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ISOTOPOMER EFFECTS ASSOCIATED WITH
NITRIFICATION AND DENITRIFICATION: IMPLICATIONS
FOR THE GLOBAL NITROUS OXIDE CYCLE

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ISOTOPOMER EFFECTS ASSOCIATED WITH NITRIFICATION AND DENITRIFICATION: IMPLICATIONS FOR THE GLOBAL NITROUS OXIDE CYCLE

By

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ABSTRACT

ISOTOPOMER EFFECTS ASSOCIATED WITH NITRIFICATION AND DENITRIFICATION: IMPLICATIONS FOR THE GLOBAL NITROUS OXIDE CYCLE

By

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Nitrifying and denitrifying bacteria carry out a vital role in soil systems by regulating supplies of inorganic nitrogen. Nitrifying and denitrifying bacteria also affect the Earth's atmosphere profoundly through the addition of the greenhouse gas, N_2O. To better understand the role of nitrification and denitrification in atmospheric greenhouse gas production we need a reliable method to distinguish N_2O formed from these two processes. Such information will be particularly valuable in agricultural environments that can be managed to foster one process or the other. The intramolecular distribution of ^{15}N in nitrous oxide (isotopomer) is emerging as a new tool in defining the sources and sinks of this trace gas (Popp et al., in press; Toyoda and Yoshida, 2000; Toyoda et al., in press, Perez et al, 2001) and is commonly expressed in terms of site preference (difference in $\delta^{15}N$ between the central and outer N atoms). Using laboratory cultures of whole soil microbes and pure cultures of an N_2O-producing denitrifier, *Pseudomonas chlororaphis* (ATCC #43928), it was demonstrated that the isotopomer fingerprint of N_2O derived from denitrification is unique from that of nitrification (Christensen and Tiedje, 1988). Furthermore, it was demonstrated that in agricultural soils the consumption of N_2O during denitrification has no affect on site preference, but in deciduous forest soils an isotopomer effect is observed. With these results we are now poised to begin to apply isotopomers to apportion the relative contribution of N_2O derived from nitrification and denitrification in agricultural soils.

DEDICATION

This work is dedicated to my grandparents Pinkus Rolnitzky, Alexander Pitt, and Margaret G. Pitt who passed before this work could be completed. I thank them for all their hard work and dedication to my family.

ACKNOWLEDGEMENTS

I would like to thank Drs. Nathaniel Ostrom, John Breznak, Hasand Gandhi, Robin Sutka, and Tim Bergsma for all of their invaluable experience and advice throughout the course of this work. I would like to especially thank my committee chairperson, Dr. Peggy Ostrom, for her patience and ability to take a chance on someone she hardly knew. I would also like to thank my parents Edward and Celina Pitt for their love and support, my grandmother Mary Rolnitzky for her amazing inspiration, and Rachel Frumkin who is my future.

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INTRODUCTION

Nitrous oxide emissions from agriculture are a significant contributor to global greenhouse gas emissions and can, in fact, contribute more to radiative forcing of climate change than CO_2 (IPCC, 2001; Robertson *et al.*, 2000). Increasing emissions of N_2O over the past century result from human-induced changes to microbial nitrification and denitrification. While these processes can be managed in agriculture, identification of their relative importance to N_2O flux is unclear. Traditionally, stable isotopes have been used for apportionment but they have not constrained the N_2O budget better than other approaches (IPCC, 2001, Sutka *et al.*, 2003). Recent field studies used isotopomeric site preference of N_2O (difference in $\delta^{15}N$ between the central and terminal nitrogen atoms of N=N=O) to differentiate N_2O arising from nitrification versus denitrification (Perez *et al.*, 2001; Yamulki *et al.*, 2001). However, accurate apportionment between reactions using site preference requires examining individual processes in pure culture. Previous work evaluated site preference during nitrification (by nitrifying and methanotrophic bacteria) (Fig. 1, reaction A) and during nitrifier-denitrification (Fig. 1, reaction B) (Sutka *et al.*, 2003). Here I evaluate site preference during denitrification (Fig. 1, reactions C, D) and show that site preference is a robust indicator of microbial origins of N_2O in agricultural soils. Trends in site preference during N_2O reduction (Fig. 1, reaction D) within mesocosms revealed differences between deciduous forest and agricultural soil, implying important differences in microbial community structure and/or function between managed and natural soils.

Bacterial nitrifiers produce N_2O by either oxidation of hydroxylamine (Fig. 1, reaction A) or reduction of nitrite (Fig. 1, reaction B) (Wrage *et al.*, 1999). These two pathways impart different site preferences on the N_2O produced (Table 1) (Sutka *et al.*, 2003) making it possible to distinguish which process was involved in N_2O production. Most denitrifying bacteria produce and consume N_2O (Fig. 1, reactions C, D). However, it is unclear if N_2O produced by nitrite reduction during nitrifier-denitrification (Fig 1, reaction B) differs in site preference from that of N_2O produced during denitrification (Fig. 1, reaction C) and if there is any change in site preference during consumption of N_2O (Fig 1, pathway D) that could confound our ability to distinguish nitrifier-denitrification from denitrification.

I used *Pseudomonas chlororaphis* to evaluate site preference of N_2O production during denitrification (Fig 1, reaction C), because this bacterium lacks the enzyme to reduce N_2O to N_2 (nitrous oxide reductase). Thus, it will only express isