces variations sera au cœur de la deuxième et la troisième parties de notre travail. Enfin des travaux récents ont montré que la nitrification peut être une source importante des émissions de N_2O. Plusieurs facteurs pouvant influencer ces émissions ont été étudiés, mais peu d'études ont caractérisé l'effet direct de l'oxygène. Ceci a motivé le travail de la quatrième partie qui traite de l'effet de la pression partielle en O_2 sur la nitrification et les émissions de N_2O associées.

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CHAPITRE 2

SIMULATION DE LA DENITRIFICATION, DE LA DYNAMIQUE DES DENITRIFIANTS ET LEUR CAPACITE PROGRESSIVE A REDUIRE N_2O : COMPARAISON MODELE - EXPERIENCE

Ce chapitre 2 correspond à un article « soumis » pour la revue *European Journal of Soil Science* : K. Khalil, P. Renault, B. Mary, N. Guérin (2002) Modelling Denitrification including the Dynamics of Denitrifiers and their Progressing Ability to Reduce Nitrous Oxide: Comparison with Batch Experiments.

SUMMARY

Nitrous oxide is involved in the global greenhouse effect and the chemistry of O_3 in the upper troposphere and lower stratosphere. In order to define a relevant model for microbial NO_3^- and N_2O reductions in the soil and estimate the involved parameters, we propose a method combining measurements of anaerobic soil slurry and simulations performed for Michaelis Menten kinetics of NO_3^- and N_2O reductions, including non enzymatic competition between NO_3^- and N_2O as electron acceptors and the microbial dynamics of two denitrifier groups that are able and unable to reduce N_2O, respectively. Three models, varying in terms of their ability to reduce N_2O through denitrification, were alternately assessed. The procedure was applied on an arable soil known for its low N_2O emissions *in situ* during wet events. The first model, i.e. accounting for microbial growth without any increase in N_2O reduction ability, was not able to reproduce experimental data. It was necessary to consider that some denitrifiers initially unable to reduce N_2O into N_2 became able to undertake this process. Models 2 and 3 account for the induction of N_2O reductase, with either the progressive synthesis of this reductase simultaneously for all N_2O reducers or the sudden synthesis of this reductase distributed over a range of times for N_2O reducers. These models were able to approximately describe experimental kinetics, although some biases remained. In addition, denitrifier biomass estimated by fitting simulated data to experimental data was consistent with biomass estimated from fumigation extraction and microbial enumeration.

INTRODUCTION

Nitrous oxide is involved in the global greenhouse effect (Smith, 1990; IPCC, 1996) and affects the chemistry of O_3 in the upper troposphere and lower stratosphere (Graedel and Crutzen, 1992). Its emission from soils results from nitrification (Groffman, 1991) and denitrification (Hénault and Germon, 1995; Conrad, 1996). The $N_2O/(N_2O+N_2)$ ratio of the terminal denitrification products varies greatly (Granli and Bockman, 1994): low values correspond to situations in which N_2O consumption nearly equals N_2O production. A small bias in the estimate of one of these two terms can therefore result in large errors in N_2O emission calculations.

It is generally admitted that the reduction of NO_3^- and NO_2^- into N_2O may be described by Michaelis Menten equations (Betlach and Tiedje, 1981; Myrold and Tiedje, 1985; Maag et al., 1997), leading to simplified descriptions, via either zero-order reactions at high NO_3^- contents (Broadbent, 1951; Wijler and Delwiche, 1954; Nömmik, 1956; Bremner and Shaw, 1958; Cooper and Smith, 1963; Kaspar, 1985) or first-order reactions at low NO_3^- contents (Bowman and Focht, 1974; Standford et al., 1975; Reddy et al., 1982). Uncertainties concerning the description of the microbial reduction of NO_3^- into N_2O include (i) the existence of an inhibitory effect of high NO_3^- levels (Renner and Becker, 1970; Avnimelech, 1971; Blackmer and Bremner 1978, Nakajima et al., 1984; Lalisse-Grundmann et al., 1988), (ii) the value of the Michaelis constant describing the microbial affinity for NO_3^- for which varied estimates have been reported (in mol m^{-3}): 6 for intact cores (Hénault, 1993), 11.4-31.4 (Malhi et al., 1990), approximately 0.22 (Klemedtsson et al., 1977) or 0.0018–0.0166 for soil and sediment slurries (Murray et al., 1989), 0.016 (Fewson et al. 1961) or 0.3-3.8 (Zumft, 1997) for purified NO_3^- reductase, and (iii) competition between NO_3^- and N_2O for the denitrifiers that can perform NO_3^- and N_2O reductions.

On the one hand, Blackmer and Bremner (1979) considered that low NO_3^- concentrations increased N_2O reduction (i.e. < 5mg $N\text{-}NO_3^-.kg^{-1}$ DW soil). On the other hand, it is generally accepted that high NO_3^- concentrations and/or high NO_2^- concentrations decrease N_2O reduction and lead to an increase in the $N_2O/(N_2O+N_2)$ ratio of the final denitrification products (Blackmer and Bremner, 1979; Guthrie and Duxbury, 1978; Firestone et al., 1979; Terry and Tate, 1980; Letey et al., 1980; Gaskell et al., 1981; Nömmik et al., 1984; Weir et al., 1993; Luo et al., 1996). Several hypotheses have been proposed to explain these observations, including (i) the induction of N_2O reductase by low NO_3^- concentrations rather

than by N_2O itself (Soohoo and Hollocher, 1990), (ii) inhibition by NO_2^- which could accumulate during the preliminary reduction of NO_3^- (Renner and Becker, 1970; Shimuzu *et al.*, 1978; Firestone *et al.*, 1979; McKenney *et al.*, 1982), (iii) direct inhibition by NO_3^- (Gaskell et al., 1981), and (iv) competition between NO_3^- and N_2O as electron acceptors (Cho and Sakdinan, 1978). In contrast to NO_3^- reduction, estimates of the Michaelis constant for N_2O reduction were higher for purified N_2O reductase (i.e. about 2-26 mmol m^{-3}; Zumft, 1997) than for soil slurries (e.g. 0.1-0.4 mmol m^{-3}; Holtan-Hartwig *et al.*, 2000). It is often assumed that limited transfer reduces $[NO_3^-]$ at the microorganism level, and explains the increase in the apparent Michaelis constant for NO_3^- reduction (Reddy *et al.*, 1978; Myrold and Tiedje, 1985); in the case of N_2O reduction, the trend probably differs, because this gas is currently produced and reduced at the same sites. Moreover, N_2O reduction is not a simple enzymatic reaction: it depends both on the affinity of the enzyme for N_2O and on the rate of electron exchange between periplasmic N_2O reductase and the membrane respiratory chain (Spanning *et al.*, 1995; Zumft, 1997).

Competition between NO_3^- and N_2O as electron acceptors seems to vary with time, suggesting soil N_2O reducers adapt to high levels of NO_3^- (Terry and Tate, 1980; Guthrie and Duxbury, 1978). It may then be necessary to account for temporal variations in the concentration of N oxide reductases (Baumann *et al.*, 1996; Otte *et al.*, 1996) and microbial dynamics (especially the denitrifier – to – N_2O reducer ratio). To our knowledge, no publication deals with any advantage conferred to denitrifiers by the ability to reduce N_2O when NO_3^- is not limiting. The energy yield of each denitrification step varies with the electron donor and other media characteristics (Stouthamer, 1988), but the translocated 'H^+ : transferred e^-' ratio appears to be similar or equal during the respiratory reduction of NO_3^- into N_2O and the reduction of N_2O into N_2, respectively (Van Spanning *et al.*, 1995). Genes involved in N_2O reduction are sometimes located on megaplasmids (Dröge *et al.*, 1999). The transfer of self-transmissible plasmids over 1-3 day periods has already been observed for other functional properties (Richaume *et al.*, 1992; Sudarshana and Knudsen, 1995); however, recipient cells generally represent only a small proportion of all present micro-organisms (Dröge *et al.*, 1999).

The aims of this work were to (i) propose a new denitrification model that accounts for microbial activities and microbial dynamics, including the progressing ability of denitrifiers to reduce N_2O, (ii) propose a method to estimate the parameters of the model, and (iii) check the model and the method with a set of experimental data obtained from an arable soil known for its low N_2O emissions during wet events.

MATERIALS AND METHODS

SOIL AND EXPERIMENTAL PROCEDURES

Soil Sampling and Treatments

Experiments were performed on an Orthic Luvisol (FAO classification) sampled in Picardie (France), 15 km from Péronne, 49°80' N and 3°60' E. The soil had been cultivated with corn in 2000. The properties of the soil were as follows: clay, 194 $g.kg^{-1}$; silt, 706 $g.kg^{-1}$; sand, 68 $g.kg^{-1}$; pH_{water}, 8.2; total $CaCO_3$, 32 $g.kg^{-1}$; organic C, 8.52 $g.kg^{-1}$; total N 1.00 $g.kg^{-1}$; and NO_3^--N, 4.70 $mg.kg^{-1}$. Clods were sampled between 10 and 30 cm on the 12th September 2000 and sieved between 2.5 and 3 cm at field moisture (18.35% dry weight basis). They were then gently dried in air for 3 days to obtain residual moisture of around 10%, sieved again at 2mm and stored at 4°C in plastic bags until the beginning of the experiments. Before experiments began, soil aggregates were placed on a suction table. They were first equilibrated at 1 m water suction for 1 day, then at 0.5 m for 1 day, then at 0.1 m for 1 day, and finally at 0.05 m for 4 days.

Batch Incubations

In order to assess the influence of $[NO_3^-]$ and $[N_2O]$ on NO_3^- and N_2O reductions, 25g fw (fresh weight) of soil preliminarily equilibrated at 0.05 m water suction were placed in closed 250 ml flasks and maintained at 20°C for 14 hours. Then 25 ml of KNO_3 solution were added and slurries were then incubated under anaerobic conditions at 20°C. Nitrate concentrations of the added solutions were 0, 0.02, 0.04, 0.1 0.2, 0.4, 0.6, 1, 2, 3, 5, 7 and 10 mol $NO_3^-.m^{-3}$ solution. Because of the initial $[NO_3^-]$ in the soil, these applications corresponded to initial NO_3^- concentrations in the slurry solutions of 0.151, 0.166, 0.182, 0.229, 0.306, 0.462, 0.617, 0.928, 1.66, 2.44, 3.99, 5.59 and 7.92 mol $NO_3^-.m^{-3}$. The slurries were placed under anaerobic conditions by alternating 3 successive cycles of 10 min vacuum and 10 min N_2 filling of the flasks.

After the final equilibration at atmospheric pressure, three types of initial conditions were obtained:

- condition 1: 31.5 ml of N_2 were replaced with 38.6 ml of C_2H_2 (i.e. approximately 28.4 ml in air and 10.2 ml in solution) and 2.9 ml of Kr in order to characterise total denitrification and check for the existence of gas leakage, respectively;
- condition 2: 2.9 ml of N_2 were replaced with 2.9 ml of Kr in order to characterise the net production of N_2O under low N_2O conditions, i.e. under conditions where N_2O production and consumption are *a priori* of the same order of magnitude;
- condition 3: 3.6 ml of N_2 were replaced with 0.7 ml of N_2O and 2.9 ml of Kr in order to characterise the net production of N_2O under N_2O enriched conditions.

One replicate was incubated at 20°C for each initial NO_3^- concentrations and initial gaseous condition (i.e. conditions 1, 2 and 3). Stirring the suspensions at 150 rpm prevented the occurrence of micro-gradients of $[NO_3^-]$, $[NO_2^-]$, $[N_2O]$ within the soil slurries.

The initial and final '$[NO_3^-]+[NO_2^-]$' and $[NH_4^+]$ concentrations in the slurries were extracted with a molar KCl solution (soil / solution ratio of 1/5). Measurements were made with a colorimeter analyzer enabling continuous flux measurements (Sampler 1000, Skalar). Nitrous oxide concentrations were measured at the beginning of incubation and (i) after 5, 12, 24, 36, 48, 60, 72, 96 and 120 hours for condition 1, and (ii) after 12, 24, 48, 72, 96, 120, 168, 240, and 312 hours for conditions 2 and 3. In practice, 3.5 ml of gas were sampled from the flasks with Venoject® vacuum tubes and replaced by 3.5 ml N_2. Venoject® air was vacuum analysed by EC gas chromatography with a HP5890 Series II fitted with a Porapak Q column (80-100 mesh, 2 m) coupled to an automatic sampler (HSS 1000, SRA-Instruments) and to integration software on a PC Workstation. Measured $[N_2O]$ values were corrected to account for progressive dilution resulting from the periodic substitution of 3.5 ml of gas mixture with the same volume of N_2. Nitrogen, CO_2, O_2, Kr, C_2H_2, and high N_2O concentrations were measured 3 and 4 times for condition 1 and for conditions 2 and 3, respectively, with a TCD gas chromatograph HP5890 Series II fitted with Porapak Q (80-100mesh, 1.8 m) and sieve molecular (1-5 Å, 1.8 m) columns coupled to the same integration software. Measurements were made after 0, 72, 120 and 312 hours. In practice, 0.3 ml of gas was sampled with a syringe and injected into the gas chromatograph. The carrier gas was Ar-CH_4 (95/5) for EC-GC and He for TCD-GC. Relative uncertainties in the gas measurements were about 1%.

Microbial Biomass Measurement; Enumeration of Heterotrophs and Denitrifiers

Three replicates of microbial biomass measurements were performed by fumigation-extraction (Chaussod *et al*., 1988). Portions of 25 g of soil were placed into 250 ml vials and fumigated for 16 h with chloroform vapors. The fumigated samples and corresponding unfumigated samples were extracted by 100 ml of K_2SO_4 (soil/solution: 1/5). Soil suspensions were then pelleted by centrifugation in disposable plastic vials and soluble organic C was measured in the supernatant by persulfate-UV oxidation (Wu et al., 1990). The microbial extractable carbon (EC) is given by the difference between fumigated and unfumigated samples. Microbial C biomass *C-BM* can be calculated using a microbial organic C biomass to microbial extractable organic C ratio of 1/0.38.

Triplicate microbial enumeration was performed. Ten grams of soil (equivalent dry weight) were homogenised in 50 ml of NaCl (0.8%) for 1.5 min with a Waring blender. Soil suspension (10 g dw were homogenised in 50 ml of NaCl (8 $mg.l^{-1}$ solution)) was serially diluted 5-fold. One Hundred microliters of dilutions were aliquoted in 8×12 wells of microliter plates containing 100 µl of Nutrient Broth (NB, Difco, Detroit, USA) concentrated twice. For denitrifier enumeration, the medium was amended with KNO_3 (10 mM). The plates were incubated at 28°C for 5 days and 10 days for heterotroph and denitrifier enumerations, respectively. For denitrifier enumeration, anaerobic conditions were obtained using the BBL GasPack Pouch System (Becton Dickinson, Sparks, USA). The presence of heterotrophs was revealed by turbidity in positive wells. The presence of denitrifiers was considered positive if neither NO_2^- nor NO_3^- was detected using Griess-Ilosway's reagent and Morgan's reagent, respectively. The most probable number of heterotrophic or denitrifying micro-organisms was estimated by Cochran's method (Cochran, 1950).

The denitrifier biomass *bm* was then estimated using the following equation:

$$bm = BM \times r \times \left(\frac{MPN_D}{MPN_H} \right) \quad (1)$$

where *BM* is total microbial biomass ($g.kg^{-1}$ soil), *r* the microbial organic C biomass – to – microbial extractable organic C ratio, MPN_D and MPN_H the number of denitrifiers and heterotrophs, respectively ($cfu.g^{-1}$ soil).

THEORY

Microbial Processes

Two groups of denitrifying bacteria have been considered and reduce NO_3^- into N_2O concomitantly to the reduction of N_2O into N_2 (Eq. (2a)), and only the reduction of NO_3^- into N_2O (Eq. (2b)), respectively:

$$2NO_3^- \rightarrow N_2O \rightarrow N_2 \tag{2a}$$

$$2NO_3^- \rightarrow N_2O \tag{2b}$$

Both of the denitrification steps *D* and *R* (i.e. $NO_3^- \rightarrow N_2O$ and $N_2O \rightarrow N_2$) are affected by the biomass of the microbial communities that perform this transformation:

$$V_D = (bm_1 \times v_{D1}) + (bm_2 \times v_{D2}) \tag{3a}$$

$$V_R = bm_1 \times v_{R1} \tag{3b}$$

where V_D and V_R are the actual rates of the first and second denitrification steps (mol $N_2O.kg^{-1}$ $soil.s^{-1}$), v_{D1}, v_{D2} and v_{R1} the specific rates of reactions *D* and *R* for groups *1* and *2* (mol $N_2O.g^{-1}$ $biomass.s^{-1}$), bm_1 and bm_2 the microbial biomass that reduces NO_3^- into N_2 and NO_3^- into N_2O, respectively (g $biomass.kg^{-1}$ soil). The specific rates v_{D1}, v_{D2} and v_{R1} depend on the microbial group through the existence or non-existence of competition between NO_3^- and N_2O reductions. The model assumes that neither the maximum specific rate v_{Dmax} (mol $N_2O.g^{-1}$ $biomass.s^{-1}$) nor the Michaelis constant Km_D (mol $NO_3^-.m^{-3}$ solution) depends on the microbial groups. For microbial group *1*, we assume (non enzymatic) competition between NO_3^- and N_2O as terminal electron acceptors:

$$v_{D1} = v_{D\max} \times \left(\frac{[NO_3^-]}{[NO_3^-] + Km_D} \right) \times f\left([NO_3^-], [N_2O]\right) \tag{4a}$$

$$v_{R1} = v_{R\max} \times \left(\frac{[N_2O]}{[N_2O] + Km_R} \right) \times f\left([NO_3^-], [N_2O]\right) \tag{4b}$$

where v_{Dmax} and v_{Rmax} are the maximum specific rates of reactions *D* and *R* for groups *1* and *2* (mol $N_2O.g^{-1}$ $biomass.s^{-1}$), $[NO_3^-]$ the concentration of NO_3^- in the solution (mol.m^{-3} solution), $[N_2O]$ the concentration of N_2O in the air (mol.m^{-3} air), and *f* a function that indirectly accounts for competition between NO_3^- and N_2O. The specific denitrification rate v_{D2} for microbial group *2* is described by a Michaelis Menten equation:

$$v_{D2} = v_{D\max} \times \left(\frac{\left[NO_3^-\right]}{\left[NO_3^-\right] + Km_D} \right) \quad (5)$$

Assuming that the flux of electrons is below a maximum value F_e (mol e^-.g^{-1} biomass. s^{-1}) depending on the consumable organic matter, we then have:

$$f\left(\left[NO_3^-\right], \left[N_2O\right]\right) = Min\left(1. ; \frac{F_e}{\left(\left(8 \times v_{D1}\right) + \left(2 \times v_{R1}\right)\right)} \right) \quad (6)$$

Where *Min* is for the lower value, 8 and 2 the numbers of electrons transferred through the respiratory chain during the reduction of 2 NO_3^- into N_2O, and N_2O into N_2, respectively. As soon as there are sufficient N oxide reductases, v_{Dmax} and v_{Rmax} can be related to F_e:

$$F_e = 8 \times v_{D\max} = 2 \times v_{R\max} \quad (7)$$

We assume that v_{Dmax} and Km_D do not vary with time, or that variations occur initially over a period short enough to neglect transient changes at the enzyme level and related respiratory chain level (Otte *et al.*, 1996; Baumann *et al.*, 1996). In contrast, three models were alternately used to account for an increase in the ability to reduce N_2O with time:

Model A: there is no induction of N_2O reductase already present, i.e. there is no change in v_{Rmax} and Km_R constants. Changes in bacteria groups *1* and *2* result only from the respective growth of these groups;

Model B: N_2O reductase that was initially absent is induced for each bacteria over a period short enough to consider that the relevant bacteria move suddenly from group 2 to group 1, this last group being characterised by one v_{Rmax} value and one Km_R value only. The flux rate of bacteria from group 2 to group 1 is described by the derivation of a rational empirical function:

$$\left(\frac{\partial bm_2}{\partial t} \right)_m = -\frac{\left(e/\alpha\right) \times \left(t/\alpha\right)^{e-1}}{\left(1 + \left(t/\alpha\right)^e\right)^2} \times \left(bm_{21}\right) \quad (8a)$$

$$\left(\frac{\partial bm_1}{\partial t} \right)_m = -\left(\frac{\partial bm_2}{\partial t} \right)_m \quad (8b)$$

where *m* index is for movement, *t* is time (s), bm_{21} the biomass of bacteria initially belonging to group 2 that progressively move to group 1 (g biomass.kg^{-1} soil), α the time at which 50% of these bacteria reduce N_2O (s),

e an exponent of which the value is as much as high as the transition is sudden at time α;

Model C: changes in the N_2O reductase concentration and other components in the respiratory chain are progressive and identical for all bacteria belonging to group 1. Some calculations show that an increase in the N_2O reductase concentration may induce a decrease in Km_R concomitantly to an increase in v_{Rmax} at low reductase concentrations, or only a decrease in the apparent Km_R value at higher concentrations (see Appendix 1). We consider here only a decrease in Km_R described empirically by a rational function:

$$Km_R = Km_{R0} + \left\{ \left(Km_{R\infty} - Km_{R0} \right) \times \left(\frac{(t/\alpha)^e}{1 + (t/\alpha)^e} \right) \right\} \quad (9)$$

where Km_{R0} and $Km_{R\infty}$ are the initial and final Michaelis constants for N_2O reduction (mol $N_2O.m^{-3}$ air).

Microbial growth first results from the balance between the energy supplied by catabolic activities and the energy required for microbial maintenance (Decker et al., 1970; Stouthamer, 1973). Assuming that the N supply does not limit microbial growth as long as the N_2O concentration evolves, and ignoring variations in organic-C really available for micro-organisms, this balance is written as so:

$$\left(\frac{\partial bm_1}{\partial t} \right)_g = bm_1 \times \left(\left\{ \left(v_{D1} \times Y_D{}^{\max} \right) + \left(v_{R1} \times Y_R{}^{\max} \right) \right\} - m_E \right) \quad (10a)$$

$$\left(\frac{\partial bm_2}{\partial t} \right)_g = bm_2 \times \left(\left\{ v_{D2} \times Y_D{}^{\max} \right\} - m_E \right) \quad (10b)$$

where *t* is the time (s), *g* index is for "growth", $Y_D{}^{max}$ and $Y_R{}^{max}$ are the maximum yields of NO_3^- and N_2O reductions (g biomass produced per mol of N_2O produced and reduced, respectively), and m_E is the energy required for microbial maintenance expressed as an equivalent default in the biomass built per unit of biomass (s^{-1}). Denitrifiers initially unable to reduce N_2O into N_2 can progressively acquire this ability (MacConnaughey et al., 1985; Holtan-Hartwig et al., 2000). The actual evolution of microbial groups *1* and *2* combines growth and fluxes between groups 1 and 2 for *Model B*:

$$\left(\frac{\partial bm_1}{\partial t}\right) = \left(\frac{\partial bm_1}{\partial t}\right)_g - \left(\frac{\partial bm_2}{\partial t}\right)_m \quad (11a)$$

$$\left(\frac{\partial bm_2}{\partial t}\right) = \left(\frac{\partial bm_2}{\partial t}\right)_g + \left(\frac{\partial bm_2}{\partial t}\right)_m \quad (11b)$$

Assuming that the microbial biomass is composed of 15% carbohydrates ($C_6H_{12}O_6$), 10% lipids ($C_{47}H_{96}O_9$) and 75% protides ($C_{16}H_3O_8N_4$) (Vavilin et al., 1994), a fraction f_N (0.1036) of its weight corresponds to N. The model assumes that N originated from NO_3^-. The actual evolution of [NO_3^-] and [N_2O] over time can therefore be written as so:

$$\omega \times \frac{\partial\left[NO_3^-\right]}{\partial t} = -\left(V_D + f_N\left\{\frac{\partial\left(bm_1 + bm_2\right)}{\partial t}\right\}\right) \quad (12a)$$

$$\left(\left(v_a / m_s\right) + \left(\left(\frac{\omega}{\rho_w}\right) \times s_{N_2O}\right)\right) \times \frac{\partial\left[N_2O\right]}{\partial t} = V_D - V_R \quad (12b)$$

where ω is the soil water content (g.g^{-1} dry soil), v_a the volume of air over the slurry in the flask (m^3), m_s the mass of dry soil in the flask (kg), ρ_w the bulk density of water (kg.m^{-3}) and s_{N_2O} the solubility of N_2O (mol N_2O.m^{-3} solution / mol N_2O.m^{-3} air).

Temporal variations in [N_2O] and [NO_3^-] therefore depend on 5 to 7 parameters: initial bm_1 and bm_2 values, specific NO_3^- reduction activity v_D, the Michaelis constant Km_D, either the Michaelis constant Km_R or the initial and final values of this constant (Km_{R0} and $Km_{R\infty}$, respectively), perhaps the biomass bm_{21} of bacteria initially belonging to group 1 that become able to reduce N_2O, and parameters α and e that characterise the induction of N_2O reductase. Total denitrifier biomass bm and the proportion p_R of denitrifiers that can reduce N_2O have been substituted by bm_1 and bm_2 in the *"Results"* and *"Discussion and Conclusion"* sections.

Numerical Simulations and Parameter Estimations

Temporal variations in [NO_3^-], [N_2O], bm_1 and bm_2 were calculated using Euler first order approximation (Nougier, 1983). The parameters to be estimated were obtained by fitting simulated data to experimental data. This was performed by minimizing the following expression, using the Marquardt-Levenberg algorithm (Marquardt, 1963):

$$\chi^2 = \sum_{i=1}^{n}\sum_{j=1}^{n}\left(\left[N_2O\right]_{\exp,i,j} - \left[N_2O\right]_{sim,i,j}\right)^2 \quad (15)$$

where $\left[N_2O\right]_{\exp,i,j}$ and $\left[N_2O\right]_{sim,i,j}$ were the experimental and simulated N_2O concentrations at time *i* for flask *j*, and *n* the total number of experimental time measurements. The following steps were performed successively:

- step 1: from the kinetics of total denitrification (condition 1), we estimated only one total denitrifier biomass value *bm*, one specific denitrifying activity value v_{Dmax} and one Michaelis constant Km_D for NO_3^- reduction (mol NO_3^-.m^{-3} solution) for all the flasks; this operation was repeated for several energy yields and maintenance coefficients. In addition and for the energy yield and maintenance coefficients (arbitrarily) used for the following steps, we estimated denitrifier biomasses in each flask using v_D and Km_D estimated previously;
- step 2: from the kinetics of N_2O net production with no initial N_2O in the flask (condition 2), we estimated only one initial proportion p_R of denitrifiers that reduce N_2O and perhaps the proportion of denitrifiers initially belonging to group 2 that become able to undertake this reduction (*Model B*), either one Michaelis constant for N_2O reduction Km_R or its initial and final values (Km_{R0} and $Km_{R\infty}$ in *Model C*), and either parameter α or parameter *e* describing the progressing ability to reduce N_2O in *Models B* and *C* for specific denitrifying activity v_{Dmax}, the Michaelis constant Km_D and the total denitrifier biomass *bm* estimated during step 1. Using all these estimates except mean microbial biomass *bm*, we then obtained a second estimate of this biomass specific to each flask;
- step 3: for the kinetics of N_2O net production with N_2O initially in the flasks (condition 3), we only compared experimental data with simulated data, using the parameter estimated during steps 1 and 2. Due to some systematic bias between experiments and simulations for this condition, additional tests were performed for higher energy yields for N_2O reduction (by doing step 2 once more). Additional simulations were obtained by estimating the denitrifier biomass specific to each flask using all the other parameters estimated previously.

RESULTS

Condition 1

Nitrous oxide concentration increases nearly linearly with time at the beginning of incubation with a slope similar for all samples (Fig. 1a-c), except for those supplemented with a 0.153 mol NO_3^-.m^{-3} solution. For the 6 flasks supplemented with solutions of low NO_3^- concentrations, [N_2O] stabilises thereafter (Fig. 1a). For these samples, at the end of incubation, NO_3^- and extractable NH_4^+ concentrations were about 0.024 and 0.196 mol N.m^{-3} solution, respectively. No trend was observed between these concentrations and the initial NO_3^- concentration. The sum of final N_2O and NO_3^- concentrations – expressed as equivalent [NO_3^-] in the soil solution – is approximately 90% of the initial NO_3^- concentration, the deviation between the sum of final N_2O and NO_3^- concentrations and the initial NO_3^- concentration increasing from 0.005 to 0.6 mol.m^{-3} with the initial NO_3^- concentrations of the soil solution. For samples supplemented with solutions of high NO_3^- concentrations, [N_2O] increases all the time (Fig. 1b-c). For all samples, the rate of N_2O emission seems to increase with time, although inflexion points could sometimes be detected.

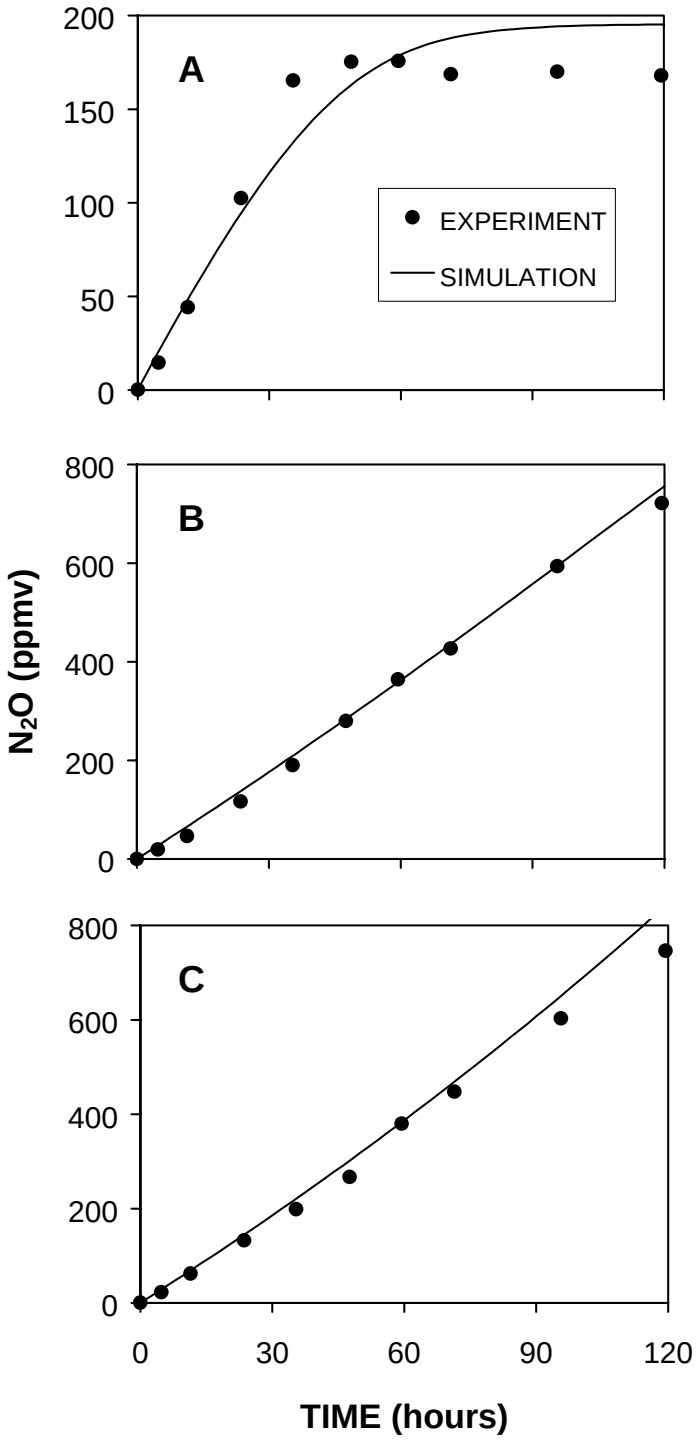

Figure 1*: comparison between experimental and simulated [N_2O] values for condition 1 (i.e. C_2H_2, no N_2O initially) for an energy yield of 1.25 mol ATP per mol of N_2O produced:*

a/ initial [NO_3^-] = 0.17 mol.m^{-3} solution;

b/ initial [NO_3^-] = 0.93 mol.m^{-3} solution;

c/ initial [NO_3^-] = 7.90 mol.m^{-3} solution.

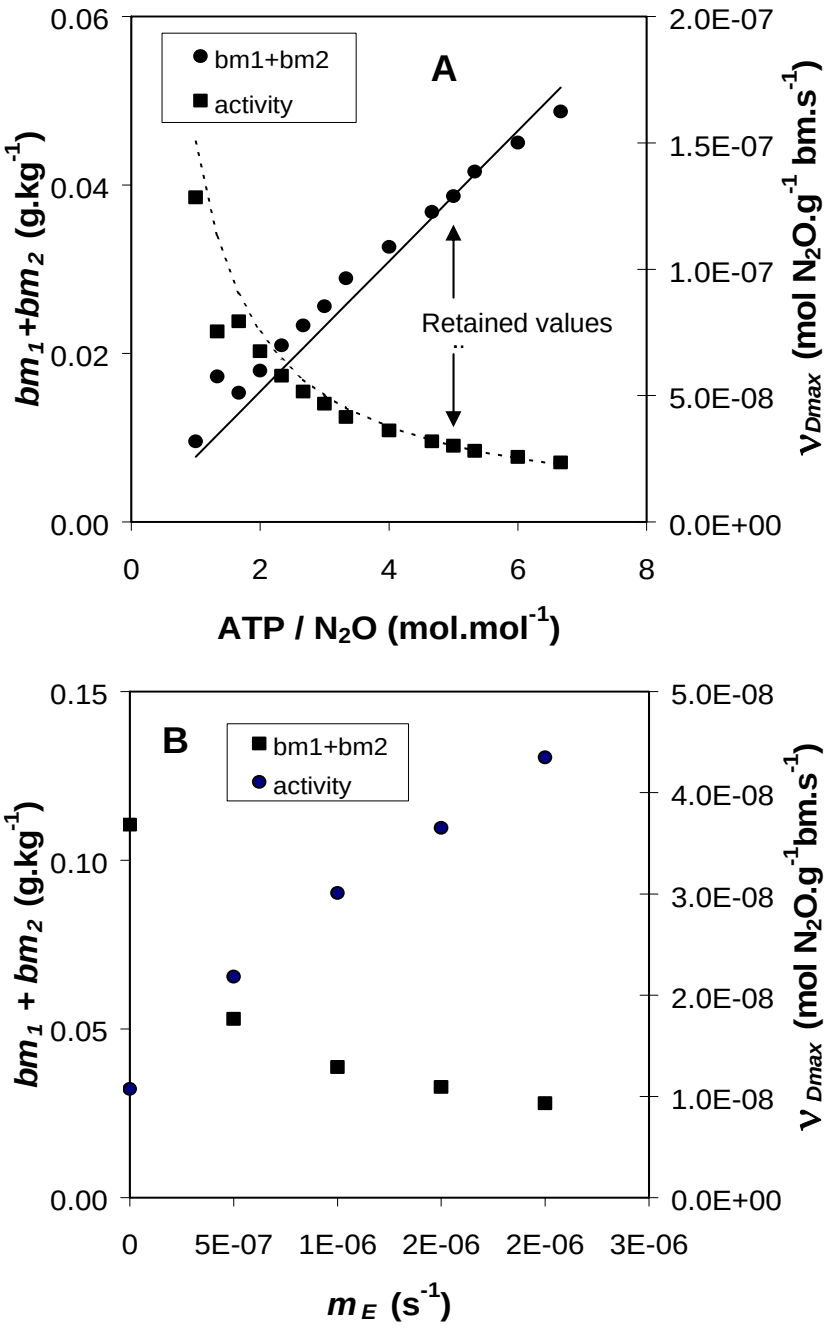

***Figure 2**: effect of the chosen energy yield for N_2O production and energy required for microbial maintenance on estimations of denitrifier biomass bm and maximum specific denitrifying activity v_{Dmax}:*

a/ effect of the energy yield Y_D^{max} for a maintenance coefficient $m_E = 10^{-6}$ s^{-1};

b/ effect of the maintenance coefficient m_E for an energy yield Y_D^{max} of 1.25 mol ATP per mol of N_2O produced.

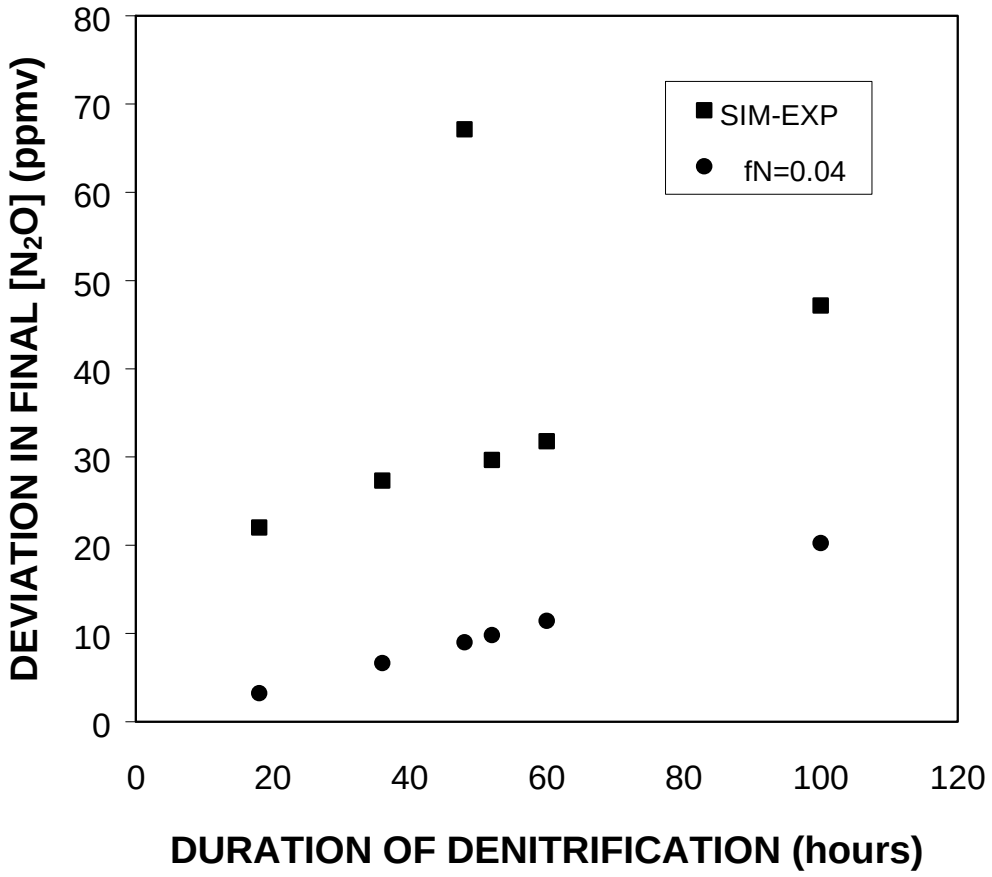

Figure 3: *Deviations between the simulated and experimental [N_2O] values as a function of the time required to reach equilibrium for [N_2O], for the 6 flasks with the lowest initial NO_3^- concentrations, and additional NO_3^- consumption expressed in equivalent defaults in N_2O production for an f_N underestimated by 0.04.*

When fitting experimental data to simulated data, for the 13 incubation flasks in condition 1 for several maximum energy yield values Y_D^{max} and maintenance coefficient values m_E, the energy yield was, as a first approximation, proportional and inversely proportional to the estimates of microbial denitrifying biomass *bm* and specific denitrifying activity v_{Dmax}, respectively (Fig. 2a). Denitrifier biomass estimates ranged between $9.57\ 10^{-3}$ and $4.87\ 10^{-2}$ $g.kg^{-1}$ soil, for the considered energy yields. Moreover, a variation in the maintenance coefficient m_E induced additional variations in the estimates of biomass *bm* and specific denitrifying activity v_{Dmax}, as illustrated in Figure 2b. For the energy yield value used in this study (i.e. 5 mol ATP produced per mol of N_2O produced), simulated [N_2O] data agreed approximately with experimental data (Fig. 1a-c). Nevertheless, two types of bias were observed between experiments and simulations. On the one hand, when [N_2O] stabilized before the end of the experiment, final simulated [N_2O] values were higher than experimental values (see for example Fig. 1a). The deviation between final experimental and simulated [N_2O] was linearly correlated with the time required to reach [N_2O] stabilisation (results not shown), with an increase of approximately 0.30 ppmv N_2O per additional hour (Fig. 3). On the other hand, additional deviations in the rate of N_2O production between experiments and simulations suggest variations in microbial characteristics between flasks. Using the previously estimated specific denitrifying activity value v_{Dmax} and Michaelis constant Km_D for the energy yield value used in this study, we estimated the microbial biomass *bm* separately for each flask: its average and standard deviation values were $4.56\ 10^{-2}$ and $2.23\ 10^{-2}$ $g.kg^{-1}$ soil, respectively; no correlation was detected between the biomass estimate and the initial [NO_3^-] of the flask (Fig. 7a). Nevertheless, one flask had a biomass estimate of $11.9\ 10^{-2}$ $g.kg^{-1}$ soil; ignoring this value would have led to average and standard deviation values of $3.95\ 10^{-2}$ and $0.32\ 10^{-2}$ $g.kg^{-1}$ soil, respectively. Considering the energy yield Y_D^{max}, the specific denitrifying activity v_{Dmax} and the Michaelis constant Km_D for NO_3^- reduction used for this study, we calculated the uncertainty in NO_3^- incorporated into the microbial biomass (expressed as an equivalent default in N_2O production) as a function of time for an increase of 0.04 in f_N: the deviation between final experimental and simulated N_2O concentrations for the 6 flasks with the lowest initial NO_3^- concentrations increases during the time required to reach equilibrium (Fig. 3) at a rate of 0.21 ppmv $N_2O.h^{-1}$; it is approximately 20 ppmv lower than the deviation between experimental and simulated N_2O concentrations after equilibrium has been reached. This last deviation corresponds approximately to 0.016 mol $NO_3^-.m^{-3}$ solution.

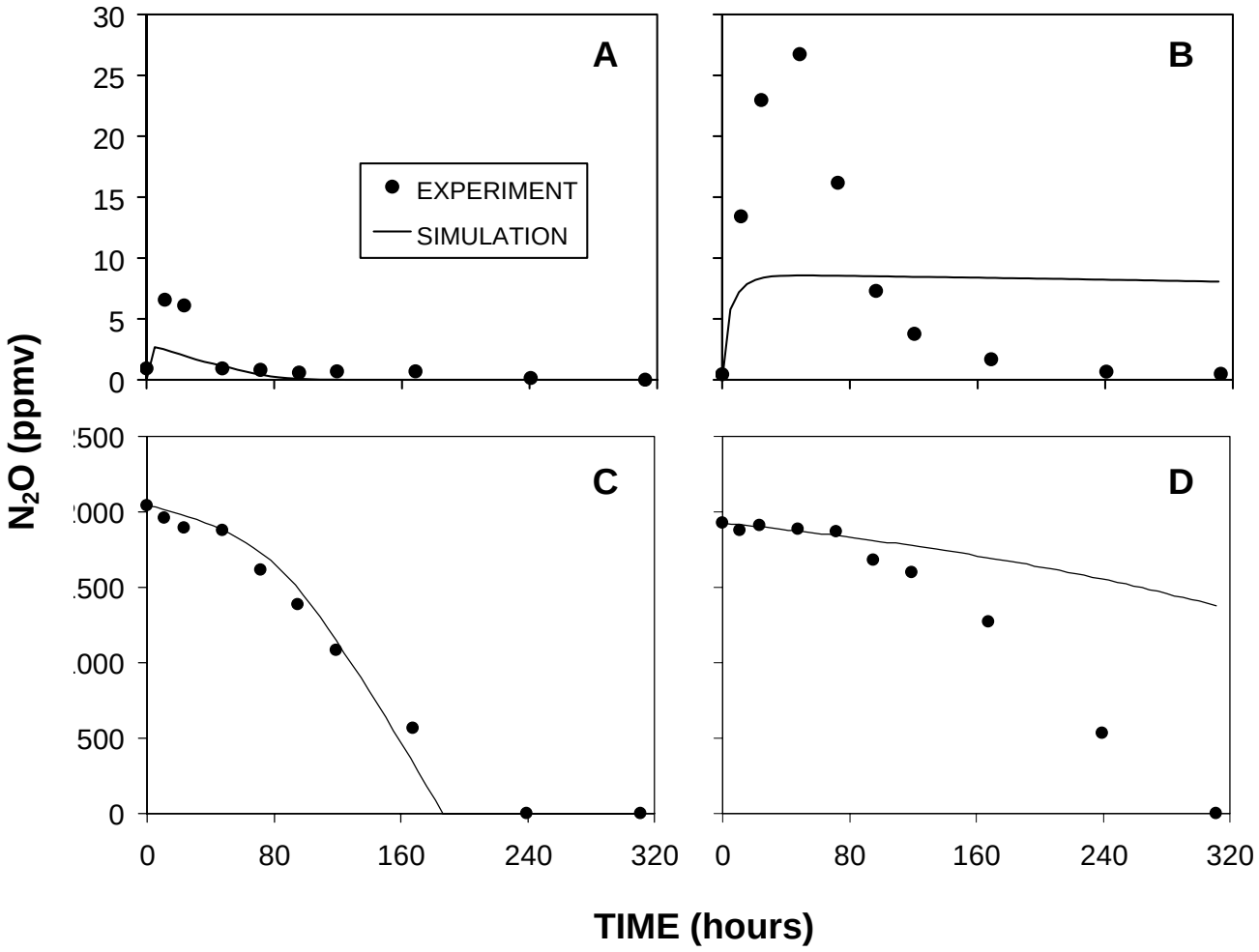

***Figure 4**: Experimental and fitted data simulated by model A, using estimated parameters reported in table 1:*

a/ condition 2, initial $[NO_3^-]$ = 0.23 $mol.m^{-3}$ solution;

b/ condition 2, initial $[NO_3^-]$ = 7.90 $mol.m^{-3}$ solution;

c/ condition 3, initial $[NO_3^-]$ = 0.23 $mol.m^{-3}$ solution;

d/ condition 3, initial $[NO_3^-]$ = 7.90 $mol.m^{-3}$ solution.

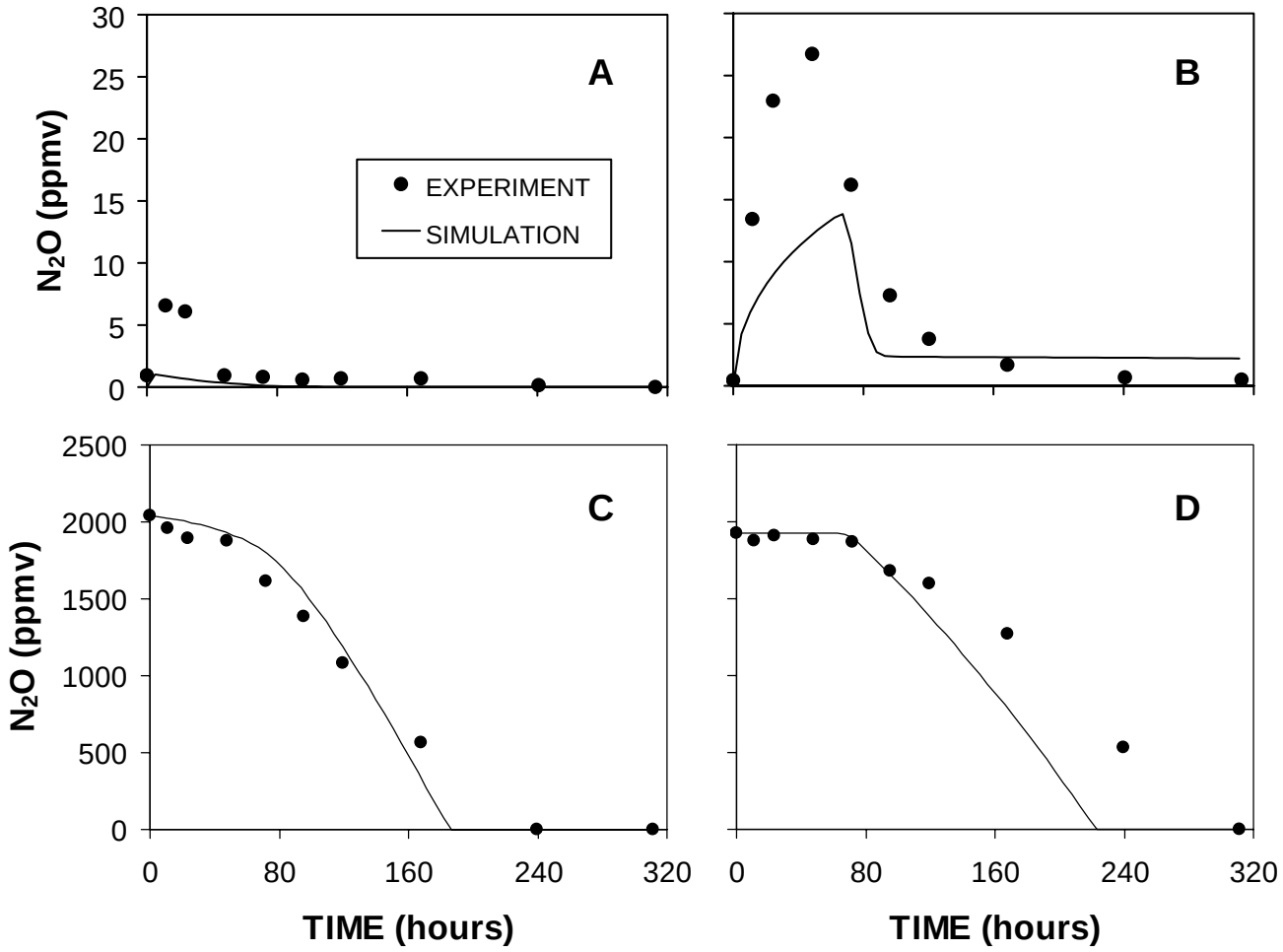

***Figure 5**: Experimental and simulated data simulated by model B, using estimated parameters reported in table 1:*

a/ condition 2, initial $[NO_3^-]$ = 0.23 mol.m^{-3} solution;

b/ condition 2, initial $[NO_3^-]$ = 7.90 mol.m^{-3} solution;

c/ condition 3, initial $[NO_3^-]$ = 0.23 mol.m^{-3} solution;

d/ condition 3, initial $[NO_3^-]$ = 7.90 mol.m^{-3} solution.

Condition 2

Two flasks in condition 2 (i.e. initial 0.125 and 1.657 mol $NO_3^-.m^{-3}$) were unfortunately enriched in N_2O (gases were sampled in these flasks with a single syringe just after sampling condition 3), and were therefore not included in the subsequent analysis. For other flasks, $[N_2O]$ increased with time at the beginning of incubation until 12-48 h, and then decreased (Fig. 4a-b). The time discriminating between the initial $[N_2O]$ increase and subsequent decrease was around 12 h for low initial $[NO_3^-]$, then progressively increased up to 48 h for the highest initial $[NO_3^-]$. The increase in $[N_2O]$ estimated over the first 12 hours varied between about 9% and 22% of the initial increase in $[N_2O]$ of corresponding samples in condition 1, for initial $[NO_3^-]$ of 0.31 and 7.9 $mol.m^{-3}$, respectively. The rate of the subsequent decrease in $[N_2O]$ was firstly of the same order of magnitude as the initial increase, and thereafter became small with near stabilisation of $[N_2O]$ at about 0.5-2 vppm. At the end of the experiment, $[NO_3^-]$ had nearly totally disappeared for samples with low initial NO_3^- concentrations, whereas it remained about 72.5% and 75.3% of the initial $[NO_3^-]$ for samples supplemented with 5.56 and 7.91 mol $NO_3^-.m^{-3}$ solution, respectively.

We successively assessed the capability of *Models A, B and C* to simulate experimental data and concomitantly estimate the model parameters, using values for mean denitrifier biomass *bm* (i.e. the sum bm_1+bm_2), specific denitrifying activity v_{Dmax} and the Michaelis constant km_D for NO_3^- reduction estimated previously from experimental data obtained in condition 1. Parameter estimates were:

- *Model A:* the proportion p_R of denitrifiers that can reduce N_2O, and the Michaelis constant Km_R for the reduction of N_2O into N_2;
- *Model B:* the initial proportion p_R of denitrifiers that can reduce N_2O and the proportion of denitrifiers that become capable of reducing N_2O, α and e coefficients that describe the rate at which the ability to reduce N_2O is acquired and the Michaelis constant Km_R for the reduction of N_2O into N_2;
- *Model C:* the proportion p_R of denitrifiers that can reduce N_2O, the initial and final Michaelis constant values Km_{RO} and $Km_{R\infty}$, respectively, and α and e coefficients that describe the rate at which the ability to reduce N_2O is acquired.

This procedure was performed for an energy yield of N_2O consumption equal to one quarter of the energy yield of N_2O production. However, for model C, it was repeated for a higher energy yield of N_2O reduction. Using *Model A*, it was not possible to correctly fit simulations

to experimental data: at low initial [NO_3^-], the simulated maximum N_2O concentrations remained lower than experimental values (Fig. 4a) and its subsequent decrease resulted from NO_3^- disappearance, whereas [N_2O] remained high (Fig. 4b) as long as [NO_3^-] was high enough for soils with high initial NO_3^- concentrations. Using all the previously estimated parameters, except "mean" denitrifier biomass *bm*, we estimated the *bm* value individually for each flask: its average and standard deviation were 0.155 and 0.197 $g.kg^{-1}$ soil, respectively. The average was thus significantly higher than the previously estimated biomass; moreover, there was a significant increase in the *bm* estimate with the initial [NO_3^-] of the flasks (Fig 7a). *Model B* fitted simulations to experimental data better than model A (Fig. 5a-b): especially at high initial NO_3^- concentrations, it simulated a decrease in [N_2O] although NO_3^- did not disappear (Fig. 5b). However, simulated [N_2O] remained lower than experimental values. Using all the previously estimated parameters, except "mean" denitrifier biomass *bm*, we estimated this last value individually for each flask: its average and standard deviation were equal to 0.088 and 0.108 $g.kg^{-1}$ soil, respectively. As for model A, the average was thus significantly higher than the previously estimated biomass, and there was a significant increase in the *bm* estimate with the initial [NO_3^-] of the flasks (Fig. 7a). In contrast to models A and B, model C made it possible to approximately simulate [N_2O] evolution over time for all the range of initial [NO_3^-] values, although simulated [N_2O] remained generally lower than experimental values (Fig. 6a-b). Increasing the energy yield of N_2O reduction induced little change in simulated [N_2O] as well as parameter estimates (Table 1), except for the exponent *e* for which large variations in this range of values only slightly affected [N_2O] evolution over time. Using all the previously estimated parameters, except "mean" denitrifier biomass *bm*, we estimated this last value individually for each flask: its average and standard deviation were $2.31\ 10^{-2}$ and 0.019 $g.kg^{-1}$ soil, respectively. In addition, trends in individual biomass estimates were noted only for low initial N concentrations (Fig. 7b); in this range of initial [NO_3^-] values, large variations in *bm* did not greatly affect simulated [N_2O] (results not shown).

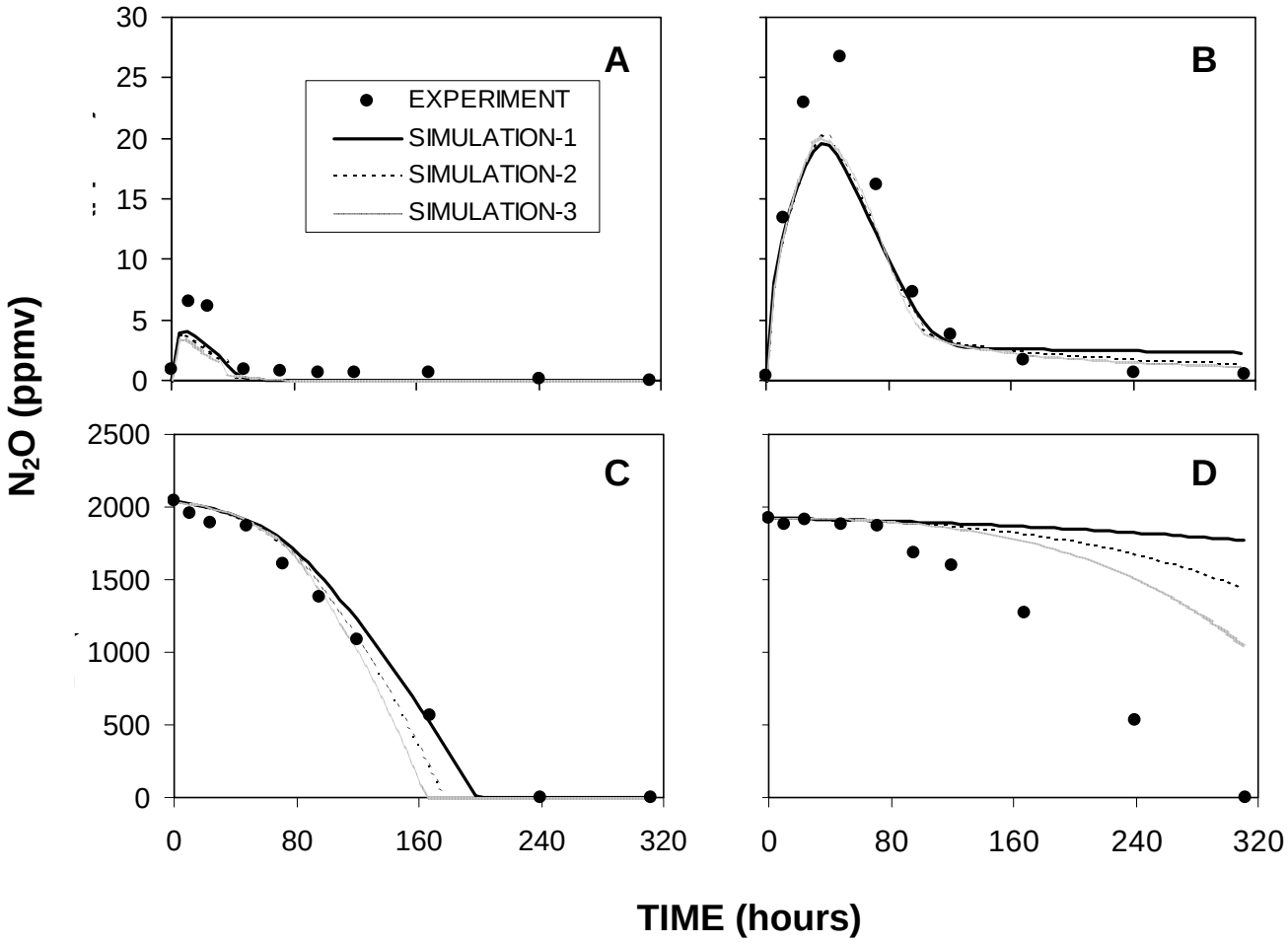

***Figure 6**: Experimental and simulated data simulated by model C, using estimated parameters reported in table 1 (simulations 1, 2 and 3 are for Y_R^{max} equal to 2.5, 3.33 and 4.17 mol ATP.mol N_2O, respectively):*

a/ condition 2, initial $[NO_3^-]$ = 0.23 mol.m^{-3} solution;

b/ condition 2, initial $[NO_3^-]$ = 7.90 mol.m^{-3} solution;

c/ condition 3, initial $[NO_3^-]$ = 0.23 mol.m^{-3} solution;

d/ condition 3, initial $[NO_3^-]$ = 7.90 mol.m^{-3} solution.

Condition	Model	v_{Dmax}	Km_D	bm	P_R	*Initial* p_R	*Final* p_R	Km_R	*Initial* Km_R	*Final* Km_R	α	e
		-°mol $N_2O.g^{-1}.s^{-1}$ -	- $mol.m^{-3}$ -	- $g.kg^{-1}$ -				- $mol.m^{-3}$ -	- $mol.m^{-3}$ -	- $mol.m^{-3}$ -	-- s --	
1	-	$3.01\ 10^{-8}$	$5.82\ 10^{-2}$	$3.87\ 10^{-2}$	-	-	-	-	-	-	-	-
2	A	-	-	-	0.468			$1.29\ 10^{-4}$	-	-	-	-
	B	-	-	-	-	0.401	0.626	$2.99\ 10^{-5}$	-	-	$2.50\ 10^{+5}$	58.10
	C†	-	-	-	0.415			-	$1.46\ 10^{-4}$	$1.12\ 10^{-5}$	$1.36\ 10^{+5}$	9.29
	C‡		-	-	0.404				$1.27\ 10^{-4}$	$2.59\ 10^{-5}$	$1.35\ 10^{+5}$	40.24
3	A	-	-	-	-			-	-	-	-	-
	B	-	-	-	-			-	-	-	-	-
	C†	-	-	-	-			-	-	-	-	-
	C‡		-	-								

***Table 1**: parameters estimated by fitting simulations to experimental data for an energy yield Y_D^{max} and a maintenance coefficient m_E of 1.25 mol ATP.mol N_2O produced and 10^{-6} s^{-1}, respectively.*

†: Y_R^{max} for N_2O reduction of 2.5 mol $ATP.mol^{-1}$ N_2O

‡: Y_R^{max} for N_2O reduction of 3.33 mol $ATP.mol^{-1}$ N_2O

Condition 3

Initially, [N_2O] slowly decreased with time at the beginning of incubation until 24-96 h, then decreased more drastically. For samples supplemented with solutions of low [NO_3^-], the duration of the initial small [N_2O] decrease and the time required for almost all N_2O to be consumed were positively correlated with the initial [NO_3^-]. The final [NO_3^-] was about 0.024 mol.m^{-3} soil solution for these samples. In contrast, at high [NO_3^-], the duration of the initial small [N_2O] decreased and the time required for almost all N_2O to be consumed did not seem to depend on the initial [NO_3^-]. The final [NO_3^-] was about 4.06 and 6.00 mol.m^{-3} soil solution for flasks at initially 5.59 and 7.90 mol.m^{-3}, respectively.

We successively assessed the capability of models A, B and C to simulate experimental data, using all the parameters previously estimated from batch experiments in conditions 1 and 2, or using all these parameters except denitrifier biomass *bm* that was then estimated once more individually for each flask. At low initial NO_3^- concentrations, [N_2O] simulated by models A, B and C were approximately in agreement with the experimental [N_2O] value and nearly identical to each other (Fig. 4c, 5c and 6c). At the end of incubation, there was nearly no more NO_3^- in these flasks. In contrast, at high initial [NO_3^-], simulated [N_2O] values varied between models: in particular, model B can simulate the decrease in [N_2O], although [NO_3^-] remains high (Fig. 5d), whereas models A and C seemed to be unable to correctly reflect this decrease (Fig. 4d, 5d and 6d). Using model C with a higher energy yield for N_2O reduction made it possible to decrease [N_2O] more rapidly (Fig. 6d). Additional simulations with higher energy yield values for N_2O reduction made it possible to accentuate this behaviour. Unfortunately, it was not possible to correctly reflect these experimental data with an acceptable value for the energy yield of N_2O reduction. Using all the previously estimated parameters in conditions 1 and 2, except "mean" denitrifier biomass *bm*, we estimated this last value individually for each flask: its average and standard deviation were 6.30 10^{-2} and 0.033 g.kg^{-1} soil respectively for model A, 4.34 10^{-2} and 0.007 g.kg^{-1} soil respectively for model B and 9.67 10^{-2} and 0.091 g.kg^{-1} soil respectively for model C. In addition, there was a significant increase in the *bm* estimate with the initial NO_3^- concentration of the flasks for models A and C (Fig. 8a). In contrast, no correlation was detected between the biomass estimate and the initial [NO_3^-] of the flask for model B (Fig. 8a).

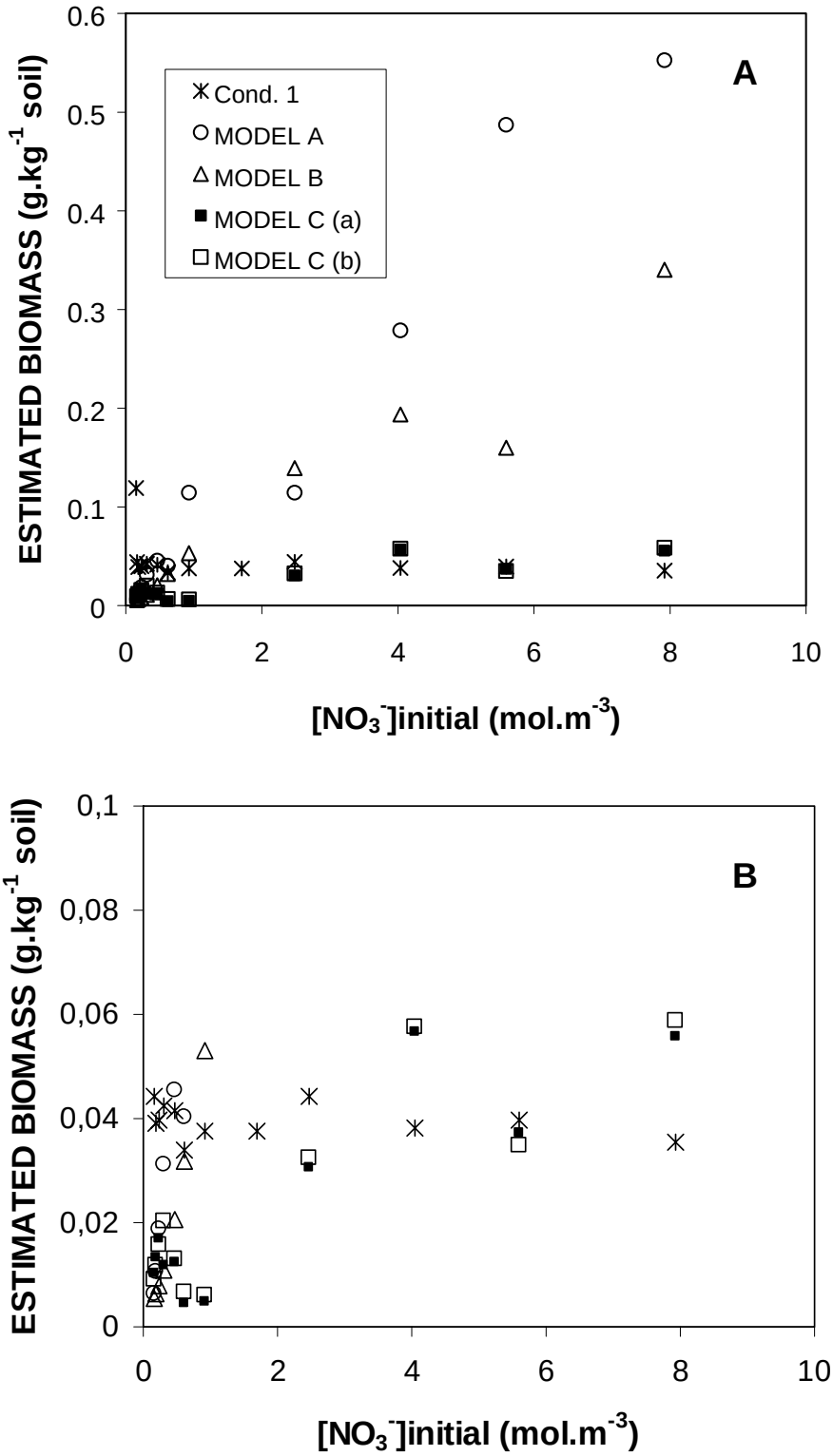

***Figure 7**: a/ total denitrifier biomass estimated by fitting simulations to experimental data for conditions 1 and 2 using models A, B and C. For model C, (a) for an energy yield Y_R^{max} for N_2O reduction of 2.5 mol ATP.mol^{-1} N_2O and (b) an energy yield Y_R^{max} for N_2O reduction of 3.33 mol ATP.mol^{-1} N_2O, respectively. b/ a zoomed-in representation of figure 7a.*

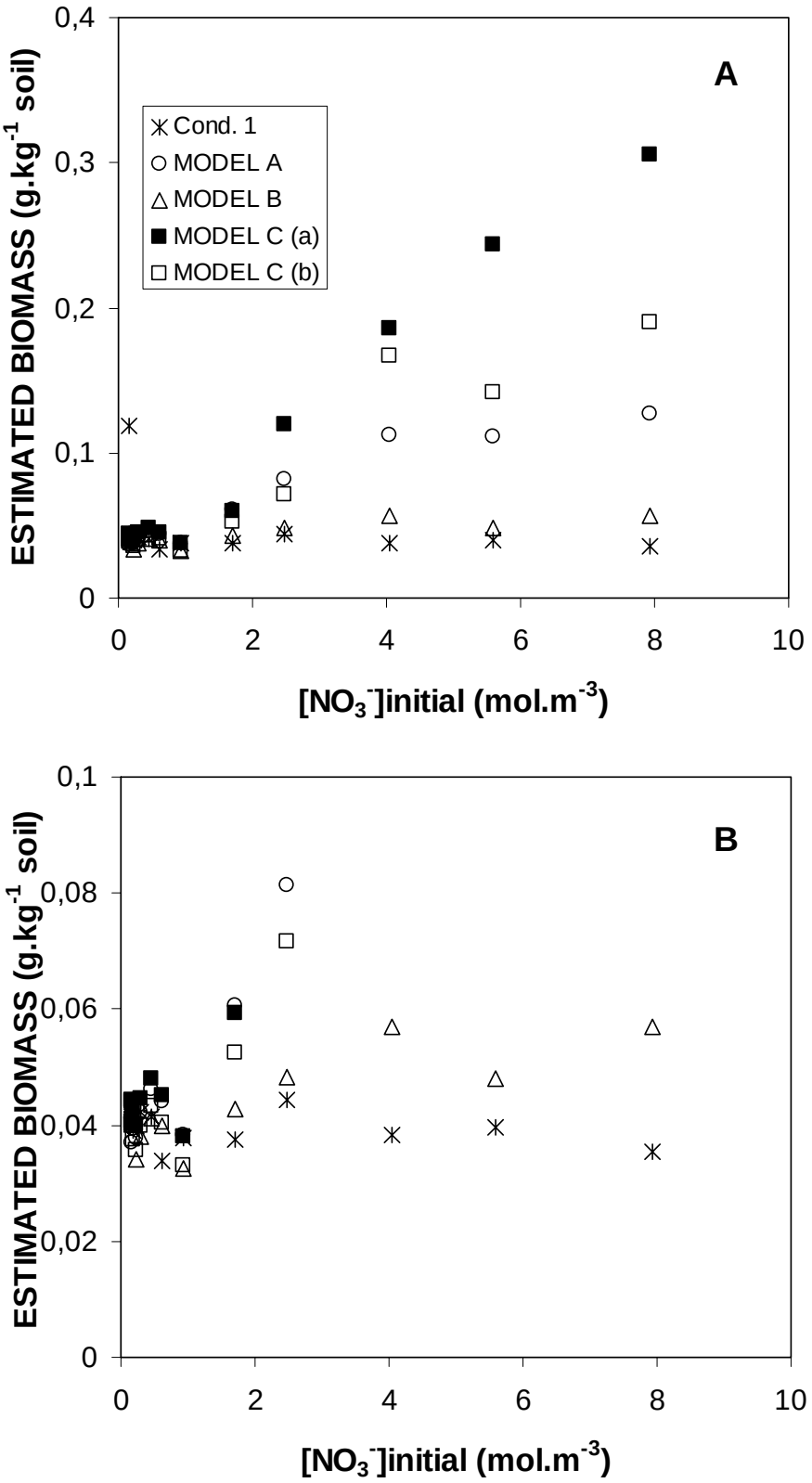

Figure 8: *a/ total denitrifier biomass estimated by fitting simulations to experimental data for conditions 1 and 3 using models A, B and C. For model C, (a) for an energy yield Y_R^{max} for N_2O reduction of 2.5 mol ATP.mol^{-1} N_2O and (b) an energy yield Y_R^{max} for N_2O reduction of 3.33 mol ATP.mol^{-1} N_2O, respectively. b/ a zoomed-in representation of figure 8a*

Direct Estimate of Denitrifier Biomass

The measured organic-C in the microbial biomass was 154 (±6) mg C. kg^{-1} soil, corresponding approximately to 300 mg total biomass per kg of dry soil. Microbial enumeration gave an indirect estimate of 4.32% of denitrifiers; this is the ratio of 6.56 10^6 denitrifier cfu.g^{-1} soil (standard deviation: 2.78 10^6) to 1.52 10^8 heterotroph cfu.g^{-1} soil (standard deviation: 3.08 10^7). Combining total microbial biomass with the number fraction of denitrifier in this biomass gave a denitrifier biomass value of about 13.7 mg.kg^{-1} soil.

DISCUSSION

The aims of this study were to (i) propose a denitrification model that accounts for microbial activities and microbial dynamics, including the progressing ability of denitrifiers to reduce N_2O, (ii) propose a method to estimate its parameters, and (iii) check the model and the method with a set of experimental data. Several conclusions from this study result from experimental data, whereas others result from the assessment of model relevancy.

Experimental Data

Although only 25 g fw soil were placed in each flask, we did not observe any irregular trends of N_2O emissions with initial $[NO_3^-]$, except for a small number of flasks, suggesting that the experimental procedure made it possible to minimize variability between flasks. During batch incubation, denitrification explained all evolved NO_3^-, since no trend was observed between final extractable $[NH_4^+]$ and initial slurry $[NO_3^-]$. Therefore, NH_4^+ production resulted probably only from mineralization, and $[NH_4^+]$ had not to be accounted for in order to balance evolved NO_3^-. Due to the imperfect characterisation of final $[NO_3^-]$ and $[NH_4^+]$ for conditions 2 and 3, the balance was calculated only for condition 1: the deviation between the sum of final N_2O and NO_3^- concentrations and the initial NO_3^- concentration increased from 0.005 to 0.6 $mol.m^{-3}$ with the initial NO_3^- concentration of the soil solution. This probably resulted from N-immobilisation due to the increase in denitrifier biomass. Assuming 10% N within the dry biomass, it would correspond to a biomass increase of between 1.2 and 149 mg $biomass.kg^{-1}$ soil during batch incubation. This could explain an increase in the rate of N_2O emission with time, and suggests that microbial dynamics should be accounted for in models over periods greater than about 100 h. It seems that soil can reduce N_2O from the beginning, since the increase in $[N_2O]$ estimated from the first 2 measurements in condition 2 was approximately only 9-22% of the initial increase in $[N_2O]$ for corresponding dates and samples in condition 1. For incubation in condition 2 with the two highest initial NO_3^- concentrations, the final NO_3^- concentrations were approximately 72.5% and 75.3% of initial $[NO_3^-]$, respectively: assuming that these final NO_3^- concentrations did not limit the first denitrification step (i.e. $NO_3^- \rightarrow N_2O$) due to their high values with regard to literature estimates of Km_D (Malhi *et al.*, 1990, Zumft *et al.*, 1997) and did not

significantly affect possible competition between NO_3^- and N_2O as terminal electron acceptors for respiratory chains. It is not possible to explain why [N_2O] suddenly fell after approximately 12 to 48 h, without accounting for induced additional N_2O reductase synthesis, as has already been evidenced by direct measurements (Otte *et al*., 1996). Such an interpretation seemed to be confirmed by similar observations in condition 3 for the two highest initial NO_3^- concentrations: after approximately 72 h, there was a great increase in N_2O net consumption, although [NO_3^-] remained high at the end of incubation for the two highest initial [NO_3^-] values. Although an increase in enzyme concentration is often accounted for by an increasing maximum rate of reaction, the actual situation for N_2O reductase may be more complex due to (i) possible competition between NO_3^- and N_2O as terminal electron acceptors for respiratory chains (Cho and Sakdinan, 1978), and (ii) N_2O reduction that not only involves periplasmic N_2O reductase but also membrane-bound electron carriers that reduce the enzyme. As an indication of the complex reality, Michaelis constant values Km_R estimated experimentally from soil slurries (Holtan-Hartwig *et al*., 2000) were lower than Km_R values estimated from purified enzymes (Zumft, 1997).

Modeling Denitrification Processes

Model Hypotheses

Some model assumptions were made in order to describe the effect of NO_3^- on N_2O reduction (Blackmer and Bremner, 1979; Terry and Tate, 1980; Gaskell *et al*., 1981; Nömmik *et al*., 1984; Weir *et al*., 1993; Luo *et al*., 1996) and the possible effect of N_2O on NO_3^- reduction (Guérin, 1999): (i) respiration through denitrification is bound by a maximum flux of e^- through the respiratory chain, which is distributed to all e^- acceptors, and (ii) competition between NO_3^- and N_2O reductions exists when the e^- flux calculated without accounting for this competition exceeds its maximum value. In this situation, both NO_3^- reduction and N_2O reduction is proportionally reduced. In addition, the model was simplified with regard to actual processes in order to facilitate parameter estimation. Nitrite was ignored as the intermediate product in the reduction of NO_3^- into N_2O; NO_3^- reducers that only undertake NO_3^- reduction were therefore ignored. Nitrate reducers are often more abundant than denitrifiers (Vinther et al., 1999). We can assume that NO_3^- reducer activity can increase the NO_2^- reduction activity of actual denitrifiers and thus lead to biased estimates in *bm* and/or v_{Dmax}, see to have v_{Dmax} values specific to groups 1 and 2. It is possible to ignore NO_2^- as the

intermediary product if either [NO_2^-] is negligible (i.e. NO_3^- reduction into NO_2^- limits NO_3^- reduction into N_2O) (Mariotti *et al*., 1981; Dendooven and Anderson, 1994) or NO_3^- reduction into NO_2^- is fast enough to consider equilibrium between [NO_3^-] and [NO_2^-] (Stevens et al, 1998). However, it has been observed that NO_2^- temporarily accumulates in soils, particularly at high levels of ammonium and high pH (Burns et al., 1996; Van Cleemput and Samater, 1996; Smith et al., 1997 and Ellis et al, 1998); Nakajima et al. (1984) and Almeida *et al*. (1995) observed, both in pure and in mixed culture, that the NO_3^- reduction rate was twice as fast to the NO_2^- reduction rate independently of NO_3^- or NO_2^- concentration. In addition, we simultaneously neglected micro-organisms that only reduce N_2O, as they seem to be really insignificant (Firestone and Tiedje, 1979). We ignored the induction time required for NO_3^- and NO_2^- reductions, see the lag phase for NO_3^- reduction into N_2O, whereas we explicitly described this process for N_2O reduction. Indeed, NO_3^- and NO_2^- reductases are synthesized under aerobic conditions (Patureau *et al*, 1996); in contrast, *de novo* synthesis of N_2O reductase only starts 16-33 h after depletion of O_2 (Firestone and Tiedje, 1979; MacConnaughey *et al*., 1985; Dendooven and Anderson, 1994). In addition, it has often been observed that initial N_2O production is minor in the first 2-5 h, whereas the N_2O production rate stabilises thereafter at a higher rate (Dendooven and Anderson, 1994). The duration of this pseudo lag phase was short with regard to the total duration of our batch incubation and can a priori be neglected as a first approximation. As already discussed in the *"Conclusions from Experimental Data"* section and in Appendix 1, we assume that the effective Michaelis constant Km_R decreases with an increasing N_2O reductase concentration, whereas we neglect effects on the maximum N_2O reduction rate v_{Rmax}.

Impacts of Preliminarily Fixed Parameters

Values of some of the model parameters were preliminarily fixed, including energy yield values Y_D^{max} and Y_R^{max} (i.e. 5 and 1.25 mol ATP.mol^{-1} N_2O, respectively) and the maintenance coefficient m_E (10^{-6} s^{-1}). The maximum energy yield Y_D^{max} varied with the type of C substrate (among other factors): the lowest values (about 2.7 mol ATP.mol^{-1} N_2O) corresponded to the use of substrates such as succinate, whereas the highest ones (about 5.3-6.7 mol ATP.mol^{-1} N_2O) corresponded to substrates such as glucose or malate (Stouthamer, 1988). With regard to the present knowledge on respiratory chains involved in denitrification (Stouthamer, 1888), there is no justification for the Y_D^{max} – to – Y_R^{max} ratio to differ from 4 (i.e. the ratio of the numbers of e^- transferred during the two denitrification steps): only the location of physical

binding with the respiratory chain differing between e^- donors (e.g. NADH+H^+, H_2, succinate, etc.) can affect this ratio, and it remains unproved that the reduction of NO into N_2O is linked to ATP production (Stouthamer, 1988 ; Van spanning et al., 1995, Zumft, 1997 and Richardson, 2000). The range of maintenance coefficient values (i.e. between 4.72 and 5 of 10^{-6} g-moles ATP/g dry weight of organisms/s) has often been suggested for heterotrophs (Stouthamer, 1973). This is why additional *bm* and ν_{Dmax} estimates were made for various Y_D^{max} values between 0.25 and 1.67 mol ATP.mol^{-1} N_2O. As a first approximation, it was proportional and inversely proportional to *bm* and ν_{Dmax} estimates, respectively (Fig. 2a). This can be easily understood considering that each time, (i) on the one hand, the increase in [N_2O] directly depends on the $bm \times \nu_D$ product, (ii) on the other hand, the relative increase in *bm* (i.e. $\partial bm/bm\partial t$) is a function of the $Y_D^{max} \times \nu_D$ product (see Eq. 10b). Therefore, from a theoretical point of view, it is possible to simulate nearly the same [N_2O] evolution over time by multiplying Y_D^{max} and *bm* by a single constant, and concomitantly dividing ν_{Dmax} by the same constant. The effect of the maintenance coefficient m_E first results from the need for the model to correctly reflect the relative increase in microbial biomass *bm* (i.e. $\partial bm/bm\partial t$), which explains the non linear increase in [N_2O], as long as [NO_3^-] is not a limiting factor: an increase in this maintenance coefficient thus induces an increase in maximum specific denitrifying activity ν_{Dmax} and, as a consequence, a decrease in the denitrifier biomass estimate. It was not possible to sufficiently increase N_2O reduction with regard to NO_3^- reduction by decreasing the $Y_D^{max} - \text{to} - Y_R^{max}$ ratio in *Model C* to correctly simulate experimental data for condition 3 and high initial NO_3^- concentrations. Changes in Y_D^{max}, Y_R^{max} or m_E did not improve the fit of simulated data to experimental ones. However, the energy yield of N_2O reduction slightly affected parameter estimates (Table 1) and simulations (Fig. 6a-d).

Ability of the Models to simulate Experimental Data

The capability of *Models A, B and C* to reflect experimental data were assessed for each condition. With respect to condition 1, the model is unique. We noted two types of bias between experiments and simulations. On the one hand, the simulated [N_2O] equilibriums were higher than the actual ones. This could probably not be explained by the dissimilatory reduction of NO_3^- into NH_4^+, since there was no correlation between final [NH_4^+] and initial [NO_3^-]: thus, [NH_4^+] probably results from the anaerobic mineralization of organic-N. In

contrast, the increase in the deviation between measured and simulated final N_2O concentrations was directly related to the time required to reach equilibrium, and such an increase could be explained if the weight proportion f_N of N in microbial biomass was underestimated by approximately 0.04 for the energy yield Y_D^{max} used (see Fig. 3). Such an underestimation of f_N is possible: in this study, we used 0.1036 of f_N as suggested by Vavilin et al. (1994), whereas estimates of around 0.14 have been proposed in other studies (Stouthamer, 1988). Nevertheless, a deviation of approximately 20 ppmv between measured and simulated [N_2O] equilibrium values remain unexplained by accounting for a bias in f_N estimation. This value may result from the [NO_3^-] not consumed during batch incubation, which cannot be simulated by Michaelis approximation of real microbial activities. On the other hand, simulations reflected experimental data imperfectly, due to the probable variability in microbial characteristics between flasks. With respect to condition 2, *Model A* would have been the natural candidate to easily describe N_2O emissions if there was no increase in the ability to reduce N_2O, whereas such an increase was noted from experimental data. This increase was confirmed by the incapability of *Model A* to simulate the rapid drop in [N_2O] falling down after an initial increase for all samples, including those in which [NO_3^-] remained much higher than the Michaelis constant Km_D (e.g. in Fig. 5b). *Models B* and *C* are 2 different alternatives with which to account for this increase that could result from N_2O reductase induction. The comparison between these 2 models (Fig. 6a-b, 7a-b, and 8a-b) clearly indicated that *Model C* was more appropriate for reflecting experimental data. Firstly, it better simulates [N_2O] evolution over time. Secondly, biomass values estimated separately for each flask using this model were less variable and exhibited a less pronounced trend with initial [NO_3^-]. However, we still noted an increase in these estimates with the low initial [NO_3^-]. These low estimates at low initial [NO_3^-] have to be considered carefully: in this range of initial [NO_3^-] values, simulated [N_2O] evolution over time varied only slightly with initial denitrifier biomass (results not shown). With respect to condition 3, *Models A, B and C* can approximately reflect experimental [N_2O] at low initial [NO_3^-]: the decrease in [N_2O] with time results from the disappearance of NO_3^- (Fig. 5c, 6c and 7c). In contrast, at high initial NO_3^- concentrations, *Model B* was more appropriate for reflecting experimental data. Firstly, it better simulates [N_2O] evolution over time (Fig. 5d, 6d, and 7d); secondly, biomass values estimated separately for each flask using this model were less variable and exhibited a less pronounced trend with initial [NO_3^-] (Fig. 8a-b). Using model C with a higher energy yield made it possible to decrease [N_2O] more rapidly (Fig. 7d).

Estimation of each parameter by fitting simulations to experimental data was compared to

other estimations taken from this work or from the literature. The range of *bm* values obtained (9.57-48.7 mg.kg^{-1}soil) for various energy yields Y_D^{max} was in agreement with its estimate resulting from fumigation-extraction and enumeration (13.7 mg.kg^{-1}soil), in the knowledge that there were considerable uncertainties for this last estimate (Cochran, 1949; Wu et al., 1990). Numerous authors have measured total biomass for soils, including Lensi et al. (1995) in continuous cultivation (0.885 g.kg^{-1}) and in permanent pasture (2.24 g.kg^{-1}), Kieft et al. (1987) in grassland soil (2.07 g.kg^{-1}), Ross et al. (1992) in forest soil (1.03 g.kg^{-1}), and Lovel et al. (1998) in permanent pasture (1.72 g.kg^{-1}). Some have also proposed estimates of the proportion of denitrifiers among heterotrophs of about some percent. Denitrifying activity has been preferentially referred to soil amounts rather than denitrifier amounts. It then corresponds to the product $v_D \times bm$, for which the estimate of the maximum $v_{Dmax} \times bm$ was 11.7 10^{-10} mol N_2O.kg^{-1}.s^{-1} in this work. This value was lower than values obtained in the literature: 4.56 10^{-8} (Klemedtsson *et al*., 1977), 1.52 10^{-8} - 2.99 10^{-8} mol.N_2O kg^{-1}.s^{-1} (Malhi *et al*., 1990), 1.39 10^{-8} - 8.23 10^{-8} (Holtan-Hartwig *et al*., 2000) and 42.5 10^{-10} – 8.17 10^{-8} (Murray *et al*., 1989). The Michaelis constant Km_D (0.058 mM) was (i) lower than several estimates, including some obtained with purified enzymes (e.g. 0.016 mM from Fewson et al. (1961), and 0.3-3.8 mM from Zumft (1997)), and soil slurries (about 0.22 mM from Klemedtsson *et al*. (1977), and between 11.4-31.4 mM from Malhi *et al*. (1990)), and (ii) higher than other estimates, including those of Murray *et al.* (1989) for soil and sediment slurries (0.0018-0.0166 mM). The estimates of the Michaelis constant Km_R obtained from *Model A* (0.129 µM) and *model B* (0.03 µM), as well as the range of Km_R values for Model C (0.146 and 0.0112 µM for the initial and final values, respectively), were much lower than values for the purified enzyme (2-26 µM after Zumft (1997)), but closest to values obtained by Holtan-Hartwig *et al*. (2000) for soil slurries (0.1-0.4 µM). This suggests that the induction of the N_2O reductase decreases its Michaelis constant as a consequence of either limitation in the e^- flux through respiratory chains or actual mechanisms that cannot be modelled by a simple enzymatic scheme (see for example the 3 schemes proposed in Appendix 1). In *Models B* and *C*, the estimated *e* exponent takes a high value (Table 1), indicating that the capability to reduce N_2O suddenly increases at time α. The α values (i.e. approximately 70 h and 37 h after the beginning of incubation for *Model B* and *Model C*, respectively) were respectively higher and lower than the 50 h corresponding to the highest N_2O concentration for condition 2. With regard to the literature, Otte *et al.* (1996) directly observed a considerable and sudden increase in the N_2O reductase concentration after about 25h. In their work, they did not

initially detect N_2O reductase, whereas Holtan-Hartwig *et al*. (2000) noted that there was already a low concentration of N_2O reductase initially. Around time α for *Model B*, Km_R decreased by about one order of magnitude (Table 1). The initial Km_R values ($1.46.10^{-4}$ $mol.m^{-3}$) were slightly lower than values obtained with purified enzymes (i.e. from 0.002 to 0.026 $mol.m^{-3}$, after Zumft (1997)).

Some information on the actual microbial processes can be deduced from a comparison between experimental and simulated data. With respect to the first denitrification step (i.e. $NO_3^- \rightarrow N_2O$), the modelling approach that has already been used by several other authors seems to be acceptable, since it enables us to reflect experimental trends as well as obtain relevant parameters. With respect to the second denitrification step, we noted an increase in the ability to reduce N_2O, which probably results from the induction of N_2O reductase (Firestone and Tiedje, 1979). In addition, accounting for this induction through a decrease in the Michaelis constant Km_R with respect to its actual value seems relevant, although we cannot simultaneously exclude an effect on the maximum N_2O reduction rate v_{Rmax}. Lastly, competition between NO_3^- and N_2O as terminal electron acceptors in the respiratory chain was used as a basic hypothesis for the model using previous results; neither the competition itself nor its mathematical description have been proved by this study. None of the three models used in this study were capable of perfectly reflecting all the experimental trends. Deviations remaining between experimental and simulated data may be due to reasons already stated, as well as the fact that other microbial and physicochemical processes were neglected, including for example (i) horizontal gene transfer that proceeds either through conjugation (Dröge *et al*., 1999), since nos genes – i.e. genes involved in N_2O reduction – are sometimes located on conjugative megaplasmids, e.g. pHG1 in *Alcaligenes Eutrophus* (Siedow *et al*., 1999), and (ii) geochemical changes that can affect the expression of the ability to reduce N_2O into N_2, including the bioavailability of Cu necessary for N_2O reductase to run (Zumft, 1997) and of Fe for other electron carriers such as cytochrome synthesised de novo upon the shift to anaerobiosis and expression of the denitrification system (Zumft, 1997).

Appendix 1: hypothetical kinetic schemes for N_2O reduction

Scheme 1

In this scheme, N_2O combines reversibly with N_2O reductase *E*, and the resulting complex *E(N_2O)* can then dissociate and produce N_2 and H_2O with the concomitant consumption of 2 H^+ and 2 e^-.

$$E + N_2O \underset{k_2}{\overset{k_1}{\longleftrightarrow}} E(N_2O) \qquad \text{(A.1a)}$$

$$E(N_2O) \xrightarrow{k_3} E + N_2 \qquad \text{(A.1b)}$$

Results are well known. Ignoring that the maximum flux of e^- can limit the N_2O reduction rate, we have:

$$v_R = k_3 \times [E_T] \times \frac{[N_2O]}{Km_R + [N_2O]} \qquad \text{(A.1c)}$$

where Km_R is the actual Michaelis constant for N_2O reductase (mol.m^{-3}) that equals the k_2/k_1 ratio. As long as $k_3 \times [E_T]$ is lower than $F_e/2$, the e^- flux through the respiratory chain does not limit v_R, and an increase in $[E_T]$ then only affects v_{Rmax} that equals $k_3 \times [E_T]$; the actual Michaelis constant is also the effective one. In contrast, for $[E_T]$ higher than $F_e/(2 \times k_3)$, the e^- flux through the respiratory chain F_e can limit v_R at high N_2O concentrations. v_R then cannot strictly be described with the Michaelis Menten equation; an approximation of the effective Michaelis constant $Km_{R\text{-}e}$ is the $[N_2O]$ value at which v_R equals $F_e/4$, i.e. half the maximum v_R:

$$Km_{R-e} = Km_R \times \left(\frac{F_e/4}{(k_3 \times E_T) - (F_e/4)} \right) \qquad \text{(A.1d)}$$

In this range of $[E_T]$ values, the maximum N_2O reduction rate v_{Rmax} does not vary with $[E_T]$.

Scheme 2

In this scheme, N_2O first combines reversibly with N_2O reductase *E*, and the resulting complex *E(N_2O)* then also reversibly combines with a terminal electron carrier bound to the microbial membrane (T_c). The resulting complex *E(N_2O)T_c* can then dissociate and produce N_2 and H_2O with the concomitant consumption of 2 H^+ and 2 e^-.

$$E + N_2O \underset{k_2}{\overset{k_1}{\longleftrightarrow}} E(N_2O) \tag{A.1e}$$

$$E(N_2O) + T_C \underset{K_2}{\overset{K_1}{\longleftrightarrow}} E(N_2O)T_C \tag{A.1f}$$

$$E(N_2O)T_C \xrightarrow{K_3} E + N_2 + T_C \tag{A.1g}$$

Assuming that $[E]+[E(N_2O)]$ nearly equals the total N_2O reductase concentration $[E_T]$, it is possible to estimate $[E(N_2O)]$:

$$[E(N_2O)] = [E_T] \times \frac{[N_2O]}{K_m + [N_2O]} \tag{A.1h}$$

Similarly, it is possible to estimate $[E(N_2O)T_c]$:

$$[E(N_2O)T_C] = [T_{cT}] \times \frac{[E(N_2O)]}{K_2/K_1 + [E(N_2O)]} \tag{A.1i}$$

where $[T_{cT}]$ is the total concentration of terminal membrane-bounded electron carriers. Replacing $[E(N_2O)]$ in Eq. (A.1i) by its value in Eq. (A.1h) gives the final equation for N_2O reduction in Eq. (A.1c), as long as the e^- flux through the respiratory chain does not limit v_R:

$$\nu_R = K_3 \times [T_{cT}] \times \left(\frac{[E_T]}{[E_T] + (K_2/K_1)} \right) \times \left\{ \frac{[N_2O]}{[N_2O] + \left(Km_R \times \left(\frac{(K_2/K_1)}{[E_T] + (K_2/K_1)} \right) \right)} \right\} \quad \text{(A.1j)}$$

As long as $[E_T]$ and/or $[Tc_T]$ are low enough to prevent the e^- flux through the respiratory chain from limiting ν_R, an increase in $[E_T]$ simultaneously increases and decreases ν_{Rmax} and $Km_{R\text{-}e}$, respectively. In addition, $Km_{R\text{-}e}$ is always lower than the actual Michaelis constant Km_R. On the one hand, at low $[E_T]$ values, ν_{Rmax} greatly depends on $[E_T]$, whereas $Km_{R\text{-}e}$ nearly equals Km_R. On the other hand, at high $[E_T]$ values, ν_{Rmax} does not vary greatly with $[E_T]$, whereas $Km_{R\text{-}e}$ significantly decreases with an increase in $[E_T]$. When the e^- flux through the respiratory chain limits ν_R at high $[N_2O]$ values, ν_{Rmax} cannot vary with $[E_T]$ and equals $F_e/2$, whereas the effective Michaelis constant $Km_{R\text{-}e}$ is reduced with respect to Eq. (A.1j), for the same reason as in scheme 1.

Scheme 3

This last scheme is probably the nearest to the actual mechanisms (Zumft, 1997). Unfortunately, it was difficult to obtain simple results as before. We present this scheme to show that it may lead to various trends that can be similar or different to those for the two schemes described previously. In the latter, N_2O reductase E is first reduced by contact with a terminal electron carrier of the respiratory chain bound to the microbial membrane. The reduced enzyme then combines with N_2O, and the resulting complex $E(N_2O)$ can then dissociate and produce N_2 and H_2O, with the concomitant consumption of 2 H^+ and enzyme oxidation.

$$E^+ + T_C \underset{K_2}{\overset{K_1}{\longleftrightarrow}} ET_C \quad \text{(A.1k)}$$

$$ET_C \xrightarrow{K_3} E^- + T_C \quad \text{(A.1l)}$$

$$E^- + N_2O \underset{k_2}{\overset{k_1}{\longleftrightarrow}} E(N_2O) \quad \text{(A.1m)}$$

$$E(N_2O) \xrightarrow{k_3} E^+ + N_2 \quad \text{(A.1n)}$$

As long as the e^- flux through the respiratory chain does not limit v_R, the N_2O reduction rate is limited by the rates of reactions in Equations (A.1m) and (A.1n) when $E^+ + ET_c << E^- + E(N_2O)$, whereas this rate can be limited by the rates of reactions in Equations (A.1k) and (A.1l), when $E^+ + ET_c >> E^- + E(N_2O)$. The first condition is fulfilled when:

$$\frac{k_3}{K_3}\left(\frac{(K_2/K_1)}{[T_c]}+1\right)-1 << \frac{(k_2/k_1)}{[N_2O]} \quad \text{(A.1o)}$$

When the left term is negative this condition is always verified whereas, in other situations, it is verified only at low N_2O concentrations. The Nitrous oxide reduction rate can then be described with a standard Michaelis Menten equation, using the actual Km_R and v_{Rmax} values. When the left term is positive and at high N_2O concentrations, it can be demonstrated that the N_2O reduction rate does not depend on N_2O but only on the total T_c and E contents. A priori, the resulting effective Michaelis constant may be higher or lower than the actual Km_R [N_2O], depending on the extent of the [N_2O] domain where the inequality (A.1o) is valid.

If the e^- flux through the respiratory chain limits v_R, at high [N_2O] values, it will indirectly induce a decrease in the effective Michaelis constant, as noted for scheme 1.

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CHAPITRE 3

INFLUENCE D'UNE PRE-INCUBATION ANAEROBIE SUR LA RESPIRATION ET LES EMISSIONS DE N_2O PAR DES MOTTES DE SOL EN INCUBATION AEROBIE

Ce chapitre 3 correspond à un article « en cours de soumission » pour la revue *Soil Biology and Biochemistry* : K. Khalil, P. Renault, B. Mary (2003) Effect of transient anaerobic conditions on subsequent aerobic respiration and N_2O emission by soil clods

SUMMARY

The objective of this study was to assess the effect of an anaerobic pre-incubation on subsequent aerobic respiration and N_2O emission at the scale of soil clods. Nitrous oxide production was measured in intact soil clods Δ (compacted clods without visible porosity) and Γ (clods with visible porosity) incubated under oxic conditions, with or without a 6 day anaerobic pre-incubation. N_2O emissions were much higher in clods that had been submitted to anaerobic pre-incubation than in clods that did not experience this pre-incubation, although very little nitrate remained in soil after the anaerobic period. ^{15}N isotope tracing technique was used to check whether N_2O came from nitrification or denitrification. The results showed that denitrification was the major process responsible for N_2O emissions. The aerobic CO_2 production rate was also measured in intact soil clods. It was greater in clods submitted to anaerobic pre-incubation than in clods that did not undergo pre-incubation, suggesting that the anaerobic pre-incubation lead to an accumulation of small compounds including fatty acids which are readily available for microbial decomposition in aerobic conditions. This process increases the aerobic CO_2 production and favours the N_2O emissions through denitrification.

Key-words: denitrification, anaerobiosis, nitrous oxide, aerobic respiration, soil clod.

INTRODUCTION

Nitrous oxide is trace gas involved in atmospheric pollution, it contributes to the greenhouse effect (Smith, 1990; IPCC, 1996), and affects the chemistry of O_3 in the upper troposphere and lower stratosphere (Graedel and Crutzen, 1992). N_2O is mainly produced in soils during biological denitrification and nitrification (Groffman, 1991; Tortoso *et al*., 1990; Conrad, 1996).

Various models, more or less complex, have been proposed to estimate N_2O emissions through nitrification and denitrification. Most of them account for the variations with time in environmental variables such as soil water, temperature and NO_3^- content. Simplified models (e.g. Parton *et al.*, 1988; Hénault and Germon, 1995; Parton *et al*., 1996) represent N cycling without simulating microbial dynamics and assume that the different N processes can be represented as a function of the soil water, temperature and pH controls on microbial activity without representing the microbial dynamics. The more complex models (e.g. Grant, 1995; Khalil et al., 2003a) explicitly consider microbial dynamics but do not consider variations in potential microbial activities, particularly the variations in respiration, denitrification and N_2O reduction activities. The importance of these variations is questionable.

It has been shown that potential denitrification may be correlated with soluble organic matter and easily mineralisable carbon (Burford and Bremner, 1975). Anaerobic conditions may lead to the accumulation of organic compounds that may be thereafter easily consumed in aerobic conditions and that temporarily decrease the pH of the soil solution (Förstner, 1987). Acetate is the main volatile fatty acid produced by anaerobic metabolism (Tsusuki *et al*., 1987). It may be consumed under conditions that enhance H_2 consumption, i.e. conditions promoting sulphate reduction (Pelmont, 1993), homoacetogenesis (Chin and Conrad, 1995) and methanogenesis (Conrad, 1999). An initial pH decrease has been observed in alcaline soils, which results from an increase in CO_2 concentration and a transient production of volatile fatty acids (Tsusuki *et al*., 1987). This pH decrease may be minimized by the consumption of protons during the reduction of NO_2^- into N_2O (Stams, 1994). The consumption of volatile fatty acids, the dissolution of metal oxides and oxyhydroxides and the partial consumption of CO_2 during homoacetogenesis and methanogenesis tend to lead to a late increase in soil pH.

Such changes in pH and in easily mineralisable C compounds, which could occur during

the transition of anaerobic to aerobic conditions, might lead to important changes in potential respiration or denitrification activities.

The micro-scale approach to study denitrification is motivated by the fact that, in many cases, the conditions experienced by soil organisms at the microscale are not reflected by measurements on bulk soil samples (Parkin, 1987). For example, O_2 concentrations may decrease from values nearly equal to the atmospheric concentration to zero values within a few millimeters in soil clods (Sextone *et al.*, 1985; Sierra *et al.,* 1995). Other examples of anoxic sites close to air-filled pores when organic residues were present (Rappoldt, 1992) with a thin layer of covering water (i.e. thickness of about 160 μm) are sufficient for anaerobiosis to occur (Parkin, 1987).

The aim of this work was to assess the consequence of a prolonged anaerobic period (6 days) on subsequent aerobic respiration and net N_2O emission through denitrification. Experiments were performed at the soil clod level. We used C_2H_2 to inhibit N_2O reduction and ^{15}N tracing technique to assess the origin of N_2O emission.

MATERIALS AND METHODS

Soil clod sampling and conservation

Experiments were performed on an Orthic Luvisol (FAO classification) sampled in Mons-en-Chaussée in Northern France (49°80' N, 3°60' E). The soil was cultivated with maize in 2000. The properties of the soil were as follows: clay, 194 g kg^{-1}; silt, 706 g kg^{-1}; sand, 68 g kg^{-1}; pH (water), 8.2; total $CaCO_3$, 32 g kg^{-1}; organic C, 8.52 g kg^{-1}; total N 1.00 g kg^{-1}. At sampling time, it contained 4.70 mg NO_3-N kg^{-1}. Clods were sampled in the ploughed layer (10-30 cm depth) on September 12, 2000. Two sets of clods were separated: clods Δ, with a massive structure and no visible porosity (resulting from compaction due to traffic) and clods Γ, with a fragmentary structure and visible porosity (Richard *et al*., 1999). The larger clods were gently broken down immediately after sampling and then calibrated : we kept clods between 2.5 and 3 cm size. In order to reduce microbial activity during storage, the clods were air-dried during 3 days to obtain a residual moisture close to 0.10 g g^{-1} soil. They were stored at 2°C until the beginning of the experiments, i.e. until November 2000, January 2002 and May 2002, for experiments 2, 3 and 1, respectively. Water evaporation occurred during storage, so that the soil moisture content at the beginning of experiments was 0.11, 0.07 and 0.06 $g.g^{-1}$, for experiments 2, 3 and 1, respectively.

Batch incubations and measurements

Experiment 1

In order to check the effect of anaerobic condition of the subsequent aerobic respiration, we measured CO_2 production for clods Δ and Γ that had either experimented or not a 6.6 day period of preliminary anaerobic incubation.

Aerobic CO_2 production was measured using the following procedure. Δ and Γ clods were first rewetted with water at 20°C on suction tables successively at –10 kPa suction during 1 day, -5 kPa during 1 day, -1 kPa during 1 day, and –0.5 kPa during 4 days. This procedure ensured a slow rewetting process which prevented crack formation. The soil moisture content thus obtained was 0.20 $g.g^{-1}$ and 0.23 $g.g^{-1}$ for Δ and Γ clods, respectively. A set of 32 clods

of each type was then incubated in anaerobic conditions using 500 mL flasks: each flask containing 4 clods received 3 successive cycles of 3 minutes vacuum and 3 minutes of pure N_2 gas addition. A 10 mL volume of N_2 was removed and replaced by the same volume of C_2H_2 in order to create the same conditions as in experiments 2 and 3. All the flasks were incubated at 20°C in the dark during 6.6 days. After this anaerobic period, the flasks were flushed with air; the clods were transferred 3 hours later in a 1 L airtight jar with a beaker containing 10 mL NaOH 0.1 N in order to trap CO_2. Eight replicate jars (each containing 4 clods that had followed the anaerobic pre-incubation) were thus incubated at 20°C in aerobic condition during 4 days. The same procedure was applied to a similar number of clods that did not experience the anaerobic preincubation, i.e. that were transferred directly from the suction table to the 1 L jars. The jars were opened every 24 h during 4 days, aerated for 5 minutes and the NaOH beaker was replaced. Trapped CO_2 was precipitated as barium carbonate by adding excess of $BaCl_2$ solution. The remaining NaOH was then titrated with HCl 0.1 N at pH 8.62.

Soluble organic C was measured in the clods at the end of the aerobic incubation. It was also measured in additional clods that experienced anaerobic conditions, at the end of the rewetting phase, and at the end of the anaerobic pre-incubation, always with 8 replicates. Organic C was extracted with a solution of K_2SO_4 0.03 M and measured with a 1010 organic carbon analyzer (O.I. Analytical, College Station, Texas, USA) using the method of persulfate oxidation at 100 °C (Barcelona, 1984). At the end of incubation, one third of the 4 clods contained in each jar was air-dried and finely ground; the total C and N were measured with an automatic CHN analyzer (Carlo Erba, NA1500, Milan, Italy). The last third of the 4 clods contained in each jar was used to measure the pH of the soil solution for both clods that experimented or not experimented anaerobic conditions. pH was also measured after the rewetting phase for clods that didn't experiment anaerobic conditions, and after the anaerobic pre-incubation for clods that experimented anaerobic conditions. For each flask sample, we added a mass of ultra pure water equal to the double of soil mass. The flask was closed and shaken for 10 minutes, then transferred to a beaker and left for 2 minutes. The electrode (Calomel electrode K401, Glass electrode G202B, Copenhagen, Denmark) was plunged into the beaker and the pH was recorded every minute during 5 minutes.

Experiment 2

In order to check the effect of anaerobic condition of the subsequent N_2O emissions, we measured N_2O net emissions from clods Δ and Γ in air that had either or not experienced a 6.6 day period of anaerobic pre-incubation. Net N_2O emissions were measured using the following procedure.

Δ and Γ clods were first rewetted with either deionized water or a KNO_3 solution (4 g L^{-1}) at 20°C on suction tables during 7 days as previously described. The final moisture obtained was 0.22 and 0.24 g g^{-1} for Δ and Γ clods, respectively. Clods rewetted with deionized water were then pre-incubated in anaerobic conditions at 20°C during 6.6 days, whereas clods rewetted with KNO_3 were not pre-incubated. The pre-incubation of water rewetted clods concerned 16 Δ and 16 Γ clods, each of them being inserted in a 150 mL flask. Anaerobiosis was realised by injecting N_2 as described before; a volume of 8 mL of N_2 was removed and replaced by 7 mL of C_2H_2 and 1 mL of krypton. During the pre-incubation, gas samples were withdrawn at 1, 2, 3, 4, 5 and 6 days and analysed for N_2O, N_2, CO_2, O_2, Kr and C_2H_2 concentrations. The mineral N (NH_4^+, NO_2^- and NO_3^-) content of the soil was measured at day 0 and 6, with 6 replicates. At day 6.6, the atmosphere of the flasks was replaced with air as indicated before; the procedure was repeated 1 hour later in order to ensure that C_2H_2 has been totally removed. Water or KNO_3 rewetted clods were then incubated aerobically for 7 hours, in airtight 150 mL flasks filled with air at 20°C, 1 mL of air being replaced with Kr at the beginning of the incubation, with 10 replicates. Gas samples were taken with a syringe 14, 16, 18 and 20 hours after the addition of Kr for clods rewetted with KNO_3 solution; 1, 3, 5 and 7 hours after the addition of Kr for clods rewetted with water. The samples were analysed for N_2O, N_2, CO_2, O_2 and Kr concentrations. The final mineral N (NH_4^+, NO_2^- and NO_3^-) content of the soil was determined on each of the 10 replicates.

The flasks samples, rewetted with KNO_3 solution, were thereafter opened for a few minutes in order to release the trapped gases. After closing the flasks, 7 and 1 ml of gas were replaced by C_2H_2 and Kr, respectively. The 10 Δ and 10 Γ replicates were incubated at 20°C one time more. Gas samples were withdrawn with a syringe 14, 16, 18 and 20 h after the addition of C_2H_2 and analysed for N_2O, N_2, CO_2, O_2, Kr and C_2H_2 concentrations. The final '$[NO_3^-]$+$[NO_2^-]$' and $[NH_4^+]$ were measured on all the Δ and Γ replicates.

Nitrous oxide concentration was determined by gas chromatography equipped with an electron capture detector (HP 5890 Series II, USA) fitted with a Porapak Q column (80-100

mesh, 2 m) coupled to an integration software on a PC Workstation. The carrier gas was Ar-CH_4 (95/5); the oven and detector temperatures were set at 50 and 300°C, respectively. N_2, CO_2, O_2, Kr and C_2H_2 concentrations were measured on a TCD gas chromatograph (HP 5890 Series II, USA) fitted with Porapak Q (80-100mesh, 1.8 m) and molecular sieve (1-5 Å, 1.8 m) columns coupled to the same integration software. The carrier gas was He; the oven and detector temperatures were fixed at 50 and 120 °C, respectively. The relative precision of each chromatograph was 0.5-1%. The mineral N content of clods were extracted with a 1 M KCl solution (soil:solution ratio = 1:5). Measurements were performed with a continuous flow colorimeter (Skalar Analytical, Breda, The Netherlands) using the method proposed by Henriksen and Selmer-Olsen (1970).

Experiment 3

This experiment aimed at determining the origin of N_2O emissions, from nitrification or denitrification, using ^{15}N isotope tracing technique. ^{15}N isotope was applied either as KNO_3 or urea, both having a 50% atom enrichment. Urea was chosen instead of NH_4^+ due to its higher diffusion rate in soil and its rapid hydrolysis into NH_4^+ already observed in this soil (Recous *et al*., 1988). Small amounts of N were applied in order to change little the amounts of mineral N in soil: i. e. 0.5 mg N kg^{-1} soil as urea-N and 2.0 mg N kg^{-1} soil as NO_3-N.

Clods were first rewetted with water using the procedure for *Experiment 2*. We then added 0.3 mL of labelled urea or KNO_3 solution to each clod, and submitted them to anaerobiosis in presence of C_2H_2 as described for Experiment 2. For each of the two added solutions, 6 clods were thus pre-incubated at 20°C during 144 h. At the end of this pre-incubation period, 3 clods were used to measure the initial concentrations of NO_3^-, NO_2^-, NH_4^+, and the ^{15}N atom% excess of NH_4^+ and (NO_2^-+NO_3^-).The N_2 atmosphere of the flasks containing the 3 other clods was replaced with air after 14 additional hours by alternating 3 successive cycles of 3 minutes vacuum and 3 minutes air filling, this procedure being repeated 1 hour later. The clods were then incubated in aerobic condition at 20°C. Gas of the flasks was sampled after 8 h with 10 mL Venoject® vacuum tubes and dual-ended sampling flasks of 250 mL. 0.2 mL of the Venoject® tubes was later withdrawn with a syringe and analysed for N-(N_2+N_2O) measurement and ^{15}N isotopic composition. The 250 mL sampling was used for N_2O and $^{15}N_2O$ measurement. 3 clods were used to extract and measure the final soil mineral N content and ^{15}N enrichment.

N-(N_2+N_2O) concentrations and their ^{15}N composition were determined by an automatic CHN analyzer (Carlo Erba, NA 1500, Milan, Italy) coupled to a mass spectrometer (Fisons, Isochrom, Manchester, England). N_2O and $^{15}N_2O$ were analysed after automatic pre-concentration on a trace gas - mass spectrometer equipment (Micromass, Manchester, England). The soil mineral N of the clods was extracted with a 1 M KCl solution (soil:solution ratio = 1:5). Measurements of ammonium, nitrite and nitrate concentrations were performed with a TRAACS 2000 analyzer (Bran & Luebbe, Norderstedt, Germany) using the methods proposed by Kamphake *et al*. (1970) for NO_3^- and NO_2^- analysis and Krom (1980) for NH_4^+ analysis. The ^{15}N enrichments of NH_4^+ and (NO_2^-+NO_3^-) were obtained after separation by micro-diffusion towards a solid support (Brooks *et al.*, 1989) which was thereafter dried and placed in a tin capsule for combustion in the CHN analyzer coupled to the mass spectrometer.

Data treatment

Model of aerobic CO_2 production with time

We assume that C decomposition rate by microbes during the aerobic condition was proportional to the amount of substrates, i.e. decomposable organic pools. For clods without anaerobic pre-incubation, only one substrate pool S_1 (mol C kg^{-1} soil) was taken into account. It disappears at the following rate:

$$\frac{dS_1}{dt} = -k_1 S_1 \qquad (1)$$

where k_1 (h^{-1}) is a rate constant, and t the time (h). Variation of this pool with time is then:

$$S_1 = S_{10} \exp(-k_1 t) \qquad (2)$$

The rate of CO_2 production can be written:

$$v_1 = -(1-Y_1)\frac{dS_1}{dt} = k_1(1-Y_1)S_{10} \exp(-k_1 t) \qquad (3)$$

where Y_1 is the assimilation yield of substrate 1 by microbes.

In the case of clods previously submitted to anaerobic conditions, our results suggest that this pretreatment lead to the creation of an additional decomposable pool S_2 (mol C kg^{-1} soil) decomposing with a rate constant k_2 (h^{-1}):The CO_2 production rate in this treatment is then:

$$v_2 = -(1-Y_1)\frac{dS_1}{dt} - (1-Y_2)\frac{dS_2}{dt} = k_1(1-Y_1)\exp(-k_1 t) + k_2(1-Y_2)\exp(-k_2 t) \quad (4)$$

where Y_2 is the assimilation yield of substrate 2 by microbes.

We first estimated the parameters k_1 and the product $(1\text{-}Y_1)\ S_{10}$ by fitting simulated CO_2 production rates (Eq. 3) to values measured in clods Δ and Γ which had not been submitted to anaerobic pre-incubation. The optimisation was run simultaneously in the two types of clods, assuming that the decomposition rate k_1 was the same in both soils. The procedure was applied to the mean of the 8 replicates. Using these last values, we then estimated the other two parameters (k_2 and $(1\text{-}Y_2)\ S_{20}$) by fitting simulated CO_2 production rates (Eq. 4) to values measured in clods Δ and Γ which had been submitted to anaerobic pre-incubation. Again we assumed that the decomposition rate k_2 was the same in both types of clods.

Relationship between aerobic CO_2 production and soluble organic C

In order to check whether there was a relationship between the aerobic CO_2 production and the soluble organic C, we presented variation of the mean aerobic CO_2 production rate (mol kg^{-1} s^{-1}) with soluble organic C (mg kg^{-1}). Soluble organic C was measured in clods submitted to anaerobic pre-incubation: (i) after rewetting on suction table; (ii) after anaerobic pre-incubation; and (iii) at the end of the measurement periods. Soluble organic C was measured only at the last date in clods which had not been submitted to anaerobiosis. They were linked with aerobic CO_2 production rates during 24 h (i) just after rewetting, (ii) just after anaerobic pre-incubation, and (iii) corresponding to the last periods of measurements, respectively. The linear regression was applied to aerobic CO_2 production rate for clods Δ and Γ.

Estimating actual CO_2 production from its accumulation in Experiment 2

Since CO_2 is soluble and dissociated in water, gaseous CO_2 is only a part of total CO_2 present in the flask when the gas is not trapped as in Experiment 2. In this case, we have to account for its solubilisation in water, and its first dissociation (Stumm and Morgan, 1996):

$$(CO_2)_g \underset{\leftarrow}{\overset{\rightarrow}{}} H_2CO_3^* \quad (5)$$

with a solubility ratio $S = H_2CO_3^*/CO_2$ equal to 0.87 at 20°C.

$$H_2CO_3^* + H_2O \underset{\leftarrow}{\rightarrow} HCO_3^- + H_3O^+ \quad (6)$$

with a pK_a equal to 6.35 at 20°C.

At the end of the experiment, the total amount of CO_2 (mol) in the flask is equal to:

$$Q = C_f\left(V_g + V_w S\left(1 + 10^{pH_f - pK_a}\right)\right) \quad (7)$$

where C_f is the CO_2 concentration in the flask atmosphere (mol m^{-3}), V_g and V_w are the gas and soil solution volumes (m^3), respectively; the subscript *f* refers to final values.

If we neglect the contribution of calcite in the production of HCO_3^- anions, it is possible to estimate the actual CO_2 production rate P_a (mol kg^{-1} s^{-1}) as a function of the apparent production $\widehat{P}$ (mol kg^{-1} s^{-1}):

$$P_a = \widehat{P}\left(1 + \frac{V_w}{V_g}S\right) + \frac{V_w S}{m\Delta t}\left(C_f \cdot 10^{pH_f - pK_a} - C_i \cdot 10^{pH_i - pK_a}\right) \quad (8)$$

where *m* is the clod mass (kg), Δt the incubation time (s), and pH_i the initial pH.

In addition, we assume the following dependence of the pH to CO_2 concentration in the gas phase:

$$pH = pH_{\min} + \left(pH_{\max} - pH_{\min}\right)\exp\left(-\frac{C}{k_3}\right) \quad (9)$$

where pH_{max} is the maximum pH value in absence of CO_2, pH_{min} its minimum value at high CO_2 partial pressure, and k_3 a constant (mol m^{-3}) which characterises the dependence to CO_2 concentration (*C*). For the calculations, we put pH_{max} = 8.20, pH_{min} = 5.75 and we successively estimated the actual aerobic CO_2 production rate for k_3 equal to 0.83, 1.25 or 1.66 mol m^{-3}, corresponding to 2 %, 3% or 4% CO_2, respectively. These values were obtained by fitting simulations of more complete geochemical models to this simple relationship for soils with traces of calcite.

RESULTS

Experiment 1: aerobic CO_2 production with or without anaerobic pre-incubation

The CO_2 production rate during the aerobic incubation decreased slowly with time in clods which had not been submitted to anaerobic pre-incubation: from 7.0 to 5.2 nmol kg^{-1} s^{-1} in clods Δ, and from 8.2 to 6.1 nmol kg^{-1} s^{-1} in clods Γ, between 12 and 84 hours, respectively (Fig. 1). The rate decreased much faster in clods which had been submitted to anaerobic pre-incubation. It varied from 14.5 to 4.9 nmol kg^{-1} s^{-1} in clods Δ, and from 17.9 to 6.0 nmol kg^{-1} s^{-1} in clods Γ, between 12 and 84 hours, respectively. The model previously described (Eq. 1-4) could be satisfactorily fitted to the observed data, even with the assumption that the constant rates k_1 and k_2 did not differ between the two treatments (Fig. 1). The estimated parameters are reported in Table 1. They show that the anaerobic pre-incubation has resulted in the formation of a small additional decomposable pool (S_2) which decomposes much more rapidly than the decomposable C present in the control soil (S_1): its turnover time ($1/k_2$ = 14.5 hours) is 18 fold smaller than that of pool 1 ($1/k_1$ = 255 hours). Assuming a microbial assimilation Y = 0.60 g C g^{-1} C, the size of the pool 2 can be assessed at 2.45 and 2.72 mmol C kg^{-1} in clods Δ and Γ, corresponding to 29.4 and 32.6 mg C kg^{-1} soil, respectively. The rate of CO_2 production at time 0, i.e. the sum $k_1 (1-Y_1) S_{10} + k_2.(1-Y_2) S_{20}$, can also be calculated. It is equal to 26.2 and 29.4 nmol kg^{-1} s^{-1}, for clods Δ and Γ submitted to anaerobic pre-incubation, respectively (Table 1). These rates were 3.5 times higher than the corresponding rates in clods which had not experienced anaerobic pre-incubation.

Accounting for the CO_2 solubilisation in water and its first dissociation, the actual aerobic CO_2 production rates were also calculated using the apparent CO_2 values measured in experiment 2. The mean aerobic CO_2 production rates in *experiment 1* were largely higher than the actual aerobic CO_2 production rates (Table 3). These last rates were calculated for different values of k_3 equal to 0.83, 1.25 and 1.66 mol m^{-3}, corresponding to 2, 3 and 4 % CO_2, respectively.

The soluble organic C contents varied between treatments from 17.9 to 24.7 mg C kg^{-1} soil. They increased from the end of the rewetting period to the end of the anaerobic pre-incubation by 19 % and 11 % for clods Δ and Γ, respectively. We obtained nearly linear relationships between measured soluble organic C contents and the more proximal CO_2 production rate

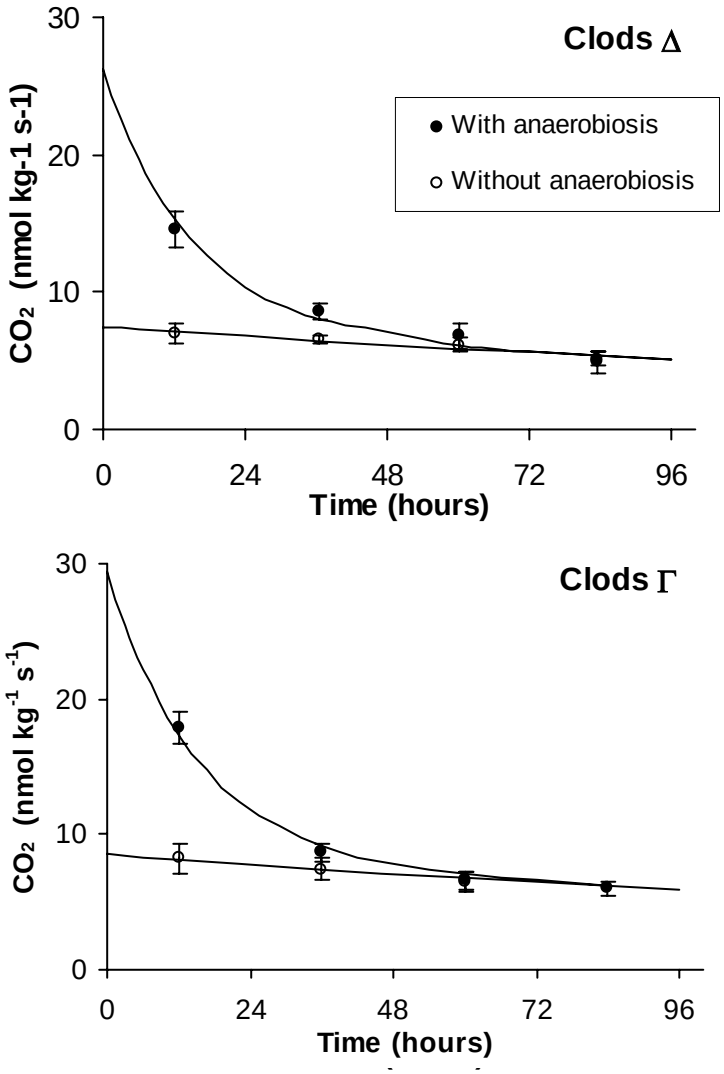

Figure 1. *Rate of CO_2 production measured (symbols) and simulated (continuous lines) during aerobic incubation following either a 6.6 day anaerobic preincubation (closed symbols), or no preincubation (open symbols), in clods Δ or Γ. Each point is the mean of 8 replicates made of 4 soil clods. The bars represent the standard errors.*

		Clods Δ	Clods Γ
$(1-Y_1)\,S_{10}$	mol kg^{-1}	$6.82\ 10^{-3}$	$7.81\ 10^{-3}$
$1/k_1$	h	254	254
$(1-Y_2)\,S_{20}$	mol kg^{-1}	$0.98\ 10^{-3}$	$1.09\ 10^{-3}$
$1/k_2$	h	14.5	14.5

Table 1. *Parameters obtained by fitting the CO_2 production rate model (Equations 3-4) to the CO_2 rates measured during a 4 day aerobic incubation with or without preliminary anaerobic incubation.*

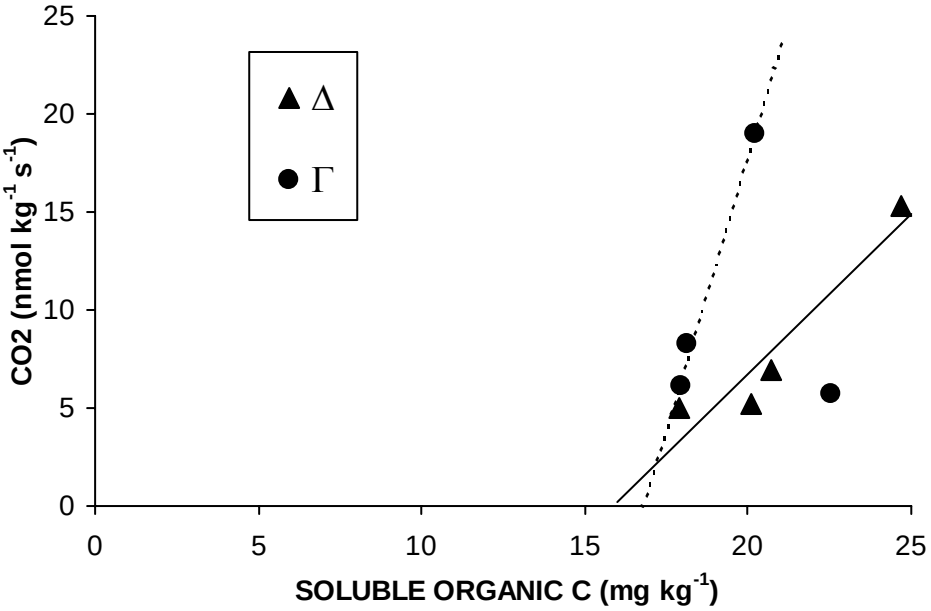

Figure 2. *Relationship between the rate of CO_2 production (10^{-9} mol kg^{-1} s^{-1}) measured during various periods of 24 hours and the soluble organic C (mg C kg^{-1}) measured at the same time, in the two types of clods. The periods are: after rewetting on suction table (day –6), after anaerobic pre-incubation (day 0), and at the end of the aerobic incubation (day 4).*

Symbol	Rewetting	Pre-treatment	Incubation	n[1]	N_2O emission rate pmol kg^{-1} s^{-1} Clods Δ		Clods Γ	
Δ, Γ	KNO_3 solution	no pre-incubation	no C_2H_2	40	**-1.0**	(0.4)	**-0.3**	(1.2)
Δ*, Γ*	KNO_3 solution	no pre-incubation	with C_2H_2	40	**5.2**	(3.2)	**3.3**	(5.4)
Δ, Γ	KNO_3 solution	no pre-incubation	no C_2H_2	10	**-1.2**	(0.8)	**-0.9**	(0.1)
Δ+, Γ+	Deionised water	Anaerobic pre-incubation with C_2H_2	no C_2H_2	10	**72.1**	(9.4)	**48.3**	(8.3)

Table 2. *Mean net N_2O emission rates (10^{-12} mol kg^{-1} s^{-1}) measured in soil clods during aerobic incubation, for various rewetting conditions and pre-treatments. Values in brackets represent the confidence intervals ($p < 0.05$).*

[1] *n= number of clods (replicates)*

measured at the same date (Fig. 2), except for one value that seemed unrealistic (the correlation coefficients were 95.4% and 97.9% for Δ and Γ clods, respectively). The linear regressions obtained suggest that there is a threshold for soluble organic C under which there is no more aerobic respiration: the difference between the initial soluble organic C and this estimation represented approximately 23 % and 9 % of the initial soluble organic C for clods Δ and Γ, respectively.

For clods that did not experiment anaerobic conditions, the mean value of pH measurements, after the rewetting phase, was 8.31 (± 0.08) and 8.24 (± 0.03), for Δ and Γ clods, respectively. After the aerobic incubation, the mean value of pH was 8.38 (± 0.12) and 8.29 (± 0.04), respectively. For clods that experimented anaerobic conditions, the mean value of pH, after the anaerobic pre-incubation, was 8.34 (± 0.02) and 8.37 (± 0.05), for Δ and Γ clods, respectively. After the subsequent aerobic incubation, the mean value of pH was 8.74 (± 0.01) and 8.47 (± 0.03), for Δ and Γ clods, respectively. The results indicated variation neither with time nor with the clod type (Δ and Γ).

Experiment 2: N_2O production with or without anaerobic pre-incubation

The net N_2O emission rates measured in experiment 2 were very small and even slightly negative in clods that did not experience anaerobic pre-incubation: –1.2 and –0.9 pmol N_2O kg^{-1} s^{-1} in clods Δ and Γ, respectively (Table 2). They were negligible in comparison to clods that had been previously submitted to anaerobic conditions: 72.1 and 48.3 pmol N_2O kg^{-1} s^{-1} in clods Δ and Γ, respectively. These values were also much higher than those measured in experiment 2 (5.2 and 3.3 pmol N_2O kg^{-1} s^{-1} in clods Δ and Γ, respectively), although these rates were measured in favourable conditions: nitrate was supplied in large amount to promote denitrification and acetylene was added to prevent N_2O reduction.

The mean N_2O emission rate following anaerobic incubation was significantly higher ($P<0.05$) for clods Δ (72.1 pmol kg^{-1} s^{-1}) than for clods Γ (48.3 pmol kg^{-1} s^{-1}). The mean clod weight was also significantly higher for Δ clods (25.8 g) than for Γ clods (20.2 g). Figure 3 suggests that N_2O emission rate does not depend directly on clod type (Δ and Γ), but would rather depend on clod mass: this relationship could be due to a larger anoxic volume in the larger clods (Renault and Stengel, 1994; Sierra et al., 1995) Measurements of mineral N content showed that the NH_4^+ and NO_3^- contents of clods that had been rewetted with water and submitted to anaerobic pre-incubation were low and not

Clods	Rewetting	Pre-treatment	Mineral N (mol N m^{-3})				Mineral N (mg N kg^{-1})			
			t = 0		**t = 24 hours**		**t = 0**		**t = 24 hours**	
			NH_4^+	**NO_3^-**	**NH_4^+**	**NO_3^-**	**NH_4^+**	**NO_3^-**	**NH_4^+**	**NO_3^-**
Δ	KNO_3 solution	No pre-incubation	**0.44**	**32.9**	**0.97**	**26.5**	**1.4**	**106.2**	**3.1**	**85.5**
			(.06)	*(3.1)*	*(.14)*	*(4.3)*	*(.2)*	*(10.1)*	*(.5)*	*(13.9)*
Γ	KNO_3 solution	No pre-incubation	**0.43**	**29.1**	**0.81**	**27.8**	**1.5**	**99.2**	**2.7**	**92.8**
			(.03)	*(1.8)*	*(.12)*	*(4.2)*	*(.1)*	*(6.1)*	*(.4)*	*(15.2)*
Δ+	Deionised water	Anaerobic pre-incubation	**0.57**	**0.38**	**0.74**	**0.35**	**1.8**	**1.2**	**2.3**	**1.1**
		with C_2H_2	*(.03)*	*(.18)*	*(.05)*	*(.03)*	*(.1)*	*(.6)*	*(.2)*	*(.1)*
Γ+	Deionised water	Anaerobic pre-incubation	**0.71**	**0.28**	**0.73**	**0.85**	**2.3**	**0.9**	**2.4**	**2.8**
		with C_2H_2	*(.09)*	*(.03)*	*(.05)*	*(.4)*	*(.3)*	*(.1)*	*(.2)*	*(1.3)*

Table 3. *Amounts of mineral N (in mol m^{-3} solution or mg kg^{-1} soil) measured at the beginning and the end of 1 day aerobic incubation following either soil water rewetting and anaerobic pre-incubation (6 days) or nitrate rewetting alone (experiment 2). Values in brackets represent the standard errors.*

Clod type	Apparent CO_2 production	Actual CO_2 production		
		k_3 = 0.83	k_3 = 1.25	k_3 = 1.66
		nmol CO_2 kg^{-1} s^{-1}		
Δ+	5.42 (1.14)	12.5	14.3	15.3
Γ+	4.23 (0.70)	8.99	10.1	10.8
Δ	1.41 (0.31)	2.72	3.12	3.36
Γ	1.63 (0.99)	2.86	3.30	3.56

Table 4. *Apparent and actual CO_2 production rates (10^{-9} mol CO_2 kg^{-1} s^{-1}) during 6 hours of aerobic incubation of clods Δ and Γ. Apparent rate is the accumulation rate measured in flasks of experiment 2; actual rate is calculated using equation 8 for three values of k_3: k_3 = 2, 3 and 4%, corresponding to 0.83, 1.25 and 1.66 mol m^{-3}. Clods Δ+ and Γ+ have been submitted to pre-treatment (anaerobiosis incubation during 6 days with C_2H_2), whereas clods Δ and Γ have not.*

Values in brackets are the confidence intervals.

different between clods or dates: the average was 2.2 ± 0.3 mg NH_4^+-N kg^{-1} soil and 1.5 ± 0.9 mg NO_3^--N kg^{-1} soil (Table 3). In clods which had been rewetted with KNO_3 solution and which did not experience anaerobic pre-incubation, the NH_4^+ concentration was similar (2.2 ± 0.3 mg N kg^{-1} soil), but the NO_3^- concentration was much higher as expected: 96.0 ± 8.9 mg N kg^{-1} soil, without significant change between the initial and final measurement ($P<0.05$). N_2O emissions were low in these clods in spite of their high NO_3^- concentration.

The apparent CO_2 production rate (i.e. without accounting for CO_2 and carbonates in soil solution) was also much higher in clods that had been submitted to anaerobic pre-incubation than in the other: 3.8 and 2.6 times higher in clods Δ and Γ, respectively (Table 4). The apparent rates were not statistically different between clods Δ and Γ not submitted to anaerobic conditions, but could be distinguished in clods submitted to anaerobic pre-incubation (significant at $P<0.10$). The calculated actual CO_2 production rates, corrected for carbonates present in soil solution, were 1.76 to 2.83 times greater. The factor depends both on the clod type and its pre-treatment and on the constant k_3 that characterises the relationship between soil pH and partial pressure of CO_2. These values remain lower than the values obtained for the same clods in experiment 1.

Experiment 3: origin of the N_2O emissions following anaerobic preincubation

Mean N_2O emission of clods supplied with $^{15}NH_4^+$ was about twice the corresponding value for clods supplied with $^{15}NO_3^-$, although the difference between these 2 values was not statistically significant ($P<0.05$).

For Δ clods submitted to anaerobic pre-incubation and thereafter enriched either in $^{15}NO_3^-$ or $^{15}NH_4^+$, the isotopic excess in ^{15}N of produced N_2O was similar to the mean excess of NO_3^- defined as the average between the initial and final excess of NO_3^- (Table 5). Unfortunately, it was not possible to estimate the $N_2O/(N_2O+N_2)$ ratio from ^{15}N data, since, the isotopic excess of $^{15}N_2$ did not significantly differ from that of air (results not shown). Mean N_2O emission of clods supplied with $^{15}NH_4^+$ was about twice the corresponding value for clods supplied with $^{15}NO_3^-$, although the difference between these 2 values was not statistically significant ($P<0.05$).

Nitrate and NH_4^+ contents were about $9\ 10^{-6}$ and $2\ 10^{-4}$, respectively before supplying the clods with either $^{15}NO_3^-$ or $15NH_4^+$. This supply lead to slightly increase NH_4^+; in contrast it lead to multiply 5 times the NO_3^- content.

		N_2O	N_2O	NH_4	NO_3	N_2O	NH_4	NO_3	$^{15}N_2O/(^{15}N_2+^{15}N_2O)$
		pmol kg^{-1} s^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	atom%	atom%	atom%	
Pretreatment									
^{15}N-urea	**No C_2H_2**	**287**	**0.10**	**3.35**	**1.45**	**0.33**	**2.02**	**0.36**	
		(283)	(.09)	(1.65)	(1.29)	(.33)	(.92)	(.15)	
$^{15}NO_3$	**No C_2H_2**	**173**	**0.06**	**2.94**	**1.32**	**3.15**	**0.66**	**3.40**	**0.04**
		(292)	(.09)	(1.06)	(.69)	(4.08)	(.11)	(1.14)	(.06)
^{15}N-urea	**C_2H_2**	**195**	**0.07**	**3.36**	**0.12**	**0.31**	**2.81**	**0.24**	
		(122)	(.04)	(.22)	(.12)	(.14)	(.25)	(.09)	
$^{15}NO_3$	**C_2H_2**	**114**	**0.04**	**2.97**	**0.65**	**4.48**	**0.70**	**4.61**	**0.78**
		(28)	(.01)	(.87)	(.14)	(.57)	(.28)	(.58)	(.39)

Table 5. *N_2O emission rate, amounts of mineral N and isotopic composition of N_2O and mineral N measured in clods Δ, during a short aerobic incubation period (7 hours) following a 6.6 day anaerobic incubation with C_2H_2. Pool sizes and isotopic composition of mineral N are the mean of values measured at t = 0 and t = 7 hours. ^{15}N labelling was made using $^{15}NO_3^-$ or ^{15}N-urea before the anaerobiosis period. Values in brackets are the standard deviations (3 replicates).*

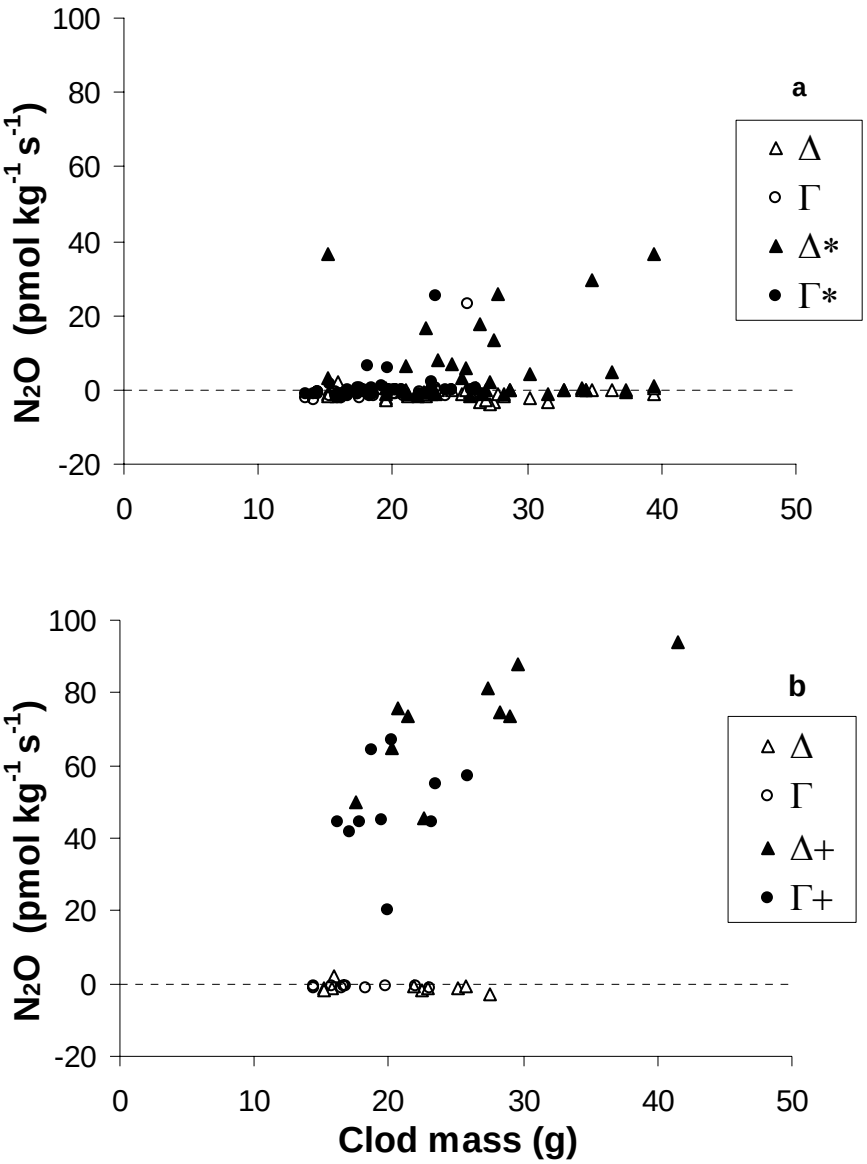

Figure 3: *Net N_2O emission rates (10^{-12} mol kg^{-1} s^{-1}) as a function of clod mass, measured:*

c) in soil clods rewetted with nitrate solution and incubated aerobically with C_2H_2 (Δ, Γ*) or without C_2H_2 (Δ, Γ);*

in soil clods rewetted with deionised water and incubated aerobically without C_2H_2 either after anaerobic pre-incubation and C_2H_2 (Δ+, Γ+) or without pre-incubation (Δ, Γ).

DISCUSSION

N_2O production with or without anaerobic pre-incubation

During the aerobic incubation of soil clods, N_2O emission rates were much higher in clods submitted to anaerobic pre-incubation than in clods that did not experience this anoxic period (Fig. 3). The mean N_2O production rate through denitrification (using C_2H_2 inhibition of N_2O reduction and nitrification) was 60.2 pmol N_2O kg^{-1} s^{-1} in the former and only 2.87 pmol N_2O kg^{-1} s^{-1} in the latter case (no pre-incubation). Isotope measurements clearly demonstrated that most of these emissions resulted from denitrification; furthermore, we found that these emissions increased with the clod size, suggesting a dependence on aeration within the clod. Surprisingly, we found that denitrification was high in clods submitted to anaerobic pre-incubation and having a small NO_3^- content (0.46 mol m^{-3} solution) and low in clods that did not experiment anaerobic pre-incubation but which had a high NO_3^- concentration (29.1 mol m^{-3} solution). The lower NO_3^- concentration is comparable or smaller than Michaelis constant values reported in the literature: 6 mol m^{-3} for intact cores (Hénault, 1993), 11.4-31.4 mol m^{-3} (Malhi *et al*., 1990), approximately 0.22 mol m^{-3} (Klemedtsson *et al*., 1977), 0.0018–0.0166 mol m^{-3} for soil and sediment slurries (Murray *et al*., 1989) and 0.3-3.8 mol m^{-3} for purified NO_3^- reductase (Zumft, 1997). Although NO_3^- concentration was likely to be a limiting factor of N_2O emissions in clods pre-incubated anaerobically, there was no correlation between individual clod emission and its final NO_3^- concentration (results not shown). Then the other factors affecting denitrification, particularly the aeration status of the clod and its content of microbial available carbon, were most important.

Why N_2O emission through nitrification should be neglected ?

Clods that experimented anaerobic pre-incubation were simultaneously submitted to C_2H_2 (approximately 5 % of flask air), in order to check whether denitrification stopped or not at the end of this pre-incubation period. It has been recognised that both NH_4^+ oxidation and N_2O production through nitrification is inhibited by C_2H_2 until the partial pressure of this gas is higher than about 1-10 kPa (Mosier, 1980). Moreover, removing C_2H_2 is not sufficient to stop its inhibitory effect: Berg *et al.* (1982) noted that C_2H_2 effect did not cease until 7 days

after removal of initially only 10 Pa C_2H_2 applied. In order to be sure that anaerobic pre-incubation with C_2H_2 inhibited nitrification in experiments 2 and 3, additional ^{15}N measurements were realised on clods submitted to anaerobic pre-incubation without C_2H_2. Nitrification rates in clods submitted or not submitted to C_2H_2 were 0.54 pmol kg^{-1} s^{-1} and 5.6 pmol kg^{-1} s^{-1}, respectively (results not shown), and confirm the effect of C_2H_2 on nitrification.

Nitrification rates without C_2H_2 would have been equal to 5.6 nmol kg^{-1} s^{-1}, and gross N mineralisation to 0.75 nmol kg^{-1} s^{-1} in the same conditions (results not shown). This low nitrification flux can be related to the low exchangeable [NH_4^+] (i.e. 0.21 and 0.24 mmol N kg^{-1} for treatments with $^{15}NO_3^-$ and $^{15}NH_4^+$ respectively) with regard to nitrification (Bremner and Blackmer, 1981; Lensi *et al.*, 1992). Nitrogen mineralisation was not significantly affected by C_2H_2, since it was estimated at 0.37 and 0.35 nmol kg^{-1} s^{-1} in clods submitted or not to C_2H_2 during anaerobic pre-incubation, respectively. Other studies reported that the proportion of NH_4^+ transformed into N_2O was about 0.1-0.5 % (Aulakh *et al.*, 1984) or less than 1% (Bremner and Blackmer, 1978, Klemedtsson *et al.*, 1988), the remainder being transformed into NO_2^- and NO_3^-. Considering its maximum value in our situation and the actual nitrification flux in absence of C_2H_2 would have led to N_2O production rate about 0.12 nmol kg^{-1} s^{-1}. This value is small with regard to the actual value for N_2O emission through denitrification in the same conditions equal to 2.43 nmol kg^{-1} s^{-1} (results not shown).

Required conditions to increase N_2O emissions through denitrification

Since total denitrification rate of clods that did not experiment anaerobic pre-incubation was negligible compared to the N_2O production rate by clods submitted to anaerobic pre-incubation, it appears necessary that the aeration status of clods placed in air-filled flasks is affected by the anaerobic pre-incubation. Indeed, experimental measurements of aerobic CO_2 production showed that submitting clods to anaerobic pre-incubation lead to multiply this production by a factor of 2.2 and 1.9 (experiment 1), and 3.8 and 2.6 (experiment 2) in clods Δ and Γ, respectively. However, these 2 methods lead to different rates of aerobic CO_2 production. As shown in Table 3, the main difference between these two experiments lies in the method (trapping CO_2 in NaOH solution or accumulation in the gas phase), since when CO_2 accumulates in the gas phase, it also partly accumulates in the solution mainly as hydrated H_2CO_3 and HCO_3^- (Stumm and Morgan, 1996). The calculation of the actual rate of CO_2 production from its apparent value measured in experiment 2 was performed using unproved hypotheses on (i) the relationship between pH and air CO_2 concentration that was

proposed from geo-chemical simulations for hypothetical calcareous and non calcareous soil using the AQUA model (Vallès and Bourgeat, 1988), and (ii) the dissolution of calcite was neglected assuming that its kinetics behaviour limits its importance for only 6 h CO_2 accumulation (Stumm and Morgan, 1996). Moreover, since aerobic CO_2 production for clods Δ was lower (versus higher) than that of clods Γ in experiment 1 (versus experiment 2), other causes are surely to be invoked, including the conservation duration of clods before measurements, and their water content during storage which differed between experiment 1 (0.06 g g^{-1}) and experiment 2 (0.11 g g^{-1}). In our calculations, the retained actual aerobic CO_2 production rates were those corresponding to k_3 equal to 1.25 mol m^{-3}. These values were the same as O_2 consumption rates measured in soil aggregates samples with 0% of CO_2 and at different O_2 concentrations (1, 2, 5, 10 and 20% of O_2) (results not shown).

In addition to an increase in aerobic respiration, it is possible to invoke an increase in the $N_2O/(N_2O+N_2)$ ratio; however such possible (but unproved) increase could not have been sufficient alone to explain the difference between clods submitted or not to anaerobic pre-incubation, since total denitrification of clods that did not experiment anaerobic conditions was greatly lower to the N_2O emissions of other clods. An increase in $N_2O/(N_2O+N_2)$ ratio could *a priori* result from a decrease in the pH of the soil solution (Knowles, 1982), an increase in $[NO_3^-]$ (Blackmer and Bremner, 1979), and/or a decrease in aerobic respiration (Parkin, 1987). In our context, the last 2 hypotheses can not be retained, since $[NO_3^-]$ and aerobic respiration were lower and greater, respectively, for clods submitted to anaerobic pre-incubation. Therefore, only pH decrease can be evoked and could have resulted simultaneously from the accumulation of fatty acids during anaerobic pre-incubation (Dassonville and Renault, 2002) and from an increase of CO_2 (in experiment 2) that can affect the pH (Tsusuki *et al.*, 1987; Stumm and Morgan, 1996). However, it is not certain that pH has significantly decreased since (i) measurements (after re-equilibration of soil solution CO_2 with atmospheric ones) did not change with the anaerobic pre-incubation, in agreement with unpublished simulations that show that fatty acids can decrease soil pH of only about 0.4 unit for high concentrations of acetate and butyrate (40 mol m^{-3} solution for a calcareous soil), and (ii) maximum values of CO_2 at the end of the measurement period was 1.65 10^{-3} mol m^{-3} air, that can decrease the pH of the soil solution of less than about 0.6 unit. In addition, the presence of C_2H_2 during anaerobic pre-incubation not only inhibit N_2O reduction, but also the synthesis of N_2O reductase (Klemedtsson *et al.*, 1977): this compound can have maintained the $N_2O/(N_2O+N_2)$ ratio at a high value, even have lead to an increase of this ratio if denitrifiers have significantly developed during the anaerobic pre-incubation.

Biogeochemical changes that could appear during the prolonged anaerobic period

Anaerobic condition often lead to the incomplete degradation of organic matter and the accumulation of small compounds, including fatty acids (Tsusuki *et al*., 1987) that can thereafter be easily mineralised in aerobic conditions (Burford and Bremner, 1975; Förstner, 1987). This accumulation of small organic compound results from the concomitant realization of the following conditions:

- the lack of electron donors such as N and metal oxides that can enable complete microbial oxidation of organic compounds (Pelmont, 1993). This was the case for N oxides in our experiment;
- the possibility of fermentative bacterial population to develop in anaerobic conditions, even if it is initially poorly represented (Dassonville and Renault, 2002);
- the lack of SO_4^{2-} reducing bacteria and methanogene archaea – especially in soils not subjected to frequent anaerobic conditions - that can consume volatile fatty acids, but develop slowly with regard to fermentative populations (Tsusuki *et al*., 1987; Conrad, 1999).

This accumulation of small organic compounds may be indirectly reflected by the increase of soluble organic C during the anaerobic pre-incubation. It justified our choice to consider 2 organic pools in equation (4) to explain aerobic CO_2 production: the first pool being for native organic C, and the second one for these small organic compounds. In agreement with this interpretation, the period constant $1/k_1$ for mineralisation of the first pool was about 200-260 h; in contrast, the corresponding value $1/k_2$ was only about 4-20 h, indicating that some organic compound can easily be mineralised in aerobic conditions after the anaerobic pre-incubation. Dealing with the nature of organic C, it is generally accepted that soluble compounds are better used than non-soluble ones (Moore, 1998; Kalbitz *et al.*, 2000). Although, the most abundant dissolved organic compounds (DOC) are poorly consumed (Gaffney *et al*., 1996), whereas hydrophilic acids and simple compounds are better used: consequently, only a small fraction of DOC is really mineralised (Thurman, 1985).

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CHAPITRE 4

MODELE SIMPLIFIE DES EMISSIONS DE N_2O PAR DENITRIFICATION PRENANT EN COMPTE LA STRUCTURE DES MOTTES DE SOL ET LA PREINCUBATION ANAEROBIE

Ce chapitre 4 correspond à un article « en cours de soumission » pour la revue *Soil Science Society of America Journal* : K. Khalil, P. Renault, B. Mary (2003)

A simplified model of N_2O emission by denitrification that accounts for soil clod structure and anaerobic pre-incubation

SUMMARY

Nitrous oxide emissions by soils through denitrification depend on denitrifying activity, soil respiration and the anaerobic fraction of soil. We have previously found that anaerobic pre-incubation of soil clods stimulates both microbial respiration and N_2O emissions through denitrification during a subsequent aerobic incubation of these clods. In this work, we have checked whether the observed increases in aerobic respiration and denitrification activities could explain the extra N_2O production by taking into account the biological activities and the actual structure of soil clods. Soil structure was characterized in 10 intact clods 'Δ' (with a massive structure) and 'Γ' (with visible porosity) by image analysis of thin sections. Three descriptors were calculated for each thin section: the number of air-filled holes (n_h), the maximal distance between any point and the nearest air-filled pore and the mean radius of the clod (r_a). Only the last descriptor was significantly correlated with the clod mass ($p<0.05$). The consistency of the estimates of n_h and r_a was checked by comparing the observed and predicted clod mass, the latter being dependent on these parameters. There was a good agreement between the simulated and experimental clod weights. We propose a simple model which accounts for clod structure and microbial activities to predict the anoxic soil volume and denitrification rate. The model was able to predict both the marked effect of anaerobic pre-incubation and the effect of clod mass on N_2O emissions. The variations in aerobic respiration and maximum denitrification activities that result from an anaerobic pre-incubation could explain the stimulation in N_2O emissions, accounting for the actual structure of soil clods.

Key-words: denitrification, structure, anaerobiosis, nitrous oxide, respiration, clod, aggregate.

INTRODUCTION

Nitrous oxide is trace gas which play a central role in atmospheric chemistry, it is involved in ozone decomposition in the stratosphere and exerts a significant greenhouse effect (Graedel and Crutzen, 1992; Smith, 1990; IPCC, 1996). Its emission from soils results from nitrification (Groffman, 1991) and denitrification (Hénault and Germon, 1995; Conrad, 1996).

Numerous studies have been devoted to the production of N_2O by either denitrification (Firestone *et al*. 1979; Maag *et al*., 1997, Ambus, 1998) or nitrification (Li *et al*., 1992; Zanner and Bloom, 1995; Stevens *et al*., 1997, Arah, 1997). The micro-scale approach to study and describe denitrification is motivated by the fact that, in many cases, the conditions experienced by soil organisms in micro-sites are not reflected by measurements on bulk soil samples (Parkin 1987). Oxygen concentrations may decrease from atmospheric concentration to zero values within a few millimeters in soil clods (Sextone *et al*. 1985, Sierra *et al*. 1995), and less than 1 mm in the vicinity of particulate organic matter (Parkin, 1987; Parry *et al*., 2000) and active root tips in locally saturated conditions (Bidel *et al*., 2000).

Several models have been proposed to describe respiration, anaerobiosis, total denitrification and/or N_2O emissions through denitrification at this scale (Rappoldt, 1992; Renault and Stengel, 1994; Parry *et al*., 1999). However, none of these model accounts for variations with time in respiration, denitrification and N_2O reduction, although it is well recognized that these activities vary, as the consequence of microbial dynamics and/or enzyme inductions and activations (MacConnaughey *et al*., 1985; Holtan-Hartwig *et al*., 2000). Recently, it has been shown that N_2O emissions by clods placed in aerobic atmosphere were markedly stimulated when these clods had been previously submitted to anaerobic conditions (Khalil *et al*., 2003b). These authors showed that these emissions resulted from denitrification and that O_2 consumption rate was also stimulated by the anaerobic pre-incubation. Such an increase may be explained by the accumulation of intermediate organic compounds which are not completely mineralized in anaerobic conditions and possibly by the death of strictly aerobic bacteria and fungi that cannot survive in such conditions (Förstner, 1987; Tsusuki and Ponnamperuma, 1987; Chin and Conrad, 1995; Conrad, 1999).

The aim of this work was to check whether variations in aerobic respiration and maximum denitrification activities (i.e. N_2O production and consumption) that result from an anaerobic pre-incubation are sufficient to explain the variations in N_2O emissions, accounting for the

actual structure of soil clods. The use of model is a tool to fulfill this objective. It requires an adequate description of the clods structure with regard to their aeration and denitrification behavior.

MATERIALS AND METHODS

Soil Clod Sampling

Experiments were performed in an Orthic Luvisol (FAO classification) sampled in Mons-en-Chaussée, in Northern France (49°80' N; 3°60' E). This arable soil has been following a wheat-maize-wheat-sugarbeet rotation for 12 years and was cropped in maize in 2000. The properties of the soil were as follows: clay, 194 $g.kg^{-1}$; silt, 706 $g.kg^{-1}$; sand, 68 $g.kg^{-1}$; pH (water), 8.2; total $CaCO_3$, 32 $g.kg^{-1}$; organic C, 8.52 $g.kg^{-1}$; total N 1.00 $g.kg^{-1}$; and NO_3^--N, 4.70 $mg.kg^{-1}$. Clods were sampled in the ploughed layer (10-30 cm depth) on September 12, 2000. Two sets of clods were separated: clods Δ, with a massive structure and no visible porosity (resulting from compaction due to traffic) and clods Γ, with a fragmentary structure and visible porosity (Richard *et al*., 1999). The larger clods were gently broken down immediately after sampling and then calibrated : we kept clods between 2.5 and 3 cm size. In order to reduce microbial activity during storage, the clods were air-dried during 3 days to obtain a residual moisture close to 0.10 g g^{-1} soil. They were stored at 2°C until the beginning of the experiments, i.e. until November 2000. At this time, the soil moisture was about 0.11 $g.g^{-1}$.

Measurements of N_2O Emissions

After storage, the soil clods Δ and Γ were rewetted with either water (treatment A) or KNO_3 solution at 4$g.L^{-1}$ (treatment B). Rewetting was realized at 20°C on suction tables at -10 kPa suction during 1 day, then –5 kPa during 1 day, -1 kPa during 1 day, and finally –0.5 kPa during 4 days. This procedure ensured a slow rewetting process that prevented the occurrence of additional clod cracks. The water content thus obtained was 0.22 and 0.24 $g.g^{-1}$ soil for clods Δ and Γ, respectively. The mineral N content (NH_4^+, NO_2^- and NO_3^-) of the clods was measured at the end of the wetting period on 6 replicates for each clod type and each treatment.

Clods rewetted with water (treatment A) were then submitted to generalized anaerobiosis: they were placed in closed 150 mL flasks under anaerobic conditions by alternating 3 successive cycles of 3 minutes vacuum and 3 minutes N_2 filling in the flasks. Seven mL of

gas were removed and replaced by the same volume of C_2H_2 and 1 mL of gas was replaced by Kr. Thirty two flasks containing either Δ or Γ clods were incubated at 20°C in order to follow denitrification during this first incubation period. The final mineral N content (NH_4^+, NO_2^- and NO_3^-) was measured on 6 replicates of clods Δ and Γ. After 6 days and 14 h (day 6.6), clods were placed in aerobic condition by alternating 3 successive cycles of 3 minutes vacuum and 3 minutes of air filling in the flasks. In order to ensure that all C_2H_2 has been removed, the flasks were subjected to the same procedure after 1 additional hour. 1 mL of air was then replaced by Kr. For treatment B, 10 clods Δ and 10 clods Γ were placed in closed 150 mL flasks under aerobic conditions just after the wetting period on suction tables; 1 mL of air was replaced by Kr, and the clods were pre-incubated during 15 h.

Gas samples of treatments A and B were then withdrawn after 1, 3, 5 and 7 hours and analyzed for N_2O, N_2, CO_2, O_2, Kr and C_2H_2 concentrations. Mineral N was extracted in treatment A at 7 hours and analyzed for NH_4^+, NO_2^- and NO_3^-.

The flasks used in treatment B were thereafter opened for a few minutes in order to release the trapped gases. After closing the flasks, 7 mL and 1 mL of gas were replaced by C_2H_2 and Kr, respectively. Clods Δ and Γ (10 replicates) were incubated at 20°C for another 6 hours. Gas samples were withdrawn 14, 16, 18 and 20 hours after the addition of C_2H_2 and analyzed for N_2O, N_2, CO_2, O_2, Kr and C_2H_2 concentrations. Mineral N was extracted in treatment B at 20 hours and analyzed for NH_4^+, NO_2^- and NO_3^-.

Nitrous oxide concentration was analyzed by gas chromatography equipped with an electron capture detector (HP 5890 Series II) fitted with a Porapak Q column (80-100 mesh, 2 m) coupled to an integration software on a PC Workstation. Nitrogen, CO_2, O_2, Kr and C_2H_2 concentrations were measured on another gas chromatograph with TCD (HP5890 Series II) and columns filled with Porapak Q (80-100mesh, 1.8 m) and molecular sieve (0.1-0.5 nm, 1.8 m) coupled to the same integration software. The mineral N content of clods was extracted with a 1M KCl solution (soil:solution = 1:5). Measurements were performed with a continuous flow analyzer (Skalar, Breda, The Netherlands) using the method proposed by Henriksen and Selmer-Olsen (1970).

Model of N_2O Emission by Soil Clods

Actual clods are assimilated to a set of n_a independent spherical aggregates having a

unique radius r_a (m). Similar hypothesis was made earlier by Rappoldt (1990) to estimate the soil anaerobic fraction, and by Parry *et al*. (2000) to estimate total denitrification within soil clods. The anaerobic fraction (v/v) of an aggregate may then be estimated, considering the diffusion of O_2 and its consumption which is assumed to be constant as long as O_2 is available (Renault and Stengel, 1994). A spherical aggregate has an anaerobic center when its radius r_a is greater than the critical radius r_c (m) given by the formula:

$$r_c = \sqrt{\frac{6\, D_{O_2}\, s_{O_2}\, [O_2]_e}{M}} \tag{1}$$

where D_{O_2} is the O_2 diffusion coefficient in soil aggregates ($m^2.s^{-1}$), s_{O_2} is the O_2 solubility (mol $O_2.m^{-3}$ water/mol $O_2.m^{-3}$ air), $[O_2]_e$ is the external O_2 concentration (mol $O_2.m^{-3}$ air) and M is the microbial respiration rate (mol $O_2.m^{-3}$ soil.s^{-1}). The larger aggregates have a spherical anaerobic center whose radius r_0 is:

$$r_0 = -r_a \frac{\cos(\theta)}{2\cos(\theta/3)} \tag{2a}$$

with

$$\theta = \pi - \arcsin\left(\frac{r_c}{r_a}\right) \tag{2b}$$

The anaerobic fraction f_0 of an aggregate is then:

$$f_0 = \left(\frac{r_0}{r_a}\right)^3 \tag{3}$$

Assuming that total denitrification rate is affected neither by NO_3^- concentration nor by competition with N_2O as alternate electron acceptor in respiratory chain, total denitrification rate is then proportional to f_0:

$$E_{N_2+N_2O} = A_d\ f_0 \tag{4}$$

where $E_{N_2+N_2O}$ is the emission rate of all denitrification products (mol N_2+$N_2O.kg^{-1}$ soil.s^{-1}), and A_d the denitrifying rate (mol.kg^{-1} soil.s^{-1}) in anaerobic conditions.

Assuming that the $N_2O/(N_2O+N_2)$ ratio of denitrification products (r_N) does not vary, the N_2O emission rate E_{N_2O} (mol $N_2O.kg^{-1}$ soil.s^{-1}) can then be written:

$$E_{N_2O} = A_d\, r_N\ f_0 \tag{5}$$

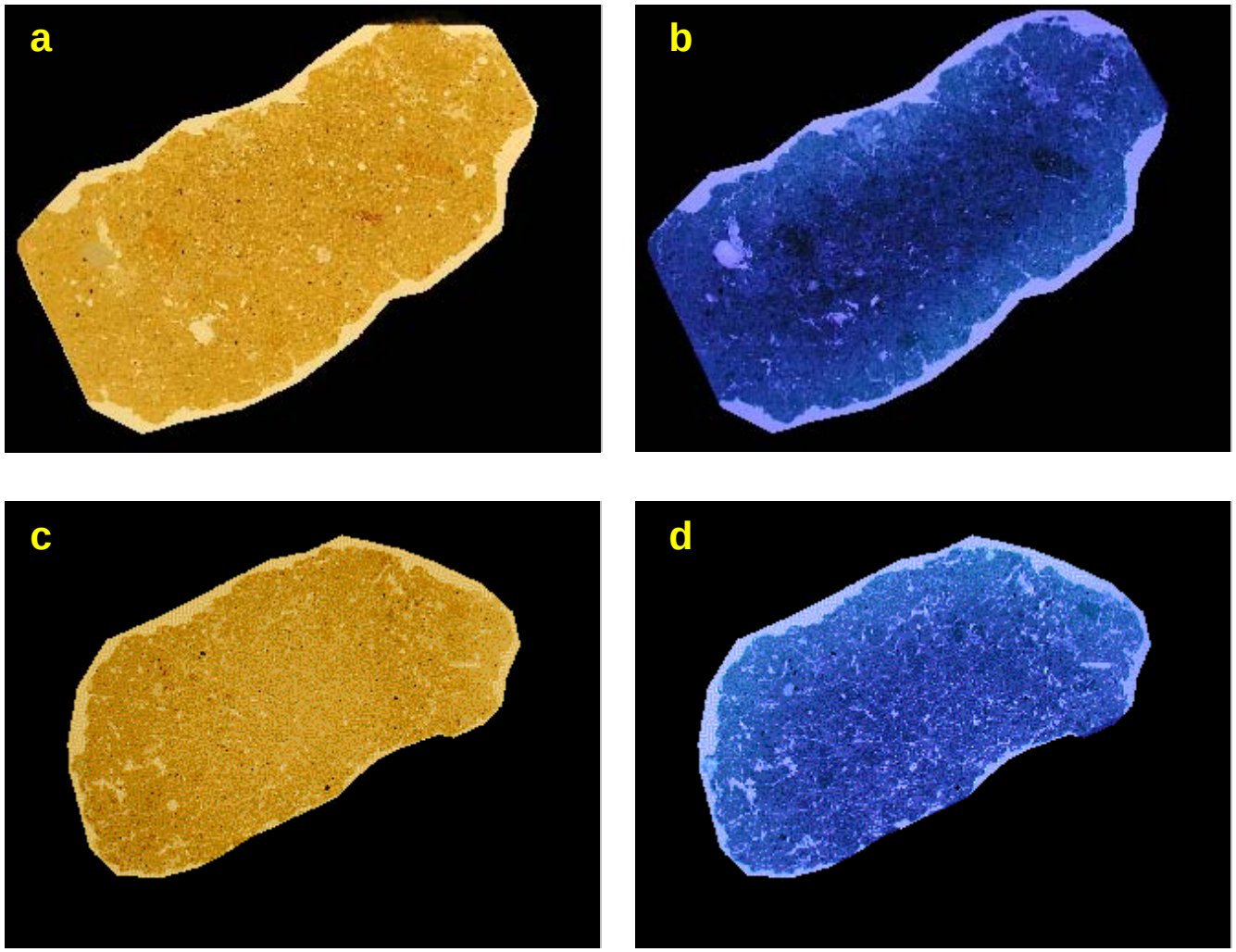


Photo 1. *Clod sections photographs: a/ and c/ under transmitted visible light of clods Δ and Γ, respectively; b/ and d/ under reflected UV light of clods Δ and Γ, respectively.*

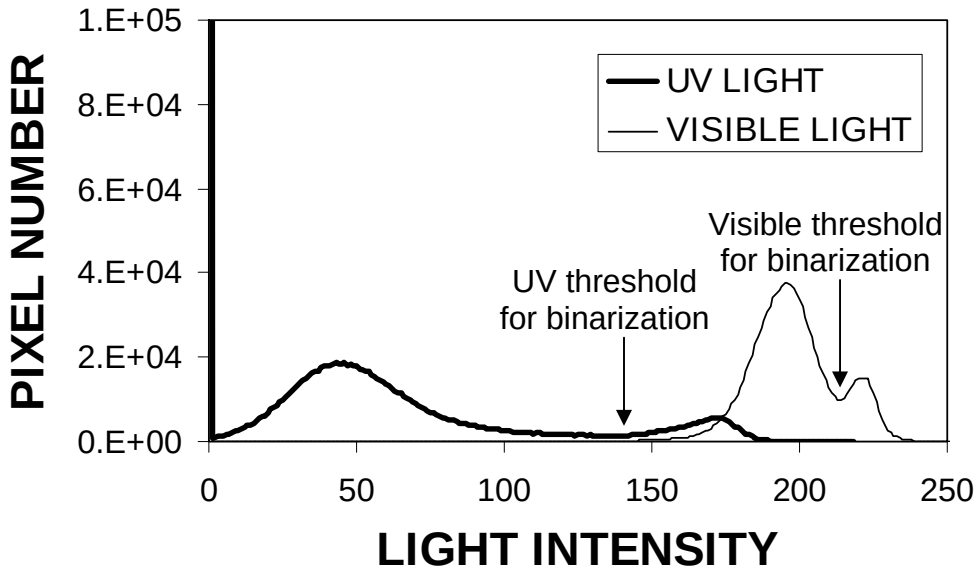
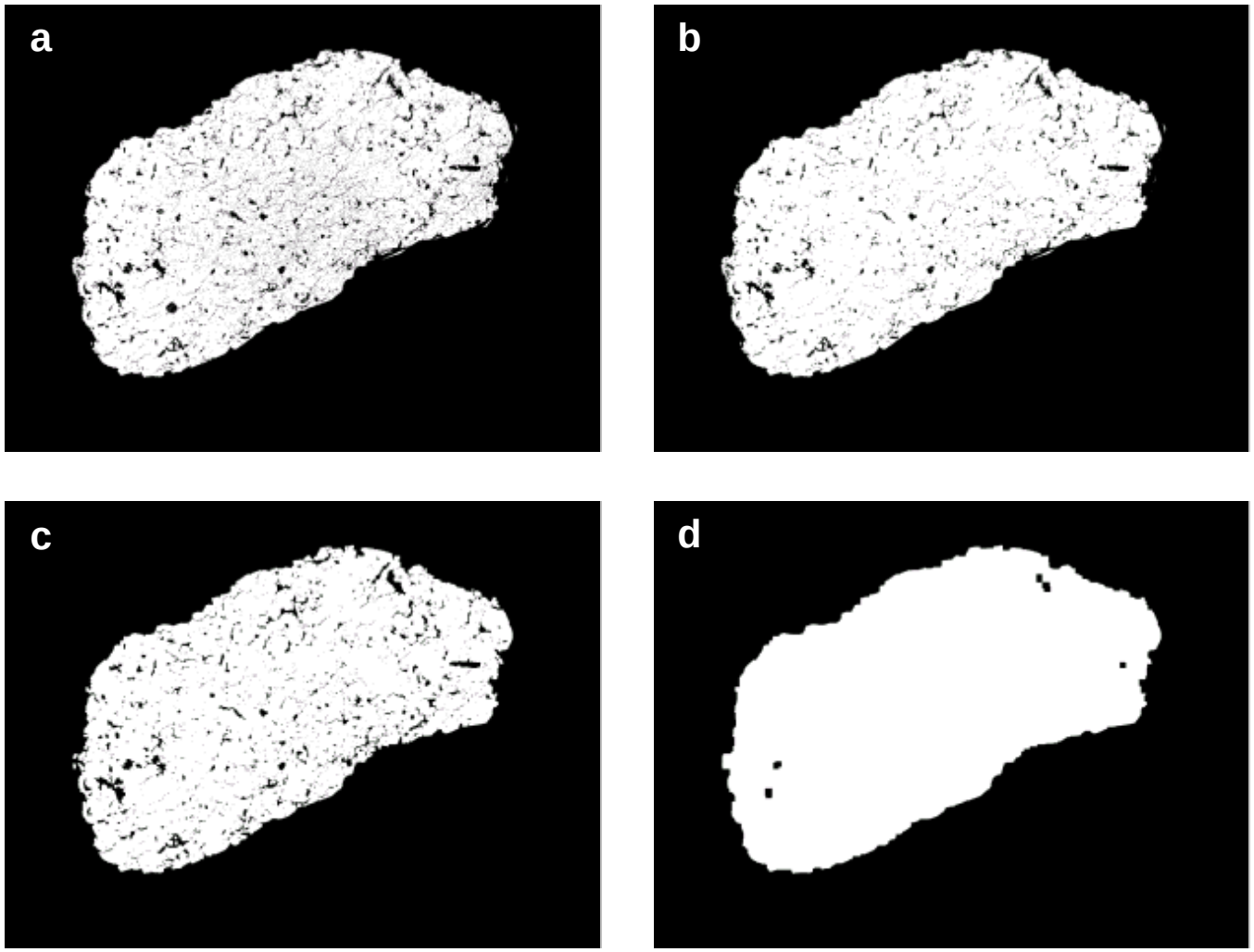


Figure 1: *Histogram of light intensity for a Γclod corresponding to Photos 1a-b.*



Figures 2a-d: *Example of morphological operations performed prior image characterization for a Γ clod corresponding to Photos 1a-b: a/ and b/ binarization of visible and UV photos, respectively; c/ logical sum of binarized sections; d/ simulation of saturation at 5 cm water suction.*

For a clod composed of n_a aggregates having the same diameter, N_2O emission rate, expressed per unit of clod mass, is also equal to E_{N_2O}.

The model includes 7 parameters: (i) physical and chemical parameters (D_{O_2} and s_{O_2}), (ii) microbial parameters (M, A_d, and r_N), and (iii) structural parameters (n_a and r_a).

Estimating Parameters

Clod Structure

Morphological characterization of clods Δ and Γ clods was realized on 10 replicates in order to (i) obtain the effective radius r_a for gas diffusion and the number n_a of spherical aggregates that constitute the virtual clods assumed to be equivalent to actual ones, and (ii) assess the variations of these parameters with clod weight and morphological type.

The clods were dried, weighted and included in resin with a fluorescent dye (Bruand *et al*., 1996). A thin section was made on each clod, care being taken to realize the section at the center of the clod. Photographs taken under transmitted-visible and reflected-UV light were scanned (1 pixel = 29 µm × 29 µm) and analyzed with VISILOG software (Noesis, Paris, France). Both visible and UV pictures highlight macropores; furthermore, quartz particles appear with visible radiations and large particulate organic matter are revealed by UV radiations (Photos 1a-d). The scanned sections were transformed into binary ones, using minima of light intensity between peaks as thresholds to distinguish voids and solid phase (see example in Fig. 1). For each clod, the binary data resulting from UV and visible acquisitions were summed up, considering that actual voids were those which were recognized as voids in UV light and voids in visible light. At the -0.5 kPa water potential, the pores having diameters lower than 500 µm were water saturated. These pores were 'closed' in the image analysis in order to simulate their saturation with water (Serra, 1982). An example of binarization of visible and UV images, subsequent summing up of these images, and water saturation is reported in Figure 2, for the clod Γ which is presented in Photo 1. The image thus obtained enables to calculate three variables characterizing clod structure:

- the number of air-filled holes (n_h). We checked whether this number is proportional to clod mass, and the structure is independent from this weight;
- the maximal distance (r_m) between any point of the clod section and the nearest air-filled pore. It is computed using successive morphological 'erosion' operations up to

Parameter	Unit	Treatment *	Value	Origin of the estimate
D_{O_2}	$m^2.s^{-1}$	A and B	$3.00\ 10^{-10}$	Renault and Stengel (1994)
s_{O_2}	-	A and B	$3.40\ 10^{-2}$	Renault and Stengel (1994)
M	$nmol.kg^{-1}.s^{-1}$	B	3.18	Khalil *et al*. (2003b) [1]
		A	12.2 (8.2-16.5)†	Khalil *et al.* (2003b) [1]
A_d	$nmol.kg^{-1}.s^{-1}$	B	1.00	Measured in this work
		A	1.60	Estimated in this work [2]
r_N	-	A and B	0.13	Khalil *et al*. (2003a)
n_a	$clod^{-1}$	A and B	4.65 (3.10-6.20)†	Estimated in this work
$\overline{n}_h$	$Clod^{-1}$	A and B	4.80	Measured in this work
α	$mm.g^{-1/3}$	A and B	3.0	Estimated in this work
β	$mm.hole^{-1}$	A and B	-0.29	Estimated in this work
ρ_b	$kg.m^{-3}$	A and B	1700	Fies and Stengel (1981) [3]

Table 1: *Parameters of the N_2O emission model by soil clods through denitrification.*

* *Treatments A and B are with and without anaerobic pre-incubation, respectively;*

† *minimum and maximum values used corresponding to mean values reduced or increased by 33%, respectively.*

[2]*: calculated from A_d value measured in treatment B and accounting for the growth of denitrifier biomass as estimated by Khalil et al. (2003a).*

[3]*: using the linear correlation between textural bulk density and clay content of natural soils.*

the disappearance of the image (Serra, 1982). r_m may be regarded as a first estimate of the equivalent radius of an aggregate constitutive of virtual clods in the model;

- the actual radius of the clod r_a (m). It is calculated using the perimeter P (m) and the area A (m^2) of the clod section given by the image analysis, as follows:

$$r_a = \frac{2A}{P} \tag{6}$$

Microbial Respiration, Denitrification and $N_2O/(N_2O+N_2)$ ratio of Denitrification Products

In order to simulate N_2O emission rates, we first estimated microbial parameters of clods, i.e. O_2 consumption rate (M), denitrifying activity (A_d) and $N_2O/(N_2O+N_2)$ ratio of denitrification products (r_N) (Table 1). The microbial respiration rate was deduced from measurements of CO_2 production corrected to account for CO_2 solubilisation and dissociation, as explained by Khalil *et al*. (2003b), using the intermediate case of pH dependence to CO_2 partial pressure.

The denitrifying activity A_d (i.e. the activity in anaerobic condition without NO_3^- limitation) was estimated from measurements made with another set of 10 clods Δ and Γ submitted to exactly the same history than in treatment B. Each clod was placed into 150 mL flasks, and supplemented with the same weight of KNO_3 solution (4 $g.L^{-1}$). The obtained slurries were put under anaerobic conditions by alternating 3 successive cycles of 3 minutes vacuum and 3 minutes N_2 filling. 7 and 1 mL of gas were then replaced by C_2H_2 and Kr, respectively. Closed flasks were incubated at 20°C for 6 hours. Gas samples were withdrawn 3, 4, 5 and 6 hours after the addition of C_2H_2 and analyzed for N_2O, N_2, Kr and C_2H_2 concentrations. These measurements were used to characterize the denitrifying activity of clods Δ and Γ in treatment B. The denitrifying activity in treatment A was obtained by multiplying this value by 1.6 in order to take into account the increase in denitrifier biomass during the 6 day anaerobic pre-incubation, as was estimated previously for this soil by Khalil *et al*. (2003a).

The $N_2O/(N_2O+N_2)$ ratio r_N. was estimated from measurements performed by Khalil *et al.* (2003a) on slurries of the same soil with and without C_2H_2 (Table 1).

Oxygen Diffusion Coefficient and Solubility in Water

In order to simulate N_2O emission rate on Δ and Γ clods, we need to have an estimate of the O_2 diffusion coefficient D_{O_2} in saturated aggregates, and the O_2 solubility s_{O_2}. These parameters were deduced from Renault and Stengel (1994) (Table 1).

RESULTS AND DISCUSSION

Experimental Nitrous Oxide Emissions

The net N_2O emissions of clods that did not experiment anaerobic pre-incubation (treatment B) and did not receive C_2H_2 were negligible and even negative (Fig. 3): the mean rate was –0.7 ± 0.6 pmol $N_2O.kg^{-1}.s^{-1}$ (confidence interval at $p<0.05$). The measurements performed on the same clods after having supplemented the flasks with C_2H_2 confirmed that total denitrification was very small: 4.2 ± 3.1 $pmol.kg^{-1}.s^{-1}$. In contrast, the mean N_2O emission of clods that had experienced anaerobic pre-incubation (treatment A) was much higher: 60.2 ± 8.1 $pmol.kg^{-1}.s^{-1}$. The N_2O emission rate was significantly higher ($P<0.05$) in clods Δ (72.1 ± 9.4 $pmol.kg^{-1}.s^{-1}$) than in clods Γ (48.3 ± 8.3 $pmol.kg^{-1}.s^{-1}$). The mean clod mass was also higher for clods Δ (25.8g) than for clods Γ (20.2 g), the difference being significant at $p<0.10$. Considering all Δ and Γ soil clods in treatment A, the correlation coefficient between N_2O emission and clod mass was equal to 0.70 ($n=20$, $p<0.05$); considering separately Δ and Γ soil clods, the correlation coefficients were equal to 0.75 (significant at $P<0.05$) and 0.25 (not significant at $P<0.05$) for clods Δ and Γ, respectively.

Immediately before the measurement of N_2O emissions, the NO_3^- content of clods which experienced the anaerobic pre-incubation (treatment A) was much lower than that of clods which did not (treatment B): 0.067 mmol $N.kg^{-1}$and 6.4 mmol $N.kg^{-1}$, respectively. This was due to the active denitrification which had occurred during the anaerobic period. In contrast, the NH_4^+ contents did not significantly differ between treatments A and B: they were 0.15 and 0.11 mmol $N.kg^{-1}$, respectively.

Complementary measurements using ^{15}N tracing demonstrated that N_2O emission did not come from nitrification (Khalil *et al.*, 2003b). It may be surprising that in these conditions, most of N_2O emissions due to denitrification were observed in clods having the lowest initial NO_3^- content. Moreover, the initial NO_3^- content in clods of treatment A, equal to 28 $mmol.m^{-3}$ solution, was lower than the Michaelis constant for NO_3^- in denitrification proposed in the literature e.g. 6 $mol.m^{-3}$ for intact cores (Hénault, 1993), 11.4-31.4 $mol.m^{-3}$ (Malhi *et al.*, 1990) and 0.3-3.8 $mol.m^{-3}$ (Zumft, 1997) for purified NO_3^- reductase.

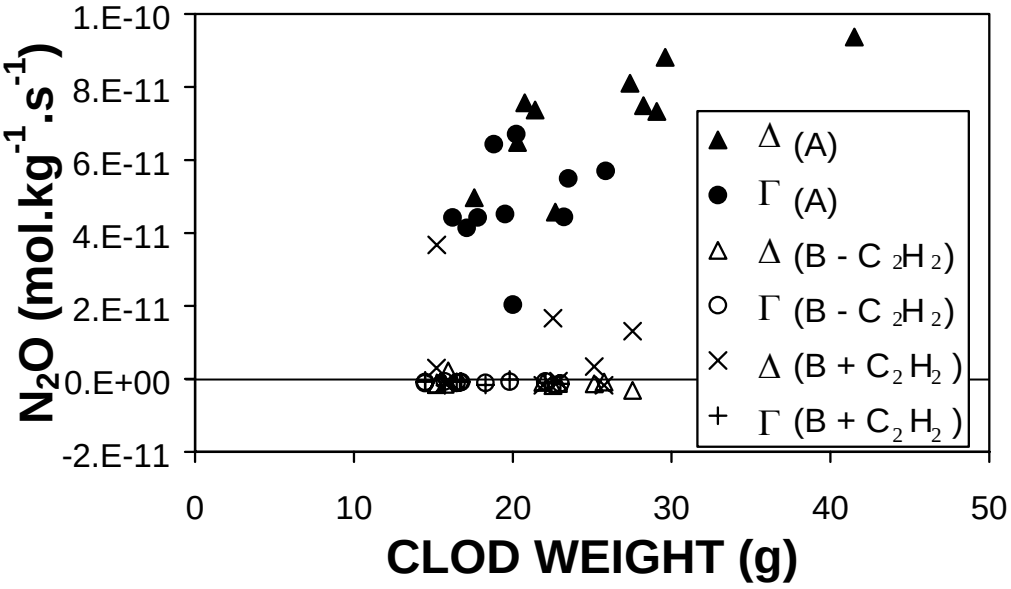


Figure 3: *Nitrous oxide emission as a function of clod weight for treatment A (i.e. with anaerobic pre-incubation) and treatment (B) (i.e. without anaerobic pre-incubation). For treatment (B), emissions were measured without and with C_2H_2 in the atmosphere.*

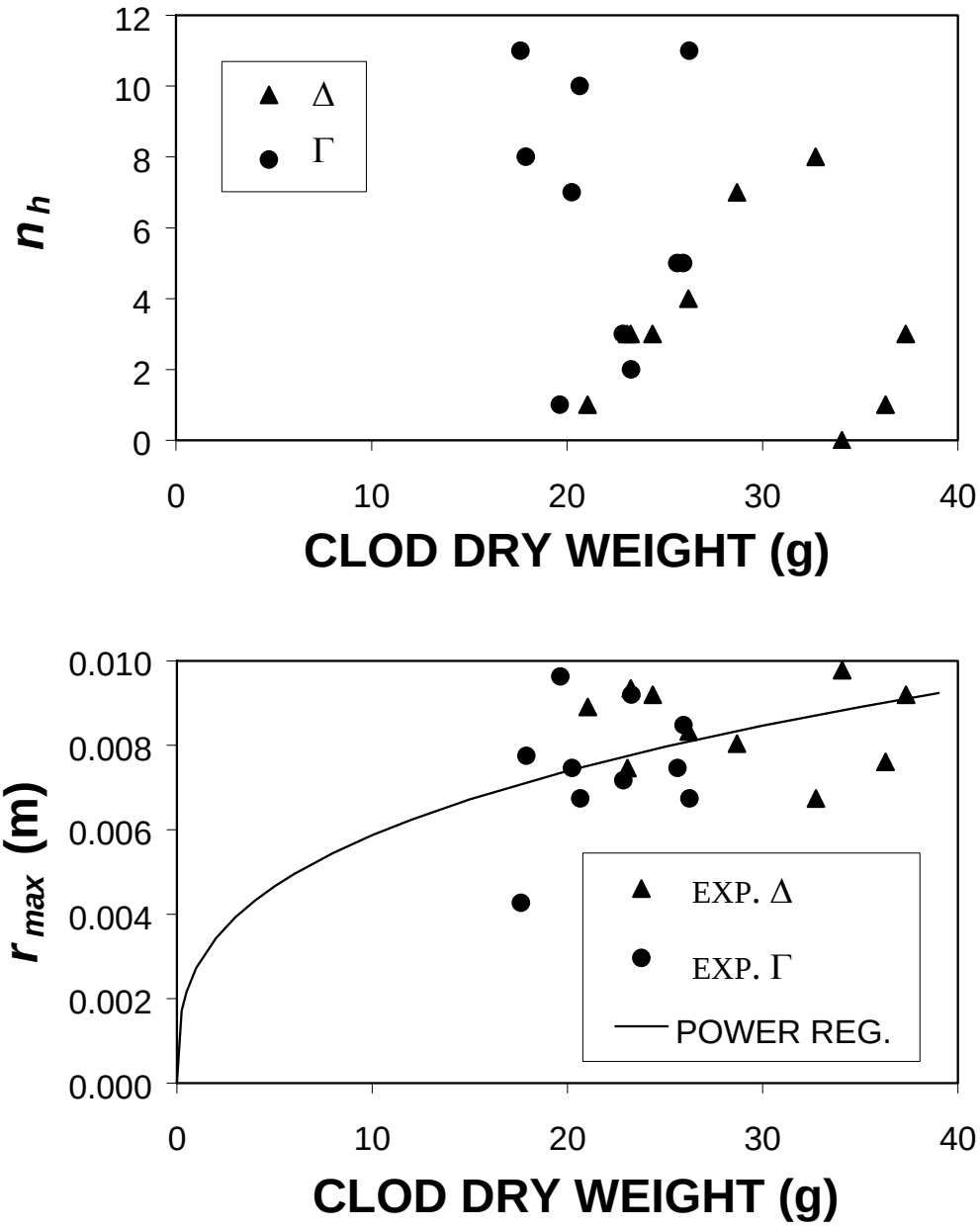


Figure 4. *Morphological characterization of centered Δ and Γ clod sections after simulating their partial saturation at 5 cm water suction: a/ number of air-filled holes per clod, as a function of clod weight; b/ maximum distance of a clod point to the nearest air-filled pore in the section, as a function of clod weight.*

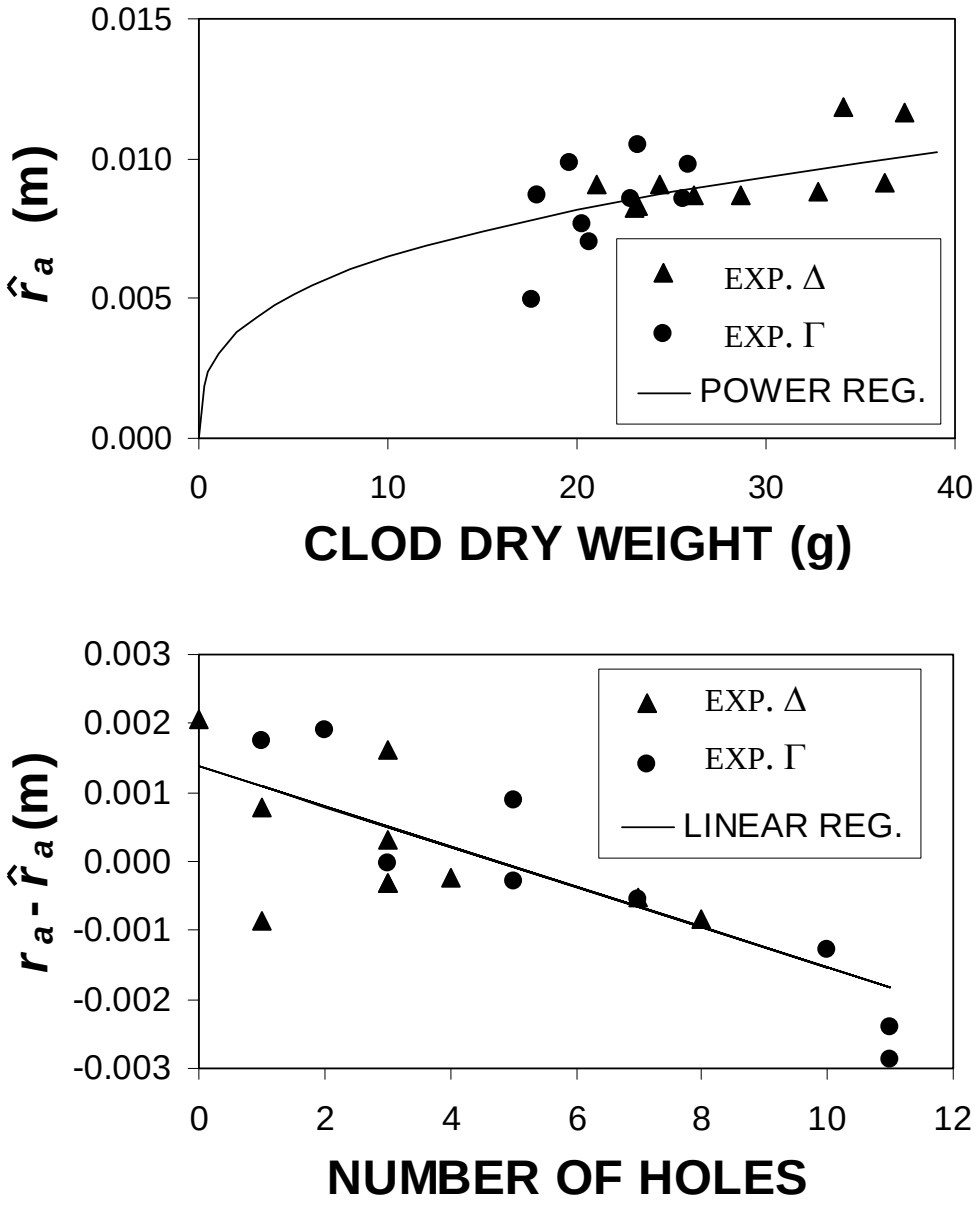


Figure 5. *Morphological characterization of centered Δ and Γ clod sections after simulating their saturation at 5 cm water suction: a/ twice surface to perimeter ratio (r_a), as a function of clod weight; b/ deviation between the actual r_a and the estimated $\hat{r}_a$, and the number of air-filled holes on a section. ($\hat{r}_a$ were obtained by fitting a 1/3 power regression to experimental r_a).*

Parameter Estimation

Relation between Clod Structure and Weight

The mean dry weight of the clods devoted to morphological characterization were 28.7 ± 3.7 and 22.0 ± 2.0 g for clods Δ and Γ, respectively (confidence interval at $p<0.05$). The number of air-filled holes did not significantly differ between the two types of clods: 4.8 ± 1.5, as well as the maximal distance to the nearest air-filled pore which was 8.0 ± 0.6 mm. There was no significant correlation ($p<0.05$) between the number of air-filled holes and the dry weight of clods (Fig. 4a). Similarly, the correlation between the maximal distance to the nearest air-filled pore and the clod weight was not significant, although it was possible to fit a cubic power regression to morphological measurements without bias (Fig. 4b). In contrast, the linear correlation coefficient between the clod mass m (g) and the estimated aggregate radius $\hat{r}_a$ was significant ($p<0.05$) (Fig. 5a).

A relationship with a similar coefficient of correlation was found between with the following power function (Fig. 5a):

$$\hat{r}_a = \alpha(m)^{1/3} \tag{7}$$

The optimized value of α was 3.0 mm $g^{-1/3}$.

Assuming that each clod is a set of n_a aggregates, its weight is then equal to:

$$m = \frac{4}{3}\pi\rho_b n_a r_a^3 \tag{8}$$

where ρ_b is the bulk textural density ($g.cm^{-3}$). Equations 7 and 8 enable to calculate the number of aggregates:

$$n_a = \frac{3}{4\pi\rho_b \alpha^3} \tag{9}$$

Assuming a bulk textural density of 1700 $kg.m^{-3}$, we can calculate that the mean number of aggregates is n_a = 5.15 per clod.

Pursuing the regression analysis, we found that the residuals (differences r_a - $\hat{r}_a$) were linearly related to the number of air-filled holes per clod (Fig. 5b). The relationship can be written:

$$r_a - \hat{r}_a = \beta(n_h - \bar{n}_h) \tag{10}$$

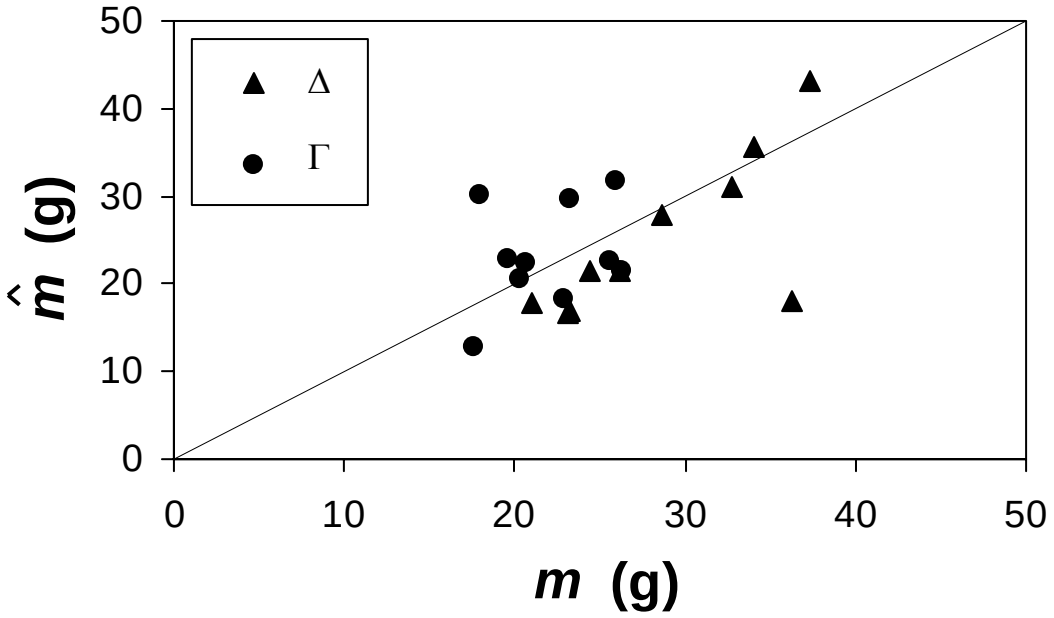


Figure 6: *Estimated clod weight as a function of actual ones, for 10 Δ and 10 Γ clods devoted to morphological characterizations. The estimated clod weight is a function of the estimated surface – to – perimeter ratio and the number of air-filled holes on a centered clod section (see Eq. (5) in the text).*

where n_h is the actual number of holes in the 2D clod section and $\overline{n}_h$ is the mean number of holes of the set of clods. The slope of the regression line is β = -0.29 mm.hole^{-1}. The mean number of holes was 4.80.The holes diameters measured by image analysis varied from 0.6 to 1.4 mm. The theoretical values of β which can be calculated using the changes in area and perimeter due to the presence of holes, were close to the measured slope since they varied from –0.11 to –0.29 mm.hole^{-1} for the 0.6 and 1.4 mm diameters, respectively. Therefore, deviations between r_a and $\hat{r}_a$ mainly result from the presence of holes, and variations in roughness of external perimeter versus clod weight have not to be invoked.

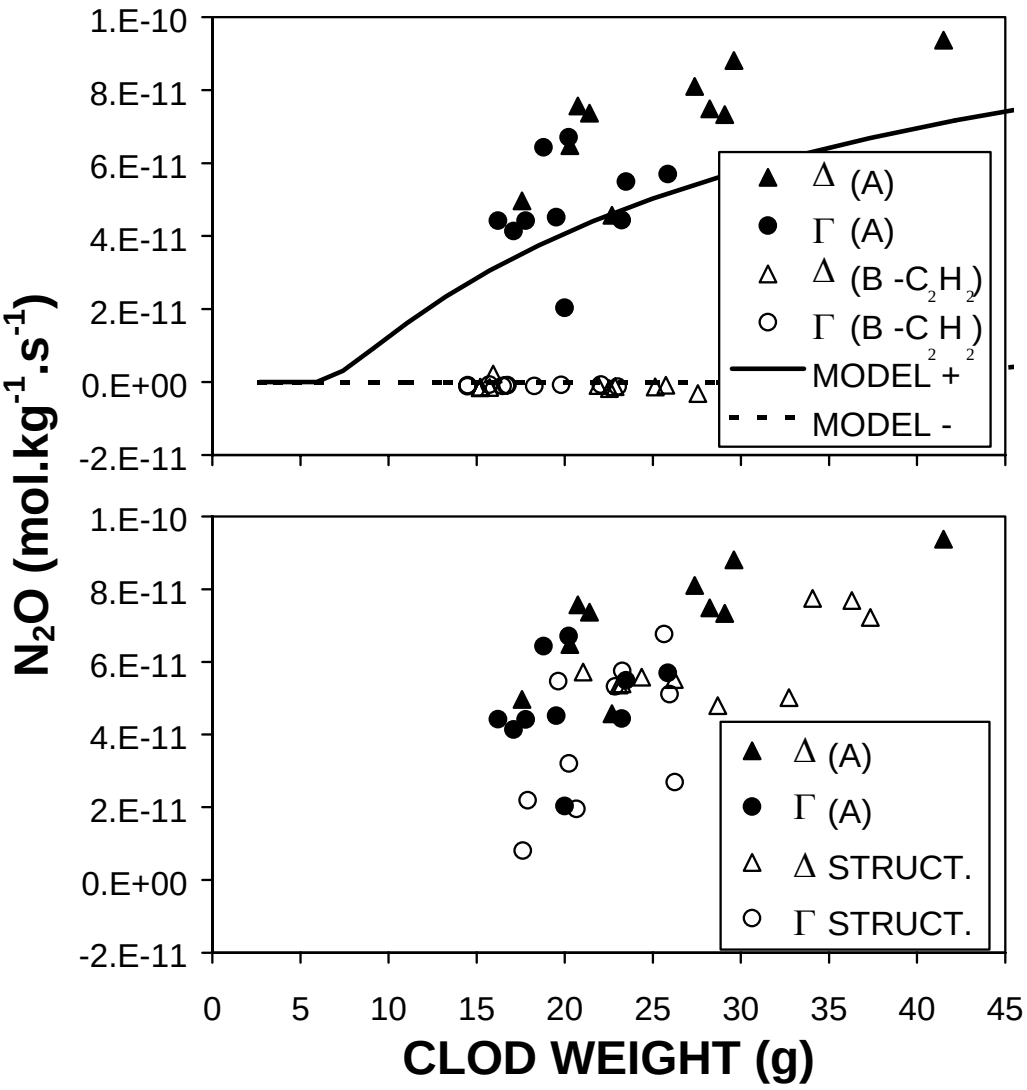
To check the consistency of the previous results, we have calculated a theoretical clod mass $\hat{m}$ (g) as a function of the theoretical radius $\hat{r}_a$ and compared it with the experimental value. The theoretical mass is:

$$\hat{m} = \frac{4}{3}\pi\, n_a\, \rho_b\, \hat{r}_a^3 \tag{11}$$

where n_a is the number of equivalent aggregates within a clod. We assume that the theoretical radius $\hat{r}_a$ is the actual radius r_a minored or majored by the effect of holes, as indicated by equation (10). Combining equations (10) and (11) yields:

$$\hat{m} = \frac{4}{3}\pi\, n_a\, \rho_b \left(r_a \left(1 - \frac{\beta\left(n_h - \overline{n}_h \right)}{r_a} \right) \right)^3 \tag{12}$$

When comparing observed and simulated values of clod mass, we fixed the parameters β and $\overline{n}_h$ to the values previously found and we optimized the parameter n_a. We found a good agreement between m and $\hat{m}$ (Fig. 6). The estimated value of the number of aggregates constitutive of a clod (n_a) was 4.65. It was close to the previous estimate (5.15) made using equation (9). Then we used β and $\overline{n}_h$ estimates and equation (12) in order to propose two estimates of the effective radius of an aggregate constitutive of a clod, using either the individual hole number measured on the clod (Eq. 13a) or the mean number of holes measured on the set of clods (Eq. 13b):



Figures 7a-b: *Comparison between simulated and experimental N_2O emissions of clods for treatments (A) and (B), when there is no C_2H_2 supply: a/ simulations performed for fictitious clods having 4.8 holes in a centered section; b/ simulations performed only for treatment (A) individually for clods devoted to morphological analyses, accounting for the observed hole numbers and surface to perimeter ratios.*

$$\tilde{r}_a = \left(\frac{m}{\frac{4}{3}\pi n_a \rho_b} \right)^{\frac{1}{3}} + \beta\left(n_h - \bar{n}_h\right) \quad (13a)$$

$$\tilde{r}_a = \left(\frac{m}{\frac{4}{3}\pi n_a \rho_b} \right)^{\frac{1}{3}} \quad (13b)$$

where $\bar{n}_h$ is the mean number of holes per clod section, i.e. 4.80 in this study.

Modeling Anaerobiosis and N_2O Emissions within Clods

Using the previously estimated parameters (Table 1) and the number of holes per clod determined by image analysis on each clod, we could simulate the net N_2O emission of soil clods in treatments A and B using equations 1-5 and 13. The observed and simulated values are given in Fig. 7. Two simulations were performed: one assuming a constant number of holes per clod in all clods, equal to the observed mean (Fig. 7a, Eq. 13a); the other corresponding to the measured number of holes in each individual clod (Fig. 7b, Eq. 13b). The model was able to predict the effect of treatments A and B on N_2O emissions; it also predicted reasonably well the variation in N_2O emission rates within each treatment. However, a bias between simulated and experimental values remains in treatment A: the model underestimated N_2O emission rates, particularly for the heaviest clods. Accounting for the variability of the number of air-filled holes between clods lead to simulate denitrification rates for clods closer from experimental values in treatment A (Fig. 7b).

However, there may be uncertainties on parameter estimates; moreover, they may vary between clods. In order to assess the effect of a change in microbial activity and clod structure, we performed additional simulations, varying either on O_2 consumption rate M alone or simultaneously on O_2 consumption rate M and the number of aggregates per clod n_a The relative variation in parameters was ± 33% (Table 1). Regarding clod structure, variations in the number of aggregates per clod n_a affects the clod mass for a given equivalent aggregate radius r_a, and therefore modify the relationship between the clod mass and equivalent radius

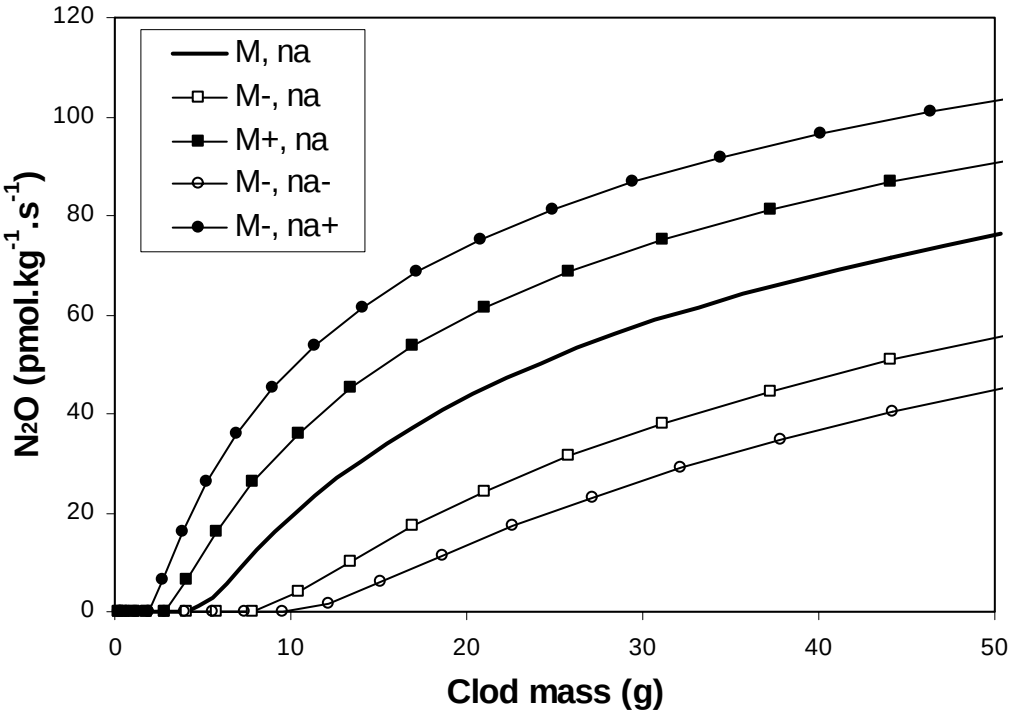


Figure 8. *Simulated N_2O emissions of clods submitted to treatment A, using either the mean values of O_2 consumption rate (M) and the mean number of aggregates per clod (n_a) or these values decreased or increased by 33%. Simulations M+ and M- were performed with M modified only; simulations (M+, n_a-) and (M-, n_a+) were performed with either M increased and n_a reduced, or M reduced and n_a increased. All clods are assumed to have 4.80 holes in a centered section.*

for clod aeration. The variations in *M* appear to greatly affect N_2O emission rate by clods (Fig. 8): for a mean clod of 25 g dry weight, the minimum and maximum N_2O emission rates correspond to a 42% reduction and a 32% increase of the mean emission rate, respectively.

If we add to this variation a variation in clod structure (i.e. either the minimum *M* with the maximum n_a, or the maximum *M* with the minimum n_a), the range of N_2O emission rates was larger (Fig. 8): for a mean clod of 25 g dry weight, the minimum and maximum N_2O emission rates correspond to a 73% reduction and a 65% increase of the mean emission rate, respectively.

Dealing with clods used in this study, its was previously shown that O_2 consumption rate was higher for clods Δ than for clods Γ (14.3 $nmol.kg^{-1}.s^{-1}$ and 10.1 $nmol.kg^{-1}.s^{-1}$, respectively): the O_2 consumption rate of clods Δ was 17% higher than the retained mean value and 17% lower in clods Γ. These differences have increased the dependency of N_2O emission rate to clod mass (see Fig. 7a), since the mean mass of clods Γ was lower than that of clods Δ. In addition, CO_2 production rates (and therefore O_2 consumption rates) varied between clods: the measured coefficients of variation were 17 % and 14 % for clods Δ and Γ, respectively. These variations lead to a variability in clod N_2O emission rates, as well as the variability in clod structure as it has already been shown, simulating the N_2O emission rates of clods that have been devoted to soil structure characterization.

The model supposed that N_2O emission through nitrification was negligible. In order to check this assumption, ^{15}N tracing technique was used for clods Δ submitted to anaerobic pre-incubation (Khalil *et al.*, 2003b). Clods that experimented such conditions anaerobic pre-incubation were simultaneously submitted C_2H_2 in order to check whether denitrification stopped or not at the end of this pre-incubation period. Previous studies have shown that both NH_4^+ oxidation and N_2O production through nitrification are inhibited by C_2H_2 if its partial pressure is higher than 1-10 kPa (Mosier, 1980). Moreover, removing C_2H_2 is not sufficient to stop its inhibitory effect: Berg *et al.* (1982) noted that C_2H_2 effect had not ceased 7 days after removal of C_2H_2, even with a partial pressure of 10 Pa.

Many studies indicated that the $N_2O/(N_2O+N_2)$ ratio could vary with various factors (Granli and Bockman, 1994) such as organic matter, NO_3^- content (Blackmer and Bremner, 1979) and competition between NO_3^- and N_2O (Baumann *et al.*, 1996; Otte *et al.*, 1996; Khalil *et al.*, 2003a). Although the model supposed that $N_2O/(N_2O+N_2)$ ratio was constant, the presence of C_2H_2 during anaerobic pre-incubation does not only inhibit N_2O reduction, but also the synthesis of N_2O reductase (Klemedtsson *et al.*, 1977): this inhibitor can have

maintained the $N_2O/(N_2O+N_2)$ ratio at a high value, even have lead to increase this ratio if denitrifiers have grown significantly during the anaerobic pre-incubation.

In addition, particulate organic matter was neglected in our model, although Parkin (1987) found that a small piece of organic matter constituting 1% of the total core weight could be responsible for 85% of the denitrification rate. Recently, Parry *et al*. (2000) suggested that the relative contribution of POM-C to clod denitrification rates depends on their specific respiratory and denitrifying activities and on the structure of the clod, which could favor or inhibit denitrification activity at POM level, as a consequence of their aeration status. Although we found POM contents and respiratory and denitrifying activities comparable to those of Parry *et al*. (2000), we could not find any significant correlation between POM content and denitrification rates (results not shown). The absence of correlation could result from the small POM dimension in our clods, but this hypothesis remains to be proven.

CONCLUSION

Khalil *et al.* (2003b) have shown that a generalized anaerobic pre-incubation period markedly stimulates the microbial respiration and the net N_2O emission of soil clods during a subsequent aerobic incubation, and that N_2O emission is due to denitrification. The objective of the present work was to check whether the increase in aerobic respiration and maximum denitrification activities could explain the stimulation of N_2O emissions, accounting for the actual structure of soil clods. This objective was fulfilled using a model that takes into account clod structure and calculates the anaerobic volume of the clod and its denitrification behavior. The model was able to simulate experimental data, although some bias remained. It showed the importance of taking into accounting clod structure. Changing model hypotheses, for example the temporal variation of $N_2O/(N_2O+N_2)$ ratio of denitrification products, could modify the model outputs. The results of this work should to explain the variability of N_2O emissions *in situ*. Nevertheless, some characteristic features retained in our experimental procedure have surely distinguished clod behavior from their behavior *in situ*. Firstly, the presence of C_2H_2 during the anaerobic pre-incubation of this study inhibited the synthesis and activation of N_2O reductase during this period, whereas an actual phase of anaerobiosis in situ would probably favor N_2O reductase and therefore decrease the $N_2O/(N_2O+N_2)$ ratio of denitrification products. Secondly, the extent of an anaerobic event may vary with time and space, and therefore affect the microbial behavior after such periods. After an anaerobic period, microbial aerobic respiration decreases in a few days from a high value to a value nearly equal to the respiration before anaerobic pre-incubation. The results obtained in this study have to be extended to various dates after generalized anaerobic periods. In order to predict these parameter variations, it is required to know the soil history and particularly the alternations of anaerobic and aerobic periods.

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CHAPITRE 5

INFLUENCE DE LA PRESSION EN O_2 SUR LA PRODUCTION DE N_2O PAR NITRIFICATION ET DENITRIFICATION PAR DES AGREGATS DE SOL

Ce chapitre 5 correspond à un article « en cours de soumission » pour la revue *Soil Biology and Biochemistry* : K. Khalil, B. Mary, P. Renault (2003) Nitrous oxide production by nitrification and denitrification in soil aggregates as affected by O_2 concentration

SUMMARY

Nitrous oxide emitted by soils can be produced either by denitrification in anoxic conditions or by nitrification in presence of oxygen. The relative importance of the two processes, particularly under varied partial pressures of O_2, is not always known. This paper focuses on the influence of O_2 availability on N_2O production by nitrification and denitrification in an arable Orthic Luvisol.

Soil aggregates (2-3 mm size), water unsaturated, received 116 mg N kg^{-1} as ammonium sulphate labelled with ^{15}N and were incubated during 2 weeks at different O_2 partial pressures: 0, 0.3, 0.7, 1.4, 4 and 20.5 kPa. A ^{15}N tracing technique was used to quantify nitrification and denitrification rates. $^{15}N_2O$ and $^{15}N_2$ were measured.

O_2 availability appeared to strongly influence both nitrification and denitrification rates and also the release of N_2O. Nitrification rates were reduced by a factor of 6-9 when O_2 decreased from 20.5 to 0.3 kPa. They were highly correlated with O_2 consumption rates. Denitrification mainly occurred in complete anoxic conditions. The proportion of N_2O emitted by denitrification was estimated by two independent methods: one based on ^{15}N tracing using isotope composition of NH_4, NO_3 and N_2O, the other based on the measurement of the $^{15}N_2O$:$^{15}N_2$ ratio. The two methods gave similar results. The highest N_2O emissions were obtained under complete anoxic conditions and were due to denitrification. However emissions almost as important were obtained at day 14 with 1.4 kPa O_2 pressure, and they were due to nitrification. Nitrification was the main source of N_2O at O_2 concentrations greater than 0.3 kPa. The N_2O emissions due to nitrification were linearly related with the amounts of N nitrified, but the slope of the regression was highly dependent on O_2 concentration: it varied from 0.16% to 1.48% when O_2 concentration was reduced from 20.5 to 0.7 kPa. Emissions of N_2O by nitrification may then be quite significant if nitrification occurs at a reduced O_2 concentration.

Key-words: denitrification, nitrification, nitrous oxide, oxygen, ^{15}N.

INTRODUCTION

Nitrous oxide is involved in the global greenhouse effect (Smith, 1990; IPCC, 1996). Its emission from soils results mainly from biological denitrification and nitrification (Groffman, 1991; Hénault and Germon, 1995; Conrad, 1996). A better knowledge of the contribution of each process could help to predict and mitigate N_2O emissions by cultivated soils.

Nitrification and denitrification in soil often occur in close vicinity so that a substantial part of the NO_3^- formed by nitrification diffuses to the anaerobic denitrification zone where it is reduced to N_2 (Nielsen *et al*., 1996). The simultaneous occurrence of nitrification and denitrification in soil associated with N fertilisation has been suggested recently (Zanner and Bloom, 1995; Nielsen *et al*., 1996; Abbasi *et al*., 1997). Bremner and Blackmer (1981) observed nitrous oxide emissions from "well-aerated soils" which were not correlated with NO_3^- but were significantly correlated with NH_4^+ concentrations. Parton *et al*. (1996) found that N_2O fluxes through nitrification could be proportional to soil N turnover and that only high levels of soil NH_4 (>3 mg N kg^{-1}soil) affected N_2O emissions.

The sources of N_2O can be identified by using selective inhibitors, sterilisation or by adding substrates. The disadvantage of nitrification inhibitors is that prevention of NO_3^- production may affect the rate of denitrification. Sterilisation can be used to separate abiotic from biotic sources. Adding NH_4^+ or NO_3^- as substrates cannot provide definitive identification of the sources of N_2O unless the substrates are ^{15}N labelled. The relative importance of nitrification and denitrification can be assessed by measuring and comparing the isotope enrichments of the N_2O, NH_4^+ and NO_3^- pools (Stevens *et al*., 1997).

The availability of O_2 in soil is one of the main factors regulating nitrification, denitrification and the release of N_2O. Oxygen pressure is the main factor controlling denitrification through the activity and synthesis of denitrifying enzymes in soil (Tiedje, 1988). Denitrification has been considered as a strictly anaerobic process, but it is now well established that it can also occur in apparently aerobic environments. Many soil denitrifying micro-organisms have been found to be able to produce N_2O over a wide range of oxygen pressures.

Conversely, nitrification is a strictly aerobic process since the NH_4^+ oxidation enzyme of nitrifying organisms requires O_2 for activation (Wood, 1987). The effect of O_2 on nitrification and associated N_2O emissions has been studied more in continuous cultures than in soils.

Bollmann and Conrad (1998) showed that N_2O emitted by nitrification in soils was important at partial pressures higher than 0.1-0.5 kPa O_2. Goreau *et al.* (1980), using sediment slurries, demonstrated that production of N_2O by nitrification reached its maximum at 0.2 kPa O_2 pressure.

The mechanism of N_2O production by nitrification is not completely elucidated (Blackmer *et al.*, 1980; Stevens and Laughlin, 1998). Three main hypotheses have been proposed:

1) a constant proportion of NH_4^+ can be converted to N_2O during nitrification, resulting from various reactions of intermediates. Conrad (1990) proposed that N_2O production could be the consequence of intermediate (HNO) formation during oxidation of NH_2OH to NO_2^-. HNO oxidation could also lead to the formation of another unknown compound which would be then oxidised to NO_2^-. This hypothesis was retained in several models (Linn and Doran, 1984; Davidson, 1993; Parton *et al*, 1996).

2) the use of NO_2^- as an alternative electron acceptor during NH_4^+ oxidation for growth of nitrifiers when O_2 pressure is not high enough to supply these microbes all the required O_2 (Ritchie and Nicholas, 1972; Goreau *et al*., 1980). The effect of O_2 pressure has been shown experimentally either directly by varying O_2 partial pressure (Bollmann and Conrad, 1998) or indirectly by varying soil moisture (Zanner and Bloom, 1995) and is accounted for in some models (Grant, 1995);

3) the partial oxidation of NH_4^+ into NO_2^- in aerobic conditions, followed by NO_2^- diffusion to anaerobic regions and its subsequent reduction into N_2O by denitrification. Poth and Focht (1985) concluded that nitrite reduction to nitrous oxide by *N. europaea* is in accordance with the definition of denitrification: nitrous oxide production by nitrifiers is not correlated with nitrate additions but is correlated with ammonium additions, which can be explained by the requirement for ammonium as an electron source for nitrifier denitrification and by the lack of nitrate reductase activity in *N. europaea*.

The aim of this work was to quantify nitrification and denitrification and their specific contribution to N_2O emissions in response to the O_2 concentration above the soil. The study was then conducted on small soil aggregates, water unsaturated, using ^{15}N isotope technique to quantify nitrification and denitrification rates and N_2 production.

MATERIALS AND METHODS

Soil sampling and conservation until measurements

Experiments were performed on an Orthic Luvisol (FAO classification) sampled at Mons-en-Chaussée in Northern France (49°80' N; 3°60' E). The soil was cultivated with maize in 2000. Its properties were as follows: clay, 194 g kg^{-1}; silt, 706 g kg^{-1}; sand, 68 g kg^{-1}; pH_{water}, 8.20; total $CaCO_3$, 32 g kg^{-1}; organic C, 8.52 g kg^{-1}; total N 1.00 g kg^{-1}. Soil clods were sampled in the ploughed layer (10-30 cm depth) after digging a trench, on September 2000. Two sets of clods were separated: clods Δ, with a massive structure and no visible porosity (resulting from compaction due to traffic) and clods Γ, with a fragmentary structure and visible porosity (Richard *et al*., 1999). The clods were gently broken down immediately after sampling and then calibrated at field moisture condition (0.184 g water g^{-1} dry soil): we kept clods between 2.5 and 3 cm size. In order to reduce microbial activity during storage, the clods were air-dried during 3 days to obtain a residual moisture close to 0.10 g g^{-1} soil (corresponding to a water suction of –2 MPa). At the beginning of the present experiment (June 2002), the clods were sieved to obtain aggregates between 2 and 3.15 mm. The soil then still contained 0.07 g water g^{-1} soil; its NO_3^- content was 10.7 mgN kg^{-1} and its NH_4^+ content was 0.1 mgN kg^{-1}.The aggregates were rewetted by spraying deionised water to obtain a residual moisture close to 0.18 g g^{-1}, and were pre-incubated at 20°C in airtight jars during 7 days.

Experiment 1

The objective was to assess the influence of NH_4^+ concentration on N_2O production. Twenty five g fw (fresh weight) of soil aggregates were put into 125 mL flasks, and various amounts of NH_4^+ were added as $(NH_4)_2SO_4$ solution to obtain 0, 80, 116 or 170 mg NH_4^+-N kg^{-1} soil. The addition of this solution rose the soil water content at 0.19 g g^{-1}. Eighteen flasks were closed and incubated at 20°C for 14 days: 6 flasks were used for gas measurements (including 3 flasks without soil used as blanks), 3 flasks were used for final soil pH measurement and 12 flasks for mineral nitrogen measurements. N_2O and mineral N were

analysed at day 2, 4, 7, 10 and 14 (3 replicates at each date). Initial soil pH and mineral N were also measured at day 0 on additional samples. At each date, the atmosphere of flasks was renewed by opening the flasks for a few minutes. The same procedure and measurements were applied for each NH_4^+ concentration.

Experiment 2

Its objective was to assess the influence of O_2 concentration on N_2O production, at a given soil NH_4^+ concentration (chosen as 116 mg N kg^{-1} soil according to results of experiment 1). Twenty five g f.w. (fresh weight) of soil aggregates were put in 125 mL flasks. A ^{15}N labelled $(NH_4)_2SO_4$ solution (50% atom enrichment) was added to each soil sample to obtain the 116 mg NH_4^+-N kg^{-1} concentration and a residual moisture of 0.21 g water g^{-1} soil. The flasks were closed and placed under 6 different O_2 concentrations (nominally 0, 0.5, 1, 2, 4.8 and 21% O_2 mixed with N_2). Each flask received 3 successive cycles of 3 minutes vacuum and 3 minutes filling with a given mixture of O_2/N_2 at atmospheric pressure. 18 flasks were incubated at 20°C for 14 days. The procedure was the same than in experiment 1, except that at each measurement date the atmosphere of flask was renewed with the corresponding O_2/N_2 gas mixture by the same procedure (3 minutes vacuum and 3 minutes gas filling, repeated three times). The same measurements were performed as in experiment 1. Additional measurements consisted in O_2 concentration, $^{15}N_2$, $^{15}N_2O$ in atmosphere and ^{15}N mineral in soil. Oxygen consumption occurred during each measurement interval, so that the mean O_2 pressure in each treatment was in fact: 0, 0.3, 0.7, 1.4, 4.0 and 20.5 kPa. These values will be used as effective O_2 concentrations in the following.

Gas measurements

For both experiments, gas samples were sampled at each date from the flasks: 1 mL of gas was sampled with a syringe for O_2 and injected into an automatic CN analyzer (Carlo Erba, ANA 1500, Milan, Italy) that was adapted to measure O_2 concentrations after replacing the previous Porapak QS column by a molecular sieve column (60-80 mesh, 1.8 m, 50°C), which separates O_2 and N_2 gas. The CN analyzer was coupled to an integrator (Shimadzu, C-R6A, Chromatopac). 0.2 mL of gas was sampled with a syringe for $^{15}N_2$ analysis, using a modified

CN analyzer. Prior to the separation of N_2 and O_2, the gas sample successively passed through a water absorbent, a CO_2 trap and a N_2O cryogenic trap and finally an oven filled with reduced copper before entering an isotope ratio mass spectrometer (Fisons, Isochrom-EA, Manchester, England).

Two hundred fifty mL gas was sampled with dual-ended flasks previously vacuumed, for N_2O and $^{15}N_2O$ analysis. The analysis was made with the mass spectrometer after pre-concentration using the 'trace gas' system (Micromass, Trace gas, Manchester, England).

Mineral N measurements

The mineral N content of clods was extracted with a KCl M solution (soil:solution ratio = 1:5). Measurements were performed with a TRAACS 2000 analyzer (Bran & Luebbe, Germany) using the methods proposed by Kamphake *et al*. (1970) for NO_3^- and NO_2^- analysis and Krom (1980) for NH_4^+ analysis. The ^{15}N-NH_4^+ and ^{15}N-(NO_3^-+NO_2^-) were separated successively by micro-diffusion and collected on a glass fibre disc (6 mm diameter) impregnated with 10 µL of 1M H_2SO_4 solution (Brooks *et al*., 1989). The discs are then placed in tin capsules and analysed in the elementary analyzer - mass spectrometer equipment.

pH measurement

For pH measurement, we added a mass of ultra pure water equal to the double of soil mass in each flask. The flask was closed and shaken for 10 minutes. The mixture was poured in a beaker and left for 2 minutes. The pH was then recorded each minute during 5 minutes using calomel/glass and pH-meter. Measurements were done at the beginning of incubation on 10 replicates and at the end of incubation in each treatment on 3 replicates.

Nitrogen rates calculations

N rates were calculated using measurements of mineral -N and –^{15}N and FLUAZ model (Mary *et al*., 1998). This model combines a numerical method for solving the differential system given by the N and ^{15}N mass equations and a non linear fitting program for optimising

the N rates parameters by minimizing the difference between observed and simulated N and ^{15}N data (amounts and isotopic excess of NH_4^+ and NO_3^-). Nitrification rate could be estimated from NO_3^- experimental accumulation, but this estimate would not be as accurate as using the model. The model was used in experiment 2 to calculate mineralisation (m), nitrification (n) and denitrification (d) for the different O_2 concentrations. We assumed that immobilisation was negligible, since no readily decomposable organic nitrogen was added. NH_3 volatilisation was also neglected, on the basis of previous measurements (results not shown). These assumptions were confirmed by the measured ^{15}N balance ($^{15}NH_4^+$ + $^{15}NO_2^-$ + $^{15}NO_3^-$ + $^{15}N_2O$ + $^{15}N_2$) that did not differ significantly from 100% in any of the treatments. We also assumed that mineralisation rate was the same in all treatments at a given time interval. This assumption allowed to calculate more precisely nitrification and denitrification rates. Nitrification rates were supposed to follow first order kinetics, whereas mineralisation and denitrification zero order kinetics during each time interval . The two stages of nitrification are the following:

$$NH_4^+ + \frac{3}{2}O_2 \rightarrow NO_2^- + 2H^+ + H_2O \quad (1)$$

$$NO_2^- + \frac{1}{2}O_2 \rightarrow NO_3^- \quad (2)$$

FLUAZ model does not directly accounts for NO_2^- compartment. It considers the sum (NO_2^- +NO_3^-) and therefore calculates the ammonium oxidation rate (rate n_i), i.e. oxidation into NO_2^-. The oxidation rate into NO_3^- (rate n_a) was calculated afterwards using measurements of NO_2^- pool (*Ni*), as follows:

$$n_a = n_i - \frac{\Delta N_i}{\Delta t} \quad (3)$$

Furthermore, we have compared O_2 consumption rate with nitrification rate. Regarding the stochiometry of the previous equations relative to O_2 consumption, we calculated the following nitrification rate:

$$n = \frac{3n_i + n_a}{4} \quad (4)$$

RESULTS

Effect of NH_4^+ concentration

The evolution of soil mineral N pools during the 14 days incubation at atmospheric O_2 concentration (experiment 1) is given in Figure 1. Ammonium and NO_3^- concentrations remained almost stable in the control (i.e. without addition of NH_4^+), whereas they decreased and increased, respectively, for the 3 NH_4^+ treatments (addition of 80, 116 and 170 mg N kg^{-1}, Fig. 1a,b). Nitrite concentration increased then decreased with time, except for the control. The higher the initial concentration of NH_4^+, the higher was the peak of NO_2^-. The maximal value was 22.7 mg N kg^{-1} for the addition of 170 mg N kg^{-1} (Fig. 1c).

The nitrification kinetics were calculated using changes in mineral N contents (Table 1). They appear to depend on the initial NH_4^+ concentrations. Without NH_4^+ addition, the nitrification was low, in average 0.27 mg N kg^{-1} day^{-1} (and equal to net mineralisation). The highest NH_4^+ and NO_2^-oxidation rates were observed with the highest NH_4^+ concentration (A170): 19.1 and 23.7 mg N kg^{-1} day^{-1}, respectively. The initial rates of NH_4^+ oxidation were greater than the initial rates of NO_2^-oxidation. Then the NO_2^-oxidation rates (n_a) increased more with time than the NH_4^+ oxidation rates (n_i).

Effect of O_2 concentration

Mineral N and ^{15}N

The evolution of soil mineral N pools during the 14 days of incubation at various O_2 concentrations (experiment 2) is given in Figure 2, for a fixed initial NH_4^+ concentration (116 mg N kg^{-1}). Ammonium concentration decreased and NO_3^- concentration increased in all treatments, except at 0 % O_2 concentration: in this case, NH_4^+ concentration remained more or less constant and NO_3^- concentration fell from 10.7 to 0.1 mg N kg^{-1} within 7 days. NO_2^- concentration increased then decreased with time, except for the lowest O_2 concentrations (0 and 0.3 kPa); in these treatments, nitrite concentration remained negligible during the whole incubation period. The mineral N content responded markedly to O_2 concentration. The higher the O_2 concentration, the faster was the disappearance of NH_4^+ and the increase of

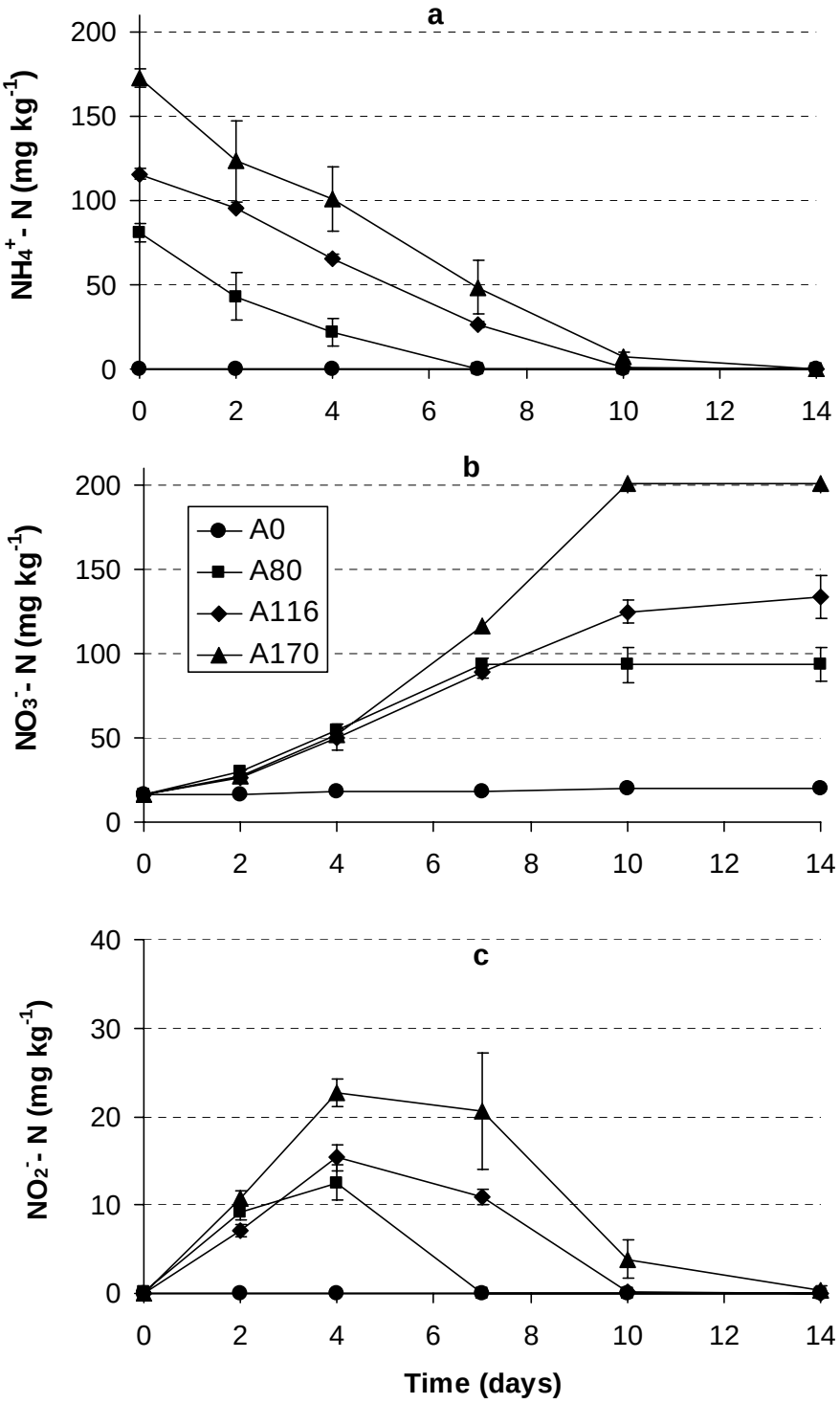

Figure 1. *Variation of mineral N (mg N kg^{-1}) with time in soil with various amounts of NH_4^+ added at time 0. A0: control (no addition), A80: 80 mg N kg^{-1}, A116: 116 mg N kg^{-1}; A170: 170 mg N kg^{-1}.* ***a)*** *NH_4^+ concentration;* ***b)*** *NO_3^- concentration;* ***c)*** *NO_2^- concentration. Vertical bars are the standard deviations of means*

Period (days)	A0		A80		A116		A170	
	n_i	n_a	n_i	n_a	n_i	n_a	n_i	n_a
0-2	0.0	0.0	15.1	10.6	9.4	5.9	17.8	12.4
2-4	0.6	0.6	12.2	10.5	15.3	11.2	14.8	8.8
4-7	0.2	0.2	8.0	12.2	12.3	13.7	19.1	19.8
7-10	0.5	0.5	0.0	0.0	8.5	12.1	18.1	23.7
10-14	0.1	0.1	0.1	0.1	1.2	1.2	0.5	1.4

Table 1. *NH_4^+ oxidation rates (n_i, mg N kg^{-1} d^{-1}) and NO_2^- oxidation rates (n_a, mg N kg^{-1} d^{-1}) in soil incubated at atmospheric O_2 concentration, for different time intervals and initial ammonium concentrations (0, 80, 116 and 170 mg N kg^{-1}).*

n_a was calculated on the basis of NO_3^- accumulation and n_i on the basis of NH_4^+ disappearance and NO_2^- variation.

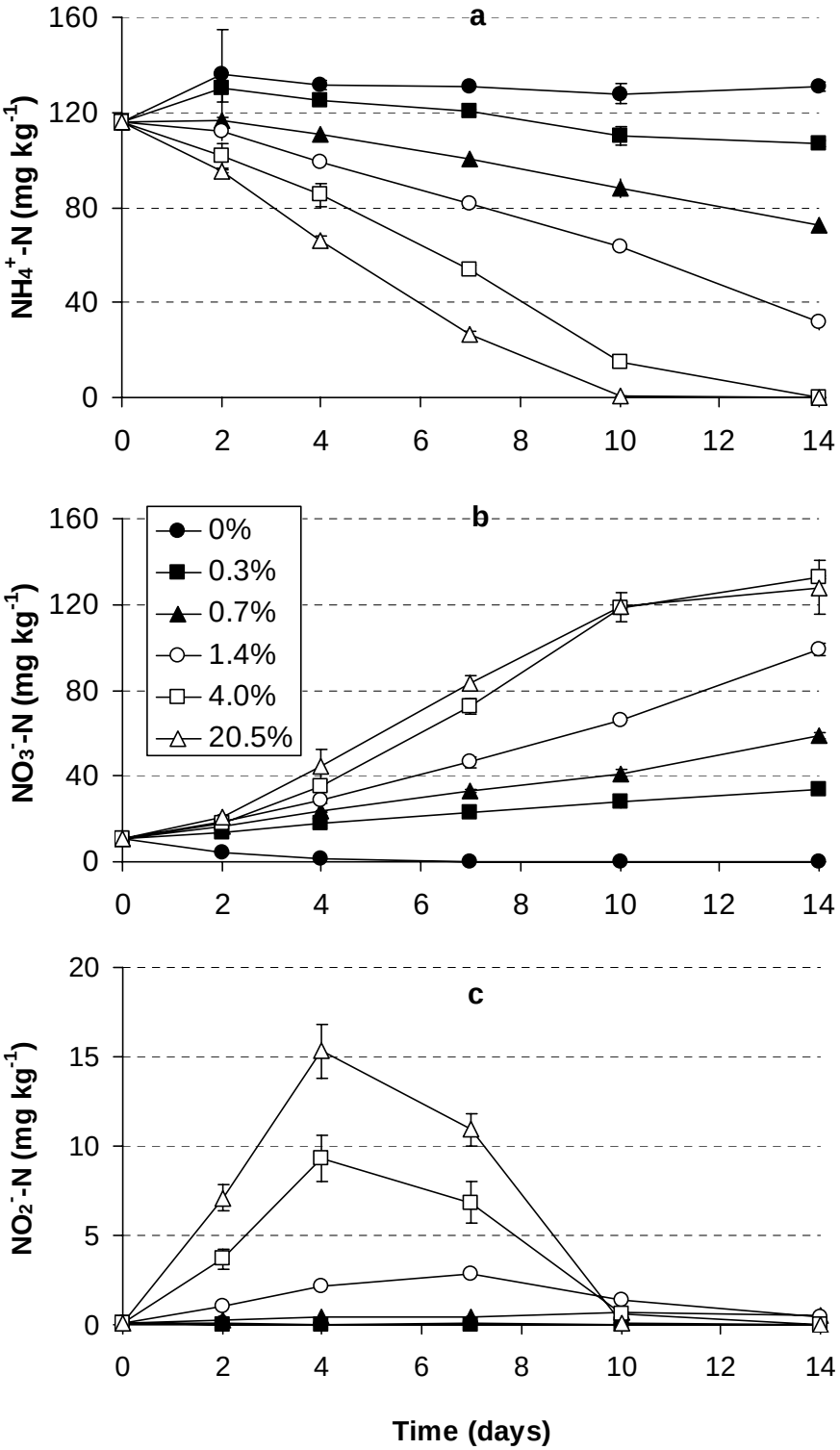

Figure 2. *Variation of mineral N (mg N kg^{-1}) with time in soil incubated at different O_2 concentrations: 0, 0.3, 0.7, 1.4, 4.0 and 20.5%:* ***a)*** *NH_4^+ concentration;* ***b)*** *NO_3^- concentration;* ***c)*** *NO_2^- concentration. Vertical bars are the standard deviations of means*

Period (days)	0%			0.3%			0.7%			1.4%			4.0%			20.5%		
	NH_4^+	NO_3^-	N_2O	NH_4^+	NO_3^-	N_2O	NH_4^+	NO_3^-	N_2O	NH_4^+	NO_3^-	N_2O	NH_4^+	NO_3^-	N_2O	NH_4^+	NO_3^-	N_2O
0 - 2	**41.24** *(1.5)*	**0.00** *(.01)*	**0.31** *(.05)*	**40.69** *(2.07)*	**4.85** *(.5)*	**4.07** *(3.43)*	**41.25** *(1.26)*	**7.08** *(3.24)*	**17.58** *(2.02)*	**40.65** *(1.97)*	**8.01** *(.78)*	**20.70** *(2.12)*	**39.73** *(2.72)*	**9.97** *(.4)*	**11.64** *(2.92)*	**41.22** *(1.29)*	**9.94** *(.01)*	**4.07** *(3.49)*
2 - 4	**41.43** *(.69)*	**0.00** *(.01)*	**0.87** *(.42)*	**39.82** *(2.86)*	**12.66** *(.44)*	**10.14** *(3.8)*	**41.66** *(.57)*	**17.21** *(1.76)*	**27.64** *(.4)*	**38.65** *(2.07)*	**20.83** *(.66)*	**37.30** *(6.83)*	**38.43** *(1.8)*	**24.34** *(1.43)*	**30.98** *(2.02)*	**40.75** *(.84)*	**28.91** *(4.29)*	**24.56** *(4.84)*
4 - 7	**38.45** *(1.02)*	**0.01** *(.01)*	**0.01** *(.01)*	**38.81** *(3.27)*	**18.04** *(.3)*	**3.70** *(2.1)*	**40.28** *(1.6)*	**23.55** *(.25)*	**24.80** *(.55)*	**38.12** *(2.29)*	**28.35** *(1.37)*	**26.08** *(2.41)*	**38.60** *(1.22)*	**32.34** *(1.53)*	**32.92** *(4.56)*	**39.66** *(1.03)*	**37.62** *(4.46)*	**31.91** *(3.13)*
7 - 10	**38.51** *(.72)*	**0.01** *(.01)*	**0.18** *(.04)*	**38.76** *(2.27)*	**23.20** *(.6)*	**2.00** *(1.45)*	**40.15** *(1.26)*	**28.49** *(.15)*	**31.56** *(1.36)*	**40.19** *(1.09)*	**32.95** *(1.17)*	**29.53** *(5.14)*	**38.25** *(2.82)*	**36.84** *(.47)*	**19.55** *(6.52)*	**26.75** *(.26)*	**37.61** *(.31)*	**8.39** *(5.67)*
10 - 14	**39.65** *(.61)*	**0.01** *(.01)*	**0.03** *(.04)*	**40.09** *(1.37)*	**27.05** *(.69)*	**26.18** *(4.75)*	**40.80** *(.38)*	**33.69** *(.11)*	**33.25** *(1.05)*	**39.94** *(.26)*	**35.76** *(.15)*	**35.87** *(1.06)*	**18.63** *(1.85)*	**37.82** *(.24)*	**11.77** *(2.5)*	**7.06** *(.01)*	**38.03** *(.24)*	**2.57** *(.71)*

Table 2. *Atom% excess of NH_4^+, NO_3^- and N_2O (mean and standard deviations values) measured in soil or gas samples at different incubation dates and different O_2 concentrations (0, 0.3; 0.7, 1.4, 4.0 and 20.5%)*

NO_3^-, and the greater was the NO_2^- accumulation. The maximal NO_2^- concentration at atmospheric pressure was 15.3 mg N kg^{-1} (Fig. 2c).

The nitrification and denitrification rates, calculated using measurements of mineral N and ^{15}N (Table 2) and FLUAZ model, are given at Table 3. Significant denitrification rates (i.e. greater than 0.2 mg N kg^{-1} d^{-1} corresponding to the accuracy of FLUAZ calculations) were only observed in the 0 and 0.3 kPa O_2 pressure treatments. Nitrification rates (both NH_4^+ and NO_2^-oxidation rates) were markedly reduced when O_2 concentration decreased: they decreased by a factor of 4-10 when O_2 pressure fell from 20.5 to 0.3 kPa. Nitrification rates increased versus time in all treatments, except for the 20.5 kPa pressure in which it reached a maximum at day 4 and then decreased although the soil still contained exchangeable NH_4^+. For the highest values of O_2 concentration, i.e. 4.0 and 20.5 kPa, the first step of nitrification (NO_2^-oxidation) was slightly slower at the beginning but increased faster than the first step (NH_4^+ oxidation), as shown previously (experiment 1). For the lowest O_2 pressures, 0.3 and 0.7 kPa, the two steps proceeded at the same rat, so that there was no NO_2^- accumulation. The reduction in O_2 concentration then affected primarily the NH_4^+ oxidation step.

N_2O and $^{15}N_2$ gas

The cumulative N_2O emissions by the soil submitted to different O_2 concentrations are presented in Figure 3a. Nitrous oxide emissions increased with time during incubation and were highly dependent on O_2 concentration in the flask atmosphere. The anaerobic situation (0% O_2) resulted in the highest production of N_2O. The emission took place rapidly, since the amount of N_2O produced at day 2 was 0.73 mg N kg^{-1}, corresponding to a rate of 30 nmol N_2O kg^{-1} s^{-1}. It levelled off after day 7, when NO_3^- concentration was very small. At day 14, the amount of N_2O evolved was 1.16 mg N kg^{-1}. The emission rate also decreased with time with the 0.3 kPa O_2 pressure but the intensity was much smaller than in the anaerobic treatment. The kinetics of emission were very different in the treatments with 0.7 and 1.4 kPa O_2 pressure: the emission rate increased with time. The amount of N_2O produced at day 14 at 1.4 kPa O_2 pressure was almost equivalent at the amount produced in anaerobic conditions: 1.09 mg N kg^{-1}. The productions of N_2O in treatments with 4.0 and 20.5 kPa O_2 were intermediate. It levelled off towards the end of the incubation, simultaneously to the nitrification process.

The corresponding measurements of the ratio $^{15}N_2O$:$^{15}N_2$ are given in Figure 3b. Knowing that N_2 emitted by the soil is exclusively derived from N_2O, we can assume that both gas have

Period (days)	0%			0.3%			0.7%			1.4%			4.0%			20.5%		
	n_i	n_a	d	n_i	n_a	d	n_i	n_a	d	n_i	n_a	d	n_i	n_a	d	n_i	n_a	d
0 - 2	0.0	0.0	3.2	1.7	1.7	0.2	2.8	2.7	0.0	4.1	3.6	0.0	7.3	5.5	0.0	10.2	6.7	0.0
2 - 4	0.0	0.0	1.4	1.7	1.7	0.0	3.2	3.1	0.0	6.1	5.5	0.0	9.8	7.0	0.0	16.0	11.9	0.0
4 - 7	0.0	0.0	0.5	1.5	1.5	0.0	3.5	3.5	0.0	6.2	6.0	0.0	11.0	11.8	0.0	13.8	15.2	0.0
7 - 10	0.0	0.0	0.0	1.6	1.6	0.4	3.3	3.2	0.0	6.5	7.0	0.0	13.3	15.4	0.0	9.2	12.8	0.0
10 - 14	0.0	0.0	0.0	1.3	1.2	0.0	4.2	4.2	0.0	8.2	8.4	0.0	4.2	4.3	0.0	0.6	0.7	0.0

Table 3. *Nitrification rate (NH_4^+ oxidation rate n_i) and denitrification rate (d) (mg kg^{-1} d^{-1}), calculated using measured N and ^{15}N pools and FLUAZ model in soil samples incubated under various O_2 concentrations (0; 0.3; 0.7; 1.4; 4.0 and 20.5%) during 14 days.*

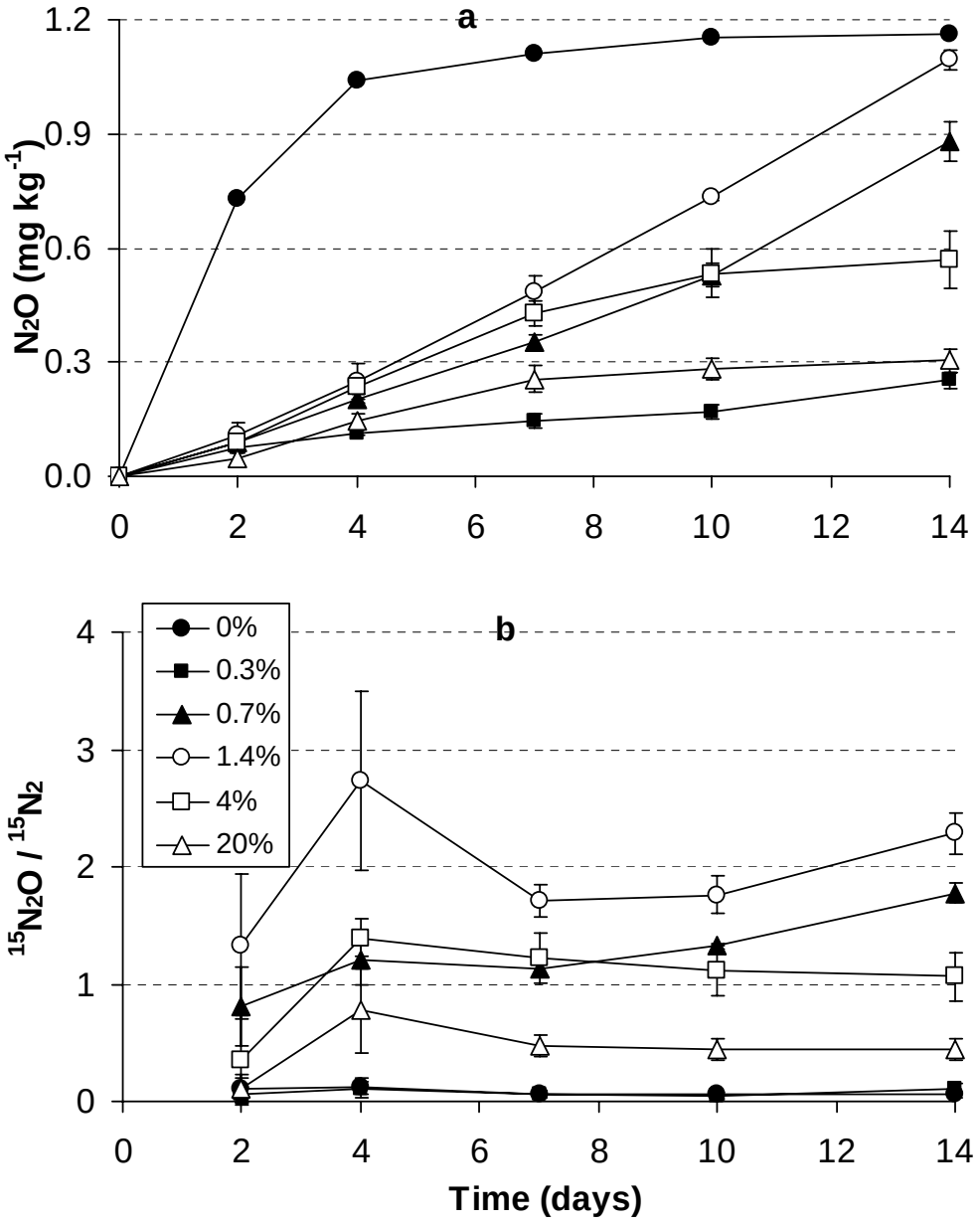

Figure 3. *Kinetics of N_2O and N_2 emissions (mg N kg^{-1}) from soil incubated at different O_2 concentrations: 0, 0.3, 0.7, 1.4, 4.0 and 20.5%:* ***a)*** *cumulative N_2O;* ***b*** *cumulative N_2 (calculated using $^{15}N_2$ measurements);* ***c)*** *cumulative N_2O : N_2 ratio (equal to the $^{15}N_2O$: $^{15}N_2$ ratio).*

the same isotopic composition. Therefore the ratio N_2O:N_2 is equal to the measured ratio $^{15}N_2O$:$^{15}N_2$. This ratio was small in the treatments with 0 and 0.3 kPa O_2, between 0.06 and 0.13. At day 14, the ratio was equal to 0.11 in the anaerobic treatment, so that the amount of N_2 evolved was estimated at 11.1 mg N kg^{-1}. The ratio was much higher in all the other treatments. The maximum N_2O:N_2 ratio was found for a 1.4 kPa O_2 pressure. These results suggest that N_2O production by denitrification (up to N_2 production) was the dominant process at 0 and 0.3 kPa O_2 pressure, whereas N_2O production mainly derived from nitrification in the other treatments (little N_2 was produced).

Proportion of N_2O derived from nitrification and denitrification

The use of ^{15}N tracing should provide another means of determining the origin of N_2O emissions. Table 2 shows the respective isotopic compositions of N_2O in atmosphere, NH_4^+ and NO_3^- in soil during the five time intervals studied. The atom% excess of mineral N forms given in this table are the average of the values measured at the beginning and the end of each time interval. The atom% excess of NH_4^+ decreased slowly with time except for the higher O_2 concentrations for which it fell at the end of the incubation due to the disappearance of NH_4^+ ions. Simultaneously, the atom% excess of NO_3^- increased due to nitrification of the labelled NH_4^+, except in the 0% treatment. In this case, the NO_3^- remained unlabelled during the whole incubation period, which confirms that there was no nitrification. The isotopic excess of N_2O was also close to 0 in this treatment, which indicates that the N_2O produced came from the unlabelled NO_3^- initially present in soil.

In the 0.3 kPa O_2 treatment, the isotopic composition of N_2O was much closer from that of NO_3^- than that of NH_4^+, which confirms that denitrification was the dominating process. However, it is noticeable in this treatment and in others (particularly at 4.0 and 20.5 kPa O_2) that the atom% excess of N_2O can be lower than the atom% excess of both NH_4^+ and NO_3^-. Such as result indicates that at least one the two pools is not uniformly labelled. This is attributed to an incomplete diffusion of the added $^{15}NH_4^+$ within soil aggregate before being nitrified due to its adsorption on solid phase. The non uniformity was much more important for NO_3^- than for NH_4^+, since the soil contained much more NO_3^- than NH_4^+ (10.7 versus 0.1 mg N kg^{-1}).

Two methods were used to calculate the proportion of N_2O from nitrification or denitrification. The first method relies on the measurements of $^{15}N_2$. It assumes that N_2 emitted by the soil has the same composition than N_2O, and that the ratio N_2O:N_2 due to

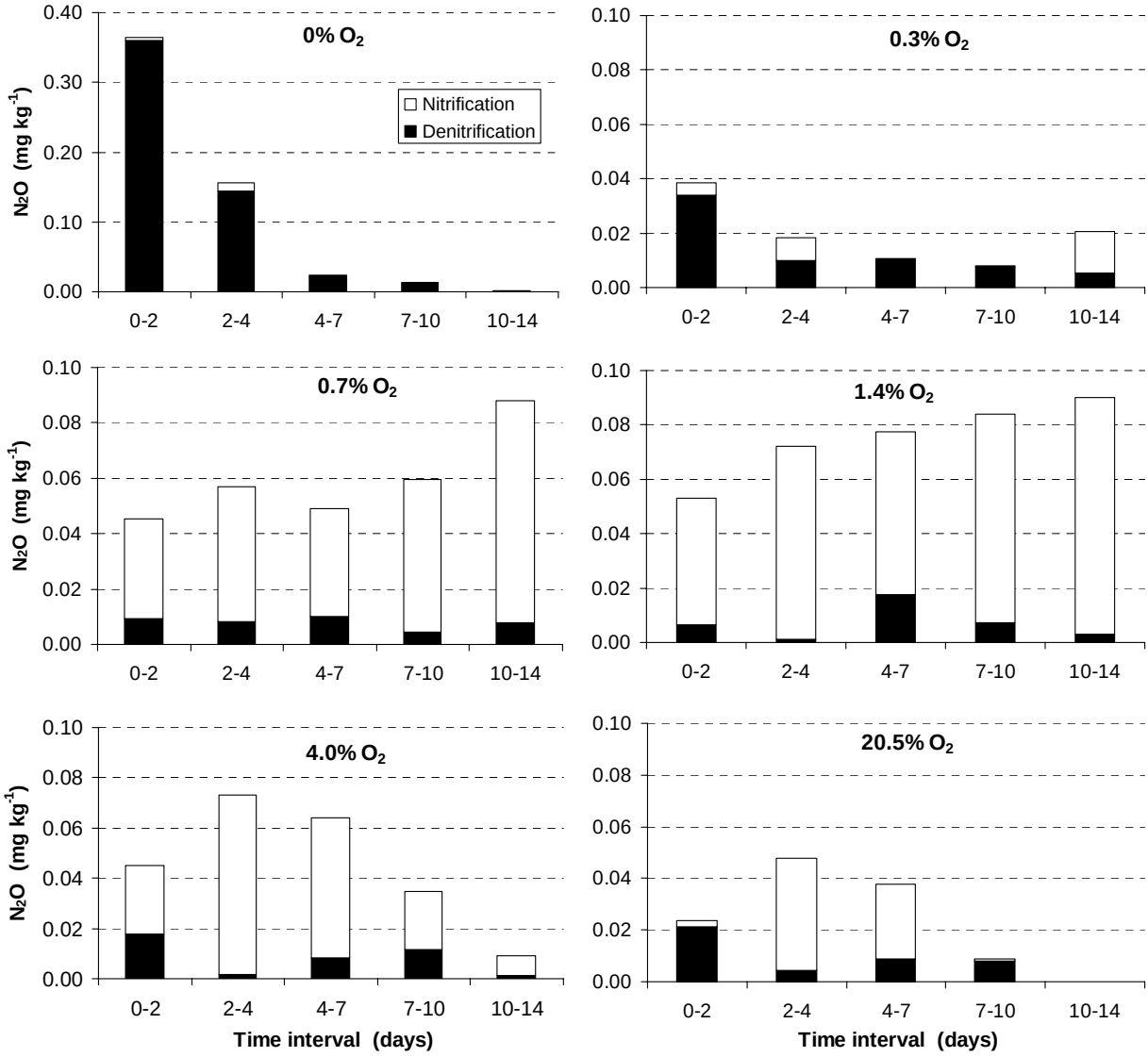

Figure 4. *N_2O emission rate (mg N kg^{-1} d^{-1}) coming from nitrification and denitrification at different time intervals in soil incubated at different O_2 concentrations: 0, 0.3, 0.7, 1.4, 4.0 and 20.5%.*

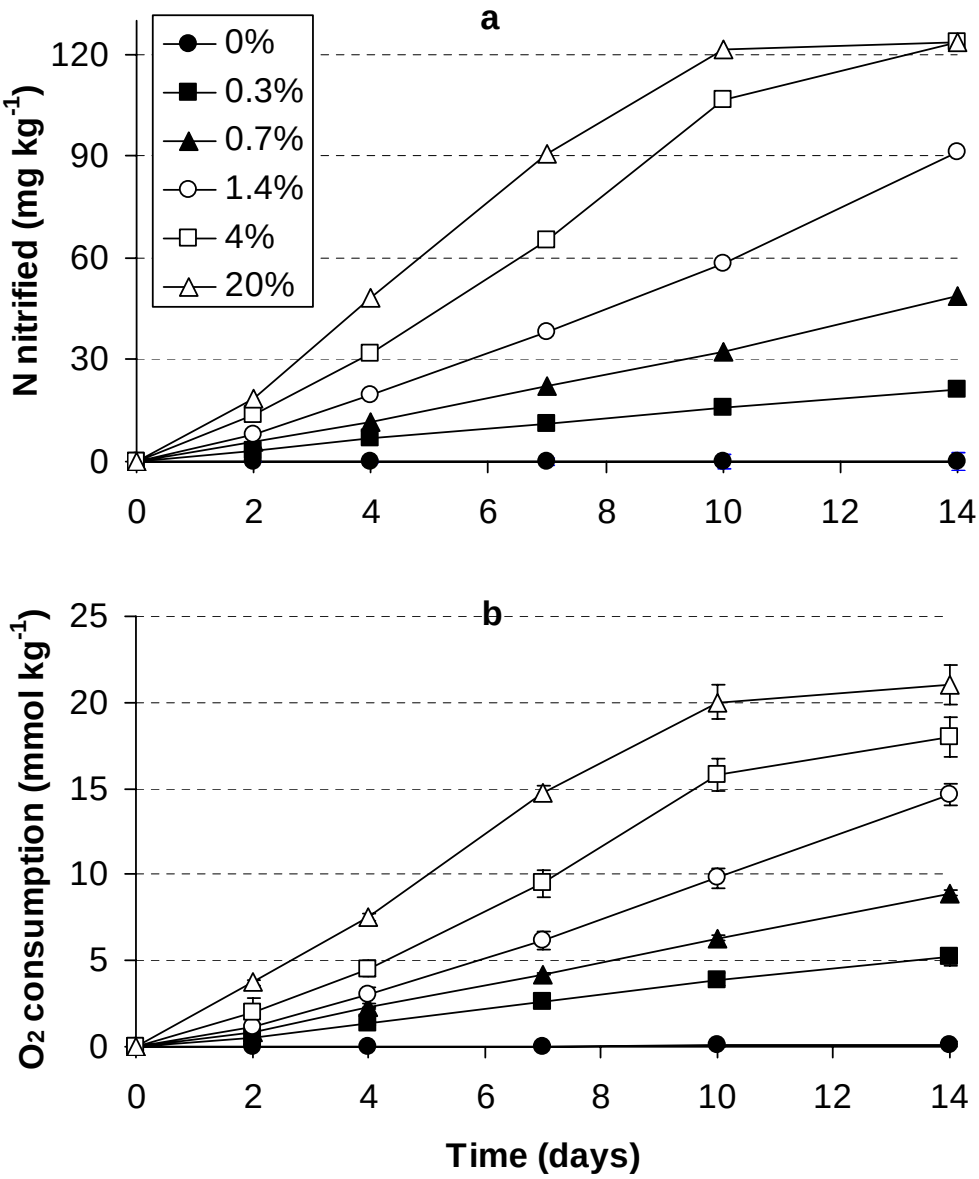

Figure 5. *Kinetics of nitrification and O_2 consumption in soil incubated at different O_2 concentrations: 0, 0.3, 0.7, 1.4, 4.0 and 20.5%.* ***a****) cumulative nitrification (mg N kg^{-1}) calculated with FLUAZ model;* ***b)*** *cumulative O_2 consumption (mmol O_2 kg^{-1}) measured. Nitrification rate is defined as $\frac{(3n_i + n_a)}{4}$, where n_i is the NH_4^+ oxidation rate and n_a is the NO_2^- oxidation rate (see text). Vertical bars are the standard deviations.*

denitrification is constant. We estimated its value in the treatment 0% O_2 at 0.12 (see Fig. 7a). The second method relies on measurements of isotopic excess of N_2O, NH_4^+ and NO_3^-. It assumes that N_2O is derived from 2 pools, one being labelled (with the composition of NH_4^+), the other pool being unlabelled. It calculates the N_2O isotopic abundance versus the abundance of each pool and their respective contribution to N_2O production. The comparison between this calculated value and the measured N_2O isotopic excess enables to estimate the proportion of N_2O emitted from the labelled pool, i.e. by nitrification. The detail of both methods is given in the appendix.

Although method 1 was based on more questionable assumptions than method 2, the two methods gave similar estimates of the proportion of N_2O emitted from nitrification, since the regression equation between the two estimates was y = 0.99x and the coefficient of determination was r^2 = 0.85 (n=30). We then took the average of the two estimates. Using these values, the amounts of N_2O emitted by nitrification and denitrification were calculated for each treatment (Fig.4). As expected, denitrification was the unique process responsible for N_2O production in anoxic conditions (0% O_2), it ceased towards the end of the incubation due to the absence of NO_3^-. At 0.3 kPa O_2, denitrification remained the major process producing N_2O, except at the end of experiment when emissions by nitrification increased. For samples with 0.7 and 1.4 kPa O_2, the proportion of N_2O by nitrification was more important than denitrification at all measurement intervals. Maximum N_2O production by nitrification was obtained for the 1.4 kPa O_2 pressure. The emissions by nitrification decreased when O_2 concentration increased from 4.0 to 20.5 kPa O_2, particularly after day 7, corresponding to the end of the nitrification process. However, emissions by denitrification took place during the first two days, even with the higher O_2 concentrations (4.0 and 20.5 kPa).

Relationship between O_2 consumption and nitrification rate

The cumulative nitrification and O_2 consumption rates are shown in Fig. 5. The oxygen consumption and nitrification rates were favoured by O_2 availability during the whole incubation period; the pattern of curves for both variables was very similar. Indeed we found a very good correlation between O_2 consumption rates and nitrification rates in the 5 treatments with oxygen (0.3, 0.7, 1.4, 4.0 and 20.5 kPa O_2): r^2 = 0.94, n = 25 (Fig. 6). The slope of the regression is 1.95 ± 0.11. It is not significantly different from 2, which is the theoretical value for O_2 consumption due to nitrification (see eq. 1-2), nitrification being calculated according to eq. (4). The O_2 consumption was the sum of the O_2 consumption by

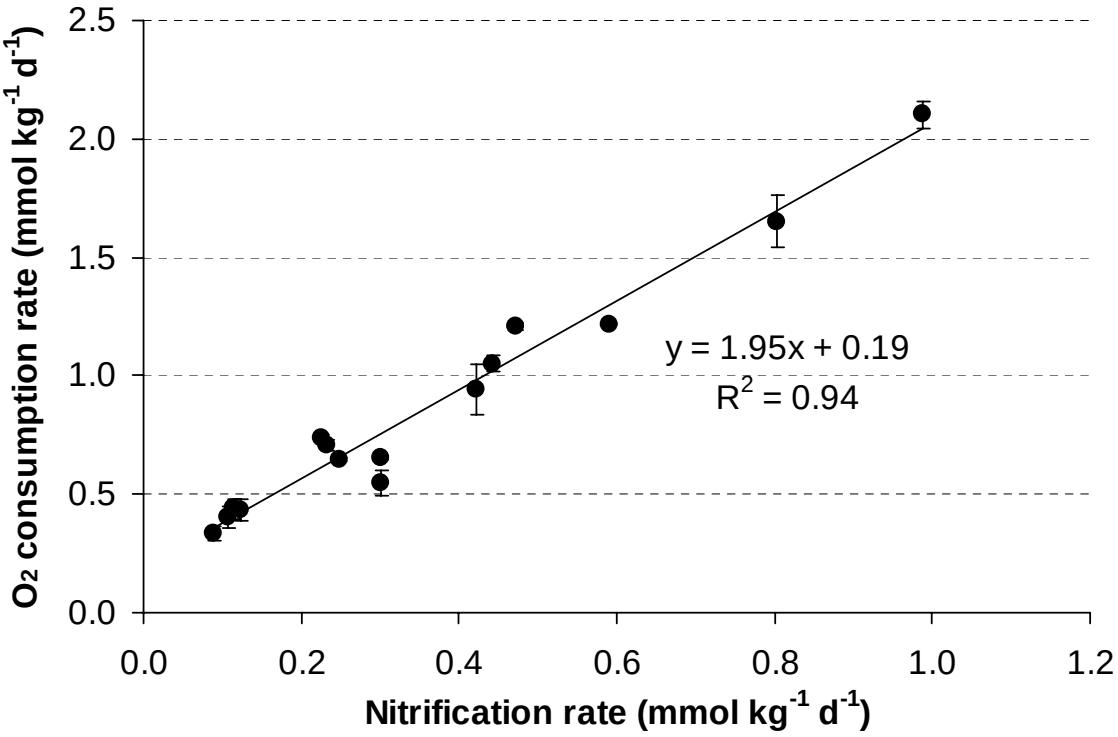

Figure 6. *Relationship between O_2 consumption rates (mmol kg^{-1} d^{-1}) and nitrification rates (mmol kg^{-1} d^{-1}) measured for the different time intervals and different O_2 treatments. Nitrification rate is defined as $\frac{(3n_i + n_a)}{4}$, where n_i is the NH_4^+ oxidation rate and n_a is the NO_2^- oxidation rate (see text).*

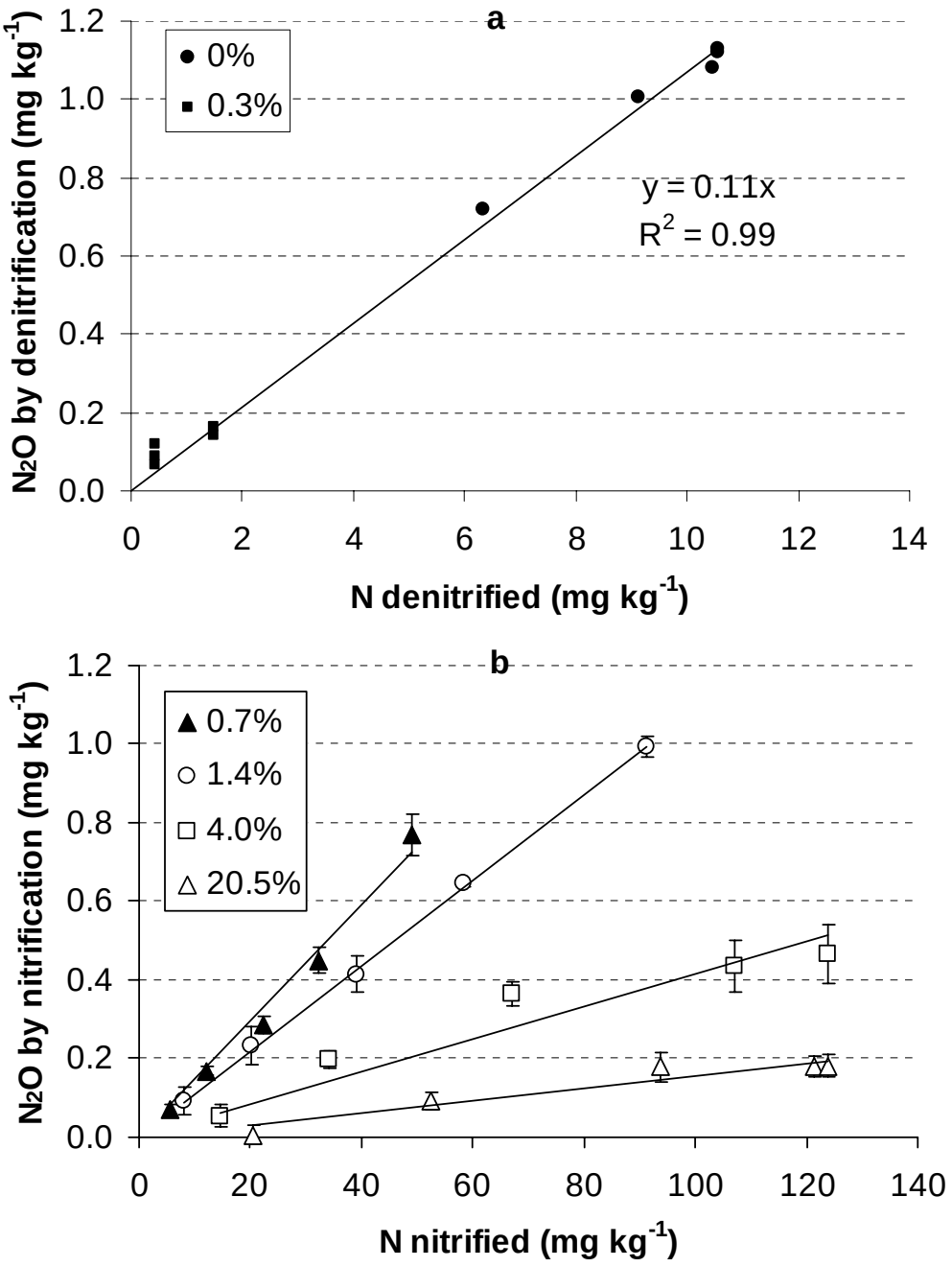

Figure 7. *Relationships between cumulative N_2O emissions (mg N kg^{-1}) and cumulative N denitrified or nitrified (mg N kg^{-1}) in soil incubated at different O_2 concentrations:* ***a)*** *N_2O emitted by denitrification versus N denitrified at O_2 concentrations equal to 0 and 0.3%;* ***b)*** *N_2O emitted by nitrification versus N nitrified at O_2 concentrations equal to 0.7, 1.4, 4.0 and 20.5%. Nitrification is defined as NO_2^- production, i.e. cumulative NH_4^+ oxidation rates. Regression equations are given at table 3.*

nitrification and respiration. The intercept of the regression line was 0.19 ± 0.06 mmol kg^{-1} d^{-1}. This value represents the O_2 consumption in absence of nitrification, due to respiration. We can assume that the respiration of treatments with nitrification is the same. The consumption of oxygen was then mainly due to nitrification, even in the 0.3 kPa O_2 treatment with a low nitrification rate.

Relationship between N_2O emissions and nitrification/denitrification rates

The amounts of N_2O produced were then compared to the amounts of N nitrified or denitrified, previously calculated with FLUAZ (Table 3). In the 0 and 0.3 kPa O_2 treatments, the amounts of N_2O emitted by denitrification were highly correlated with N denitrified (Fig. 7a). The coefficient of determination was equal to 0.99 and the slope equal to 0.11. This slope, which represents the ratio N_2O:(N_2O+N_2), was in good agreement both with the estimate previously made with $^{15}N_2$ measurements (N_2O:N_2 = 0.11) and with other estimates made with C_2H_2 inhibition in the same soil: N_2O:(N_2O+N_2) = 0.13 (Khalil *et al*., 2003a).

In the 0.7, 1.4, 4.0 and 20.5 kPa O_2 treatments, the amounts of N_2O emitted by nitrification were highly correlated with N nitrified (Fig. 7b). We obtained specific regression lines for each O_2 concentration. The slopes, intercepts and correlation coefficients are given at Table 4. Results show that the intercept was not significantly different from 0, when we consider the N_2O emitted by nitrification alone. The slope markedly increased when O_2 availability decreased: the proportion of nitrified N evolved as N_2O varied from 0.16% to 1.48% when O_2 pressure fell from 20.5 to 0.7 kPa. If we consider the total N_2O emission, the proportion varied from 0.24% to 1.80%.

The total N_2O emissions measured during each time interval were also compared with the NO_2^- concentrations found in soil at the end of each interval. The relationship was highly dependent on O_2 concentration (Fig.8). Linear relationships were found at 20.5 and 4.0 kPa O_2 pressure, but not at lower O_2 pressures. No significant correlation was found when we considered NO_2^- concentrations present in soil at the beginning of each time interval. This indicates that NO_2^- was not a single determinant of N_2O emissions by nitrification.

O_2 concentration	Total N_2O			N_2O by nitrification		
	Slope %	**Intercept** mg kg^{-1}	**r^2**	**Slope** %	**Intercept** mg kg^{-1}	**r^2**
0.7%	1.80	-0.02	0.994	1.48	0.00	0.983
1.4%	1.20	0.01	0.999	1.09	0.00	0.999
4.0%	0.43	0.07	0.951	0.42	0.00	0.892
20.5%	0.24	0.01	0.979	0.16	0.00	0.915

Table 4. *Linear regression parameters of cumulative N_2O production (mg N kg^{-1}) versus cumulative nitrification (mg N kg^{-1}). Total N_2O is the measured N_2O emission whereas N_2O 'by nitrification' is calculated (using coefficients α and β, see text). Nitrification is defined as NO_2^- production, i.e. cumulative NH_4^+ oxidation rates.*

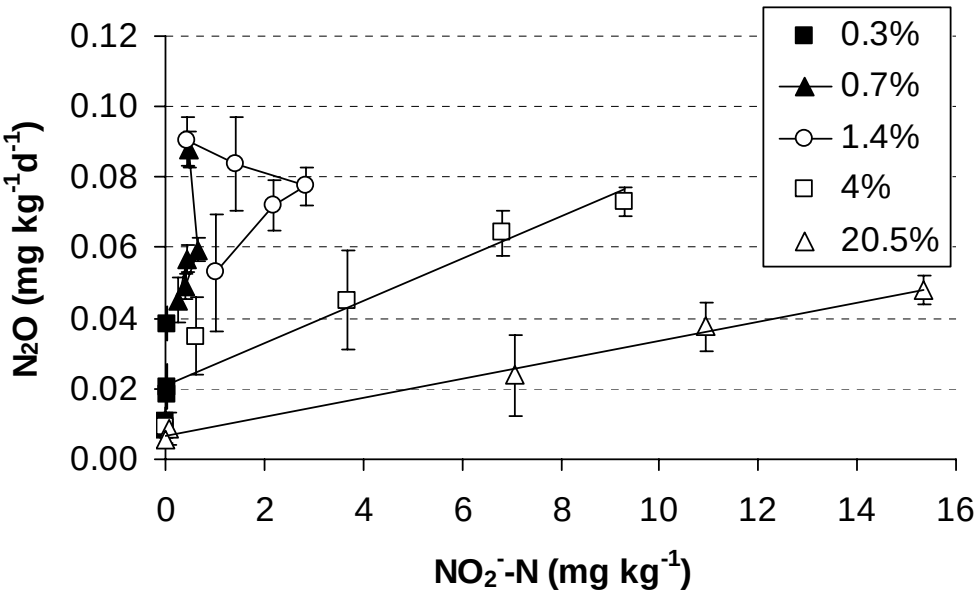

Figure 8. *Relationship between total N_2O emissions rates (mg N kg^{-1} d^{-1}) and NO_2^- concentrations (mg N kg^{-1}) measured in soil at the end of each time interval, at different O_2 concentrations: 0.3, 0.7, 1.4, 4.0 and 20.5%.*

pH evolution

The mean pH value at the beginning of incubation was 8.04 ± 0.03. At day 14, the pH had increased in soil incubated at 0% O_2, but had decreased at other O_2 concentrations. The pH decrease was greater for the highest O_2 pressure, except for the 20.5 kPa. The aerobic conditions favoured nitrification and consequently pH decrease, whereas at 0% O_2, pH had increased because there was only denitrification.

In the 20.5 kPa O_2 treatment, the nitrification was over at day 14 and the higher pH is attributed to a pH re-equilibration in soil (due to the carbonates equilibrium).

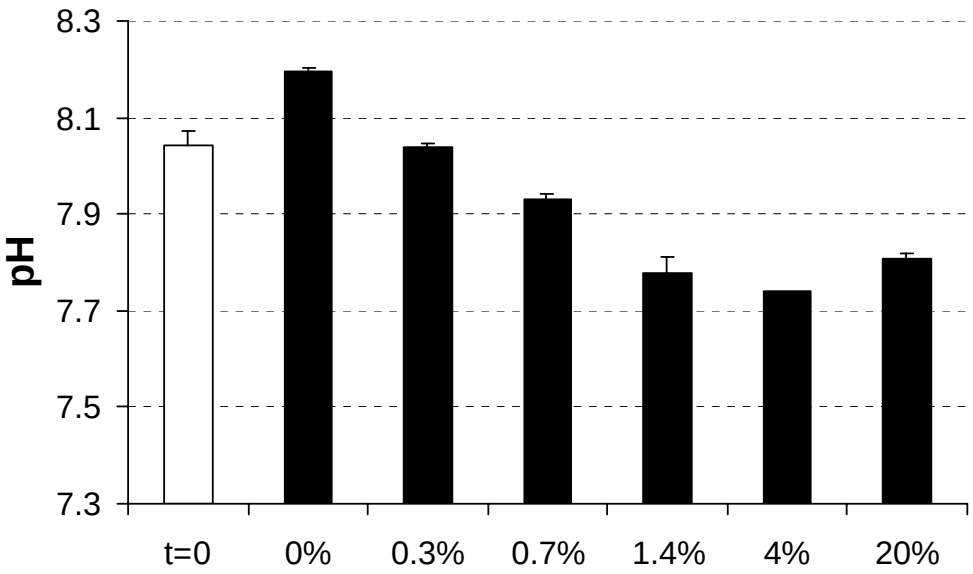

Figure 9: *Soil pH measured at the beginning (t = 0) and at the end of the incubation (t = 14 days) in soils incubated at various O_2 concentrations: 0, 0.3, 0.7, 1.4, 4.0 and 20.5%.*

DISCUSSION

Methodology of quantification and calculation

One important condition required for a successful application of isotope methods to quantify N transformations is that the added inorganic ^{15}N becomes homogeneously mixed with the native, unlabelled inorganic N in soil (Davidson *et al*., 1991). The comparison of the isotope composition of NH_4^+, NO_3^- and N_2O (Table 3) suggests that this condition was not fulfilled in our experiment. The reason for that explanation is that the added $^{15}NH_4^+$ did not diffuse rapidly within soil aggregates due to its high adsorption capacity. The $^{15}NO_3^-$ produced by nitrification was then unevenly distributed soon after its production. Stevens *et al*. (1997), using a similar ^{15}N application procedure, found that the NO_3^- pool was rather uniform, enough to perform calculations of the contribution of nitrification and denitrification to N_2O emission, using an isotope dilution equation. The difference with our experiment is that these authors used urea instead of NH_4^+ which probably diffused within soil aggregates before being hydrolysed into NH_4^+. The lack of uniformity in our experiment has different consequences: a) it should not affect the validity of nitrification estimates (using FLUAZ), because nitrification must have occurred in the soil zone where all the added $^{15}NH_4^+$ was present. In this zone, the NH_4^+ and NO_3^- can be considered as uniformly labelled. Davidson *et al*. (1991) have shown that an heterogeneous distribution of tracer in soil may have little effect on the rate estimates. The validity of our estimates of nitrification rates is also confirmed by the linear relationship with the O_2 consumption rate, the slope of which is equal to the theoretical value (Fig. 6).

b) it affected the calculation of the proportion of N_2O derived from nitrification, since the application of the isotope dilution equation was not possible. We proposed another method of calculation which accounts for two origins of N_2O, one being derived from labelled NH_4^+ and the other from unlabelled NO_3^-. The first origin is attributed to nitrification and the second one to denitrification. Although this assumption may be discussed, this calculation provided results which were in good agreement with a second independent method, based on the measured ratio $^{15}N_2O$:$^{15}N_2$. This agreement gives confidence in our conclusions.

Denitrification in well aerated aggregates

Both methods also indicated that denitrification occurred even at atmospheric O_2 pressure. This result is surprising since the moisture content was chosen (at 0.21 g g^{-1} soil) to obtain unsaturated aggregates, considering the shrinking-swelling curve previously established on that soil (Sillon, 1999). The explanation may come from the technique of adding solution to soil aggregates using pipette which does not deliver the same volume of solution to each aggregate. Some of the aggregates must have been saturated after this addition. Anoxic conditions can occur if their radius is greater than a critical value (Renault and Stengel, 1994). The critical radius was calculated using O_2 consumption rate and O_2 concentration in the atmosphere; it varied from 1.0 mm in the treatment 0.3 kPa O_2 to 3.5 mm in the treatment 20.5 kPa O_2. Therefore denitrification was possible in some of the 3 mm size aggregates.

Effect of O_2 availability on nitrification and N_2O production

Our results show that O_2 availability had a large influence on the nitrification. Nitrification rates were reduced by a factor of 6-9 when O_2 pressure decreased from 20.5 to 0.3 kPa. Only few results on O_2 effect have been reported in soils. Our results are close to those obtained by Goreau *et al.* (1980) for pure cultures of *Nitrosomonas europea*. These authors found that nitrification rate was reduced by a 7 fold factor when O_2 was reduced from 20 to 0.5 kPa.
Our results also allow to estimate the Michaelis constant of NH_4^+ oxidation relative to O_2 pressure. At the beginning of the experiment, NH_4^+ concentrations were much higher than the Michaelis constant for NH_4^+ which is about 1 mol m^{-3} (Laanbroek and Gerards, 1993), corresponding to3.0 mg N kg^{-1}. The nitrification rates versus O_2 concentration were fitted to Michaelis-Menten kinetics to obtain V_{max} and K_m at 3 time intervals (0-2, 2-4 and 4-7 days). We obtain K_m = 2.5 ± 0.6 kPa O_2, or 1.1 ± 0.3 mol O_2 m^{-3} air. This value is much greater than those reported by Laanbroek and Gerards (1993) for *Nitrosomonas europea* grown in continuous cultures : 1.3-15 mmol m^{-3}. The value of V_{max} increased with time, from 0.8 to 1.3 mmol kg^{-1} d^{-1} at 20°C, due to the growth of nitrifiers in soil.

Oxygen concentration exerted also a marked effect on N_2O production. We found that the yield of N_2O emission by nitrification, i.e. the amount of N_2O-N emitted per unit of NH_4 oxidised, increased rapidly when O_2 pressure decreased. The yield varied from 0.16% to 1.48% when O_2 pressure fell from 20.5 to 0.7 kPa. The maximum yield was obtained at 0.7

kPa but we had not enough accuracy to make calculations at 0.3 kPa O_2 pressure. Anderson *et al.* (1993) found that the optimum O_2 pressure for N_2O production in pure culture was 0.3 kPa O_2 for *Nitrosomonas europea* and 2-4 kPa for the heterotrophic nitrifier *Alcaligenes faecalis.* They obtained a yield of 1% at 5 kPa O_2 (in our study 0.42% at 4.0 kPa). Goreau *et al*. (1980) reported that N_2O production by *Nitrosomonas europea* in pure culture increased by 3-4 times when O_2 pressure fell from 20 to 1 kPa. They measured higher yields than ours: 0.3%, 0.9%, 3% and 8% at 20, 5, 1 and 0.5 kPa O_2 pressure, respectively. However they measured yields in isolates of *Nitrosomonas, Nitrosolobus* and *Nitrosospira* from soils which were similar to ours at 21 kPa O_2: 0.47%, 0.09% and 0.11%, respectively. Bollmann and Conrad (1998), working on soils, found that the maximum N_2O emission by nitrification occurred at 0.5 kPa pO2. Using the results published by Stevens *et al.* (1997), we could calculate a yield similar to ours at atmospheric pressure: 0.18 ± 0.01%, independent of the water content (40, 50 and 60% WHC). A large variability in yield has been reported at 20 kPa O_2: 0.03-1% (Garrido *et al*., 2001), 0.5-2% (Bolle, 1986), 0.09-0.28% (Breitenbeck *et al.*, 1980) and 0.02% (Tortoso and Hutchinson, 1990).

The origin of N_2O emitted by nitrification is still on debate. Ritchie and Nicholas (1972) suggested that NH_4^+ oxidisers reduced NO_2^- to N_2O to minimize intracellular accumulation of NO_2^- which is toxic. Remde and Conrad (1990) showed that N_2O could derive from nitrite produced inside the cells. Poth and Focht (1985) confirmed this hypothesis for *N. europea* cultivated in pure culture at various O_2 pressures and called it denitrification of nitrite, the nitrite being the terminal electron acceptor. They indicated that O_2 was required in NH_4^+ oxidising bacteria only for the first oxidation step: oxidation of NH_4^+ into hydroxylamine by mono-oxigenase.

Appendix

Let α be the proportion of N_2O derived from nitrification and (1-α) the proportion derived from denitrification. Two methods can be used to calculate this proportion:

Method 1: use of the ratio $^{15}N_2O$:$^{15}N_2$

Three hypotheses are made:

H1) the ratio N_2O:N_2 due to denitrification process remains constant with time and treatments;
H2) N_2O and N_2 emitted by denitrification have the same origin and isotope composition, so that the ratio N_2O:N_2 is equal to the ratio $^{15}N_2O$:$^{15}N_2$;
H3) no N_2 is produced during nitrification.

Let Q be the total amount of $^{15}N_2O$ produced (mg N kg^{-1})
Let D be the total amount of $^{15}N_2$ produced (mg N kg^{-1})
Let Q_D be the amount of $^{15}N_2O$ produced by denitrification (mg N kg^{-1})
Let R_D be the ratio $^{15}N_2O$: $^{15}N_2$ associated to denitrification
Let R be the overall ratio $^{15}N_2O$: $^{15}N_2$ measured

$$R_D = \frac{Q_D}{D} \qquad \text{(hypothesis H3)} \qquad \text{A1.1}$$

$$R_D = \frac{Q(1-\alpha)}{D} \qquad \text{(hypothesis H2)} \qquad \text{A1.2}$$

$$R = \frac{Q}{D} \qquad \text{A1.3}$$

$$R_D = R(1-\alpha) \qquad \text{(hypothesis H1)} \qquad \text{A1.4}$$

so that:

$$\alpha = 1 - \frac{R_D}{R} \qquad \text{A1.5}$$

Method 2: use of the isotope composition of $^{15}N_2O$

This method assumes that the N_2O produced has two origins: it is derived from two populations, each having a specific isotopic composition. N_2O signal is measured at mass 44 ($^{14}N^{14}N^{16}O$) and mass 45 ($^{14}N^{15}N^{16}O$).

Population	Proportion	Atom% abundance	^{14}N isotope frequency	^{15}N isotope frequency	Isotope ratio
P1	α	A_1	a_1	b_1=1-a_1	R_1
P2	1-α	A_2	a_2	b_2=1-a_2	R_2
P1+P2	1	A			R

Isotope ratio of population 1:

$$R_1 = \frac{2b_1}{a_1} \qquad \text{A2.1}$$

Isotope ratio of population 2:

$$R_2 = \frac{2b_2}{a_2} \qquad \text{A2.2}$$

Mass 44 of the mixture:

$$M_{44} = a_1^2\alpha + a_2^2(1-\alpha) \qquad \text{A2.3}$$

Mass 45 of the mixture:

$$M_{45} = 2a_1 b_1\alpha + 2a_2 b_2(1-\alpha) \qquad \text{A2.4}$$

Isotope ratio of the mixture:

$$R = \frac{M_{45}}{M_{44}} = \frac{2a_1 b_1\alpha + 2a_2 b_2(1-\alpha)}{a_1^2\alpha + a_2^2(1-\alpha)} \qquad \text{A2.5}$$

Equations A2.1 and A2.2 yield:

$$a_1 = \frac{2}{2+R_1} \qquad \text{A2.6}$$

$$a_2 = \frac{2}{2+R_2} \qquad \text{A2.7}$$

$$b_1 = \frac{R_1}{2+R_1} \qquad \text{A2.8}$$

$$b_2 = \frac{R_2}{2 + R_2} \qquad \text{A2.9}$$

Replacing a_1, b_1, a_2 and b_2 in equation A2.5 gives:

$$R = \frac{\alpha R_1 (2 + R_2)^2 + (1 - \alpha) R_2 (2 + R_1)^2}{\alpha (2 + R_2)^2 + (1 - \alpha)(2 + R_1)^2} \qquad \text{A2.10}$$

Since :

$$A = \frac{R}{2 + R} \qquad \text{A2.11}$$

it comes:

$$A = \frac{\alpha A_1 (1 - A_1) + (1 - \alpha) A_2 (1 - A_2)}{\alpha (1 - A_1) + (1 - \alpha)(1 - A_2)} \qquad \text{A2.12}$$

so that:

$$\alpha = \frac{(1 - A_2)(A_2 - A)}{(1 - A_2)(A_2 - A) - (1 - A_1)(A_1 - A)} \qquad \text{A2.13}$$

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SYNTHESE DES RESULTATS

L'objectif de notre travail était d'étudier les émissions de N_2O par nitrification et dénitrification en relation avec la structure du sol (à l'échelle centimétrique), l'aération et les activités microbiennes. Nous avons choisi de travailler sur un seul type de sol, mais dont la structure était fortement différenciée en raison des opérations de travail du sol (Richard *et al*., 1999). Cette différenciation a conduit à des proportions différentes de mottes compactes sans porosité visible à l'œil (mottes Δ) et de mottes fragmentaires à porosité visible (mottes Γ) sur lesquelles nous avons travaillé. Nous avons:

1) caractérisé les processus microbiens à l'origine de la production et de la consommation de N_2O par dénitrification,

2) évalué les émissions de N_2O par dénitrification sur des mottes soumises à 2 niveaux d'activités microbiennes (respiratoire aérobie et dénitrifiante) obtenus par une pré-incubation ou non des mottes en anaérobiose,

3) caractérisé la structure des mottes et modélisé son effet sur le volume anoxique et sur les émissions de N_2O par dénitrification, avec ou sans pré-incubation anaérobie,

4) décrit les effets de la concentration en NH_4^+ et en O_2 sur les vitesses de nitrification et d'émission de N_2O par nitrification.

I. Synthèse des principaux acquis

I.1. Fonctionnement dénitrifiant en condition anaérobie

Une majorité de travaux sur les émissions de N_2O ont porté sur la dénitrification considérée comme la principale source de ce gaz. En fait, la production nette de N_2O est le bilan entre la production brute et la consommation de N_2O. Certains travaux ne considèrent que la production nette de N_2O et ne prennent pas en compte les interactions possibles entre production et consommation qui ont été mises en évidence. D'autres études quantifient simultanément production brute et production nette (notamment en présence ou absence de C_2H_2), mais peu d'entre elles ont permis de caractériser finement les 2 processus. Dans ce travail, nous avons développé une méthode combinant des mesures sur des boues de sol incubées en anaérobiose à un modèle de simulation des réductions microbiennes de NO_3^- et N_2O dans le sol (chapitre 2). Le modèle simule la réduction de NO_3^- en N_2O et de N_2O en N_2 par des cinétiques Michaelis Menten, avec prise en compte d'une compétition non

enzymatique entre NO_3^- et N_2O comme accepteurs d'électrons terminaux, et simule la dynamique de 2 groupes microbiens l'un étant capable de réduire N_2O en N_2, l'autre non. Trois hypothèses ont été évaluées alternativement :

- *hypothèse 1* : les deux populations microbiennes (NO_3 réductase et N_2O réductase) se développent indépendamment, sans accroissement de la capacité à réduire N_2O. Avec cette hypothèse, le modèle s'avère incapable de reproduire les données expérimentales. Ceci suggère que certains dénitrifiants initialement incapables de réduire N_2O en N_2 deviennent capables de réaliser cette réduction ;
- *hypothèse 2 ou 3* : elle simule cette adaptation, en considérant soit une synthèse progressive et simultanée pour tous les dénitrifiants de la N_2O réductase (hypothèse 2), soit une synthèse brutale de l'enzyme pour certains dénitrifiants, ces derniers n'acquérant pas tous ensemble la capacité à réduire N_2O (hypothèse 3). Ces deux hypothèses permettent de beaucoup mieux décrire les données expérimentales, bien que des biais subsistent. La biomasse microbienne estimée en ajustant les données simulées aux données expérimentales est en bon accord avec la biomasse mesurée.

L'acquisition de la capacité à réduire N_2O en N_2 sur des durées de 2 à 5 jours (à 20°C) est un résultat cohérent avec d'autres résultats publiés sur la synthèse de la N_2O réductase. Ce processus entraîne donc une diminution du rapport $N_2O/(N_2O+N_2)$ au cours du temps. Cependant, le rapport peut être considéré comme stable avant la période d'induction. Nous l'avons estimé à 12% dans nos conditions expérimentales (anaérobiose totale).

L'une des causes possibles des écarts entre modèle et expérience est la validité de l'hypothèse faite dans le modèle, à savoir que le flux d'électrons traversant les chaînes respiratoires microbiennes est constant tant que les accepteurs terminaux d'électrons (NO_3^- et/ou N_2O) ne sont pas limitants et qu'il est indépendant de la nature de ces accepteurs. Il faudrait évaluer la validité de cette hypothèse, ou tester une hypothèse alternative avec le modèle.

I.2. Anoxie, respiration et dénitrification

L'anoxie dans le sol dépend simultanément de la consommation en O_2 des micro-organismes et des possibilités de transfert d'O_2, celles-ci étant affectées par la structure du sol, par sa teneur en eau et les propriétés de diffusion de l'oxygène au sein des zones saturées et des pores libres à l'air du sol. Afin de caractériser la consommation en O_2 du sol et sa

dépendance aux concentrations en O_2 et en CO_2, nous avons repris la méthode proposée par Renault et Stengel (1994).

Nous avons analysé les émissions de N_2O produites au cours d'une incubation aérobie par des mottes de sol ayant 2 niveaux d'activités microbiennes (respiratoire aérobie et dénitrifiante), obtenus par incubation préalable ou non de mottes en anaérobiose (6 jours). La pré-incubation anaérobie a été conduite en présence d'acétylène qui inhibe de façon irréversible la nitrification (Berg *et al*., 1982) ainsi que la synthèse et l'activation de la N_2O réductase, ce qui minimise la variation dans le temps du rapport $N_2O/(N_2O+N_2)$ des produits de la dénitrification. Nous avons observé que l'émission nette de N_2O par les mottes de sol en incubation aérobie était très fortement accrue par la pré-incubation anaérobie, alors que ces mottes avaient été très fortement appauvries en NO_3^- durant la période d'anoxie (contrairement à celles qui n'avaient pas subi la pré-incubation). Le marquage isotopique a prouvé que l'essentiel des émissions provenait de la dénitrification. Les mesures de teneur en CO_2 ont confirmé que la pré-incubation stimulait fortement la respiration aérobie sur 24 heures et que l'effet disparaissait après 4 jours (à 20°C). Compte tenu de la vitesse de respiration mesurée sans ou avec pré-incubation (respectivement 3.2 et 12.2 nmol O_2 kg^{-1} s^{-1}), le calcul montre qu'une motte totalement saturée doit avoir un rayon respectif de 1.0 et 0.5 cm pour qu'elle ait un cœur anaérobie. Ceci est cohérent avec nos observations, à savoir qu'une activité dénitrifiante a été détectée sur 44% des mottes (d'environ 3 cm de diamètre) n'ayant pas subi de pré-incubation et sur 100% des mottes ayant subi cette pré-incubation anaérobie.

L'effet très marqué de la pré-incubation anaérobie sur les émissions de N_2O peut avoir 2 explications:

- un fort accroissement de la vitesse de respiration aérobie suite à la période d'anaérobiose, sans doute en raison de la dégradation de petites molécules organiques accumulées en phase anaérobie (de type AGV) et facilement dégradables en conditions aérobies;
- le fait que la présence de C_2H_2 en anaérobiose inhibe la synthèse de la N_2O-réductase et qu'elle permet de maintenir le rapport $N_2O/(N_2O+N_2)$ à un niveau élevé.

A l'aide d'un modèle mécaniste, nous avons alors vérifié:

1) que l'accroissement de respiration et d'activité dénitrifiante permettait d'expliquer les écarts d'émission de N_2O entre mottes ayant subi ou n'ayant pas subi de pré-incubation anaérobie,

2) que ces résultats étaient cohérents avec les différences d'émissions de N_2O entre mottes de structures Δ et Γ (relation entre niveau d'émission et masse des mottes, corrélée à leur structure Δ ou Γ).

Le modèle prend en compte explicitement la structure du sol et la consommation d'O_2 par le sol dans le calcul de la fraction anoxique des mottes ; il simule la dénitrification, incluant la réduction de N_2O en N_2, au sein des zones anoxiques. L'analyse d'image s'est avérée être un outil pertinent pour décrire la structure des mottes de sol à partir des lames minces.

I.3. Emissions de N_2O par nitrification

Des travaux récents ont montré que la nitrification peut contribuer fortement aux émissions de N_2O. Plusieurs facteurs pouvant influencer ces émissions ont été étudiés. Toutefois, peu d'études sur l'effet direct de O_2 sur ces émissions existent. Une série d'expérimentations permettant d'étudier l'effet de NH_4^+ et O_2 sur la nitrification et les émissions de N_2O par nitrification a été réalisée. Nos résultats ont mis en évidence et quantifié l'effet de la concentration en ammonium sur l'activité nitrifiante et sur l'accumulation du nitrite. Ils ont montré que la pression partielle en O_2 influence fortement les cinétiques de nitrification. Nous avons obtenu une bonne corrélation entre nitrification et consommation de O_2, correspondant à la stœchiométrie des équations de nitrification. Ces résultats soulignent l'importance de la consommation en O_2 par nitrification en comparaison avec la consommation par respiration; cette forte consommation est souvent négligée dans l'étude des émissions de N_2O alors qu'elle peut favoriser la création des sites anoxiques et en conséquence favoriser la dénitrification. Les émissions de N_2O par nitrification sont bien corrélées avec les quantités d'azote nitrifié; les pentes des droites de régression étant très variables selon la pression partielle en O_2. La structure du sol affectant la distribution de l'oxygène dans le sol peut donc modifier la nitrification et les émissions de N_2O par nitrification, et dans une gamme de teneurs plus large que pour la dénitrification: ainsi le passage d'une concentration de 20% à 4% O_2 diminue la vitesse de nitrification d'environ 50% et augmente la perte de N_2O d'un facteur 2.5. Ces résultats montrent la nécessité de prendre en compte l'effet de O_2 pour mieux prévoir les émissions de N_2O et mieux expliquer leur variabilité.

I.4. Données complémentaires

Nous avons acquis d'autres données relatives au fonctionnement respiratoire et dénitrifiant des mottes en séparant les matières organiques particulaires (MOP) et le reste du sol (appelé matrice). Ces données restent à traiter complètement, mais il apparaît dès à présent que les teneurs en MOP sont similaires à celles obtenues dans d'autres expérimentations et que les activités respiratoire et dénitrifiante sont similaires en ordre de grandeur à celles obtenues dans des contextes cultivés (Parry *et al*., 2000). Toutefois et contrairement aux résultats de Parry *et al*. (2000), il semble que l'activité dénitrifiante puisse être négligée au niveau de ces matières organiques particulaires dans notre sol. Parmi les hypothèses permettant d'expliquer cette contradiction, nous proposons l'hypothèse "taille": la taille moyenne des MOP de notre sol pourrait être plus faible que celle des mottes étudiées par Parry *et al.* (2000) et minimiser leur caractère "hot-spot" si elle est trop faible.

II. Comparaison avec le comportement du sol *in situ*

Les résultats que nous avons obtenus sur des mottes isolées, au laboratoire, peuvent être comparés aux résultats de terrain acquis sur le même sol par les équipes INRA de Laon et Avignon (site d'Estrées-Mons, projet GESSOL). Deux périodes d'expérimentation de terrain ont été réalisées, dans des conditions agricoles classiques, l'une en automne après une culture de blé, l'autre au printemps avant une culture de maïs. Les résultats ont fait apparaître :

1) des émissions de N_2O égales en moyenne à 1.2 g $N\text{-}N_2O$ ha^{-1} j^{-1} à l'automne, au cours d'un suivi continu sur 2 mois (13/09/1999-12/11/1999), qui ont décru parallèlement à la température;
2) des émissions de N_2O égales en moyenne à 2.6 g $N\text{-}N_2O$ ha^{-1} j^{-1} au printemps, au cours d'un suivi semi-continu de 2 mois (07/04/2000-09/06/2000), qui ont atteint un maximum après l'apport d'engrais et ont diminué ensuite;
3) l'absence d'effet significatif de la structure du sol sur les émissions de N_2O, alors que les parcelles étaient très différenciées sur le plan structural, notamment par leur proportion de mottes Δ (mottes sans porosité visible à l'œil);
4) un rapport $N_2O/(N_2O+N_2)$ des produits de la dénitrification faible, estimé à 13 ± 4 % (estimation réalisée grâce au marquage $^{15}NO_3$), ce qui indique que ce sol est actif pour réduire le N_2O en N_2.

Les émissions *in situ* peuvent être comparées aux émissions mesurées au labo, en supposant une densité apparente de 1.5 g cm^{-3}, une profondeur de zone émettrice de N_2O de 30 cm (correspondant à la couche labourée) et une loi de température exponentielle (avec un

Q_{10} = 2). Les vitesses d'émission de N_2O moyennes mesurées au champ de 1.2 et 2.6 g N ha^{-1} $jour^{-1}$ correspondent à des vitesses de 0.37 et 0.70 pmol kg^{-1} s^{-1} à 20°C.

Pour un rapport $N_2O/(N_2O+N_2)$ égal à 0.13, on en déduit que les vitesses de dénitrification seraient de 2.9 et 5.4 pmol kg^{-1} s^{-1} à 20°C. Ces valeurs sont tout à fait en accord avec les vitesses de dénitrification totale (avec C_2H_2) mesurées sur mottes au laboratoire : 4.2 ± 3.7 pmol kg^{-1} s^{-1} à 20°C.

Nos travaux suggèrent que pour passer de la modélisation des processus microbiens étudiés au laboratoire à une modélisation opérationnelle des émissions *in situ*, il faut tenir compte des variations des paramètres décrivant les cinétiques microbiennes en fonction de "l'histoire" du sol, en particulier des alternances de périodes d'aérobiose et d'anaérobiose.

1) d'abord l'induction de la N_2O réductase pendant une période d'anaérobiose qui apparaît 1 à 2 jours après le début d'anoxie selon la concentration en NO_3^-
2) éventuellement pour des périodes d'anaérobiose de plusieurs jours, l'accumulation de substrats carbonés supplémentaires (acides organiques, acides gras volatils) au cours de la phase d'anoxie, qui deviennent métabolisables après rétablissement de l'aérobiose. La décomposition de ces substrats carbonés augmente alors la vitesse de consommation d'O_2 et donc le volume anoxique, favorisant à nouveau les émissions de N_2O.

Nos travaux montrent aussi qu'une forte activité nitrifiante (induite par un apport d'engrais sous forme ammoniacale) peut fortement accroître la consommation de O_2 pendant quelques jours et ainsi augmenter le risque d'anaérobiose et d'émission de N_2O par dénitrification, en plus des émissions de N_2O par nitrification. Les conséquences de ces 2 processus sur les émissions de N_2O *in situ* restent à évaluer. Cependant, ils remettent en cause une modélisation basée sur des paramètres invariants, estimés à partir d'incubations de courte durée et faites uniquement dans le sens aérobie $\rightarrow$ anaérobie.

III. Perspectives de ce travail

Les perspectives de recherche ouvertes par notre travail concernent 3 aspects:

III.1. Aspect méthodologique

Afin de distinguer nitrification et dénitrification biologiques à l'origine de la production de N_2O, nous avons utilisé la technique isotopique par un apport d'une solution ammoniacale ^{15}N. Toutefois, lors de l'apport de la solution, des zones anoxiques ont pu être créées, favorisant ainsi la dénitrification. Une autre difficulté de cette méthode est d'obtenir un mélange ^{15}N et ^{14}N aussi homogène que possible pour toutes les formes minérales azotées du sol.

Pour étudier la nitrification, nous avons cherché à obtenir des conditions expérimentales défavorables à la dénitrification et limitant les problèmes d'hétérogénéité spatiale. Pour cela, les incubations ont été réalisées sur des échantillons de sol tamisés entre 2 et 3 mm, fortement enrichis en NH_4^+. La petite taille des agrégats favorise l'aération du sol et limite les risques de présence de microsites anaérobies au cœur des agrégats (Sextone *et al.*, 1985 ; Renault et Stengel, 1994). Toutefois, aux plus faibles teneurs en O_2 testées (0.3% O_2), nous ne pouvons plus considérer que la dénitrification est absente de ces petits agrégats.

Dans tout le travail sur la dénitrification, le potentiel hydrique des mottes a été maintenu à une valeur constante. Cette condition est nécessaire car des variations de ce facteur pourraient masquer l'influence de la structure sur les émissions de N_2O par dénitrification notamment. Le potentiel choisi, -0.5 kPa, correspond à des conditions très humides, proches de la saturation que l'on rencontre au champ surtout en automne/hiver, en particulier après des opérations de travail du sol "dégradantes" (du type récolte de betterave ou de maïs). Un potentiel hydrique plus faible permettrait peut-être de mieux extérioriser les différences de structure des mottes en saturant plus ou moins de pores.

III.2 Aspect d'analyse des mécanismes

a) *Concernant la dénitrification*:

Nous avons constaté le très fort effet d'une pré-incubation anaérobie de 6 jours sur les émissions de N_2O par les mottes de sol. Qu'en serait-il si cette période d'anaérobiose était accrue ou réduite ? Une durée aussi longue est sans doute très exceptionnelle au champ, sauf en sol hydromorphe. L'effet d'une courte pré-incubation anaérobie mériterait d'être étudié.

Par ailleurs, la présence de C_2H_2 pendant cette pré-incubation inhibe la synthèse de la N_2O-réductase et permet de maintenir le rapport $N_2O/(N_2O+N_2)$ à un niveau élevé. L'absence de C_2H_2 pourrait favoriser la réduction de N_2O en N_2 et par conséquent minimiser la production de N_2O pendant la phase aérobie après la pré-incubation anaérobie. Il faudrait le confirmer.

b) *Concernant la nitrification*:

Nous avons mis en évidence un fort effet de la pression en O_2 sur l'activité nitrifiante ainsi que sur les émissions de N_2O par nitrification. Néanmoins, les mécanismes des émissions de N_2O par nitrification dans les sols restent encore mal définis. Nous pouvons envisager une meilleure description de la nitrification elle même en tenant en compte des 2 réactions : la production de NO_2^- et la production de NO_3^- ainsi que l'effet de O_2 sur chacune des 2 réactions. Une bonne compréhension de ces processus facilitera l'étude des mécanismes d'émissions de N_2O par nitrification ainsi que l'effet de O_2 sur ces émissions. Elle devrait permettre d'améliorer les modèles de prévision des émissions de N_2O. Parallèlement, il est nécessaire de passer d'un modèle d'aération des sols utilisant le critère volume anoxique à un modèle prenant en compte les gradients de concentration en O_2 dans la motte ou le profil de sol

III.3. Extrapolation à des échelles d'espace et de temps plus larges

Nous nous intéressons aux perspectives d'adaptation et d'utilisation du modèle à des problématiques agronomiques et environnementales. A l'échelle de notre travail, peut se poser le problème de la non prise en compte de la certains processus ou de leurs conséquences comme la distribution verticale des concentrations gazeuses et des concentrations en NO_3^-. Ces aspects pourraient requérir la nécessité de couplages entre des modèles établis à l'échelle de la motte et des modèles de fonctionnement à l'échelle du profil. Un premier modèle d'émission de N_2O par dénitrification a été proposé par Lafolie *et al.* (2000) à l'échelle du profil : ce modèle doit être complété par les acquis de notre travail et étendu à la prise en compte des émissions de N_2O par nitrification.

Les descripteurs de la structure tels que les distances d'un point d'une motte au pore libre à l'air le plus proche pourraient être utilisés pour caractériser les possibilités d'oxygénation locales. Toutefois, si de tels critères sont utiles pour les études de mécanismes à l'échelle locale, ils sont inadaptés à l'approche régionale des émissions de N_2O de par leur mesure délicate et de par l'absence de méthodes fiables d'extrapolation spatiale et temporelle. A ce

type de critères, il semble possible (tout au moins à l'échelle de la parcelle) de substituer des critères simples tels que la taille des éléments structuraux du sol, la nature morphologique des éléments constitutifs du sol ainsi que les travaux visant à analyser la proportion des différentes types de mottes et leur localisation au sein du profil.

A l'heure actuelle, les modèles sont généralement des produits de fonctions décrivant l'action individuelle des différents facteurs (teneur en eau, NO_3^-, matière organique, température, pH, ...) sur les émissions de N_2O. Ces modèles prennent en compte les effets moyens de ces paramètres et ne cherchent pas à décrire les processus élémentaires liés à ces émissions. Ces modèles sont fonctionnels à de grandes échelles (parcelle), mais souvent ils ne sont pas généralisables à d'autres sites. Lors de notre travail, nous avons montré que la réduction de N_2O, la nitrification et les émissions de N_2O par nitrification ainsi que la structure des mottes, ne sont pas négligeables dans les prévisions des émissions de N_2O. La prise en compte de ces facteurs dans les modèles pourrait être une base à l'élaboration de nouveaux modèles simplifiés.

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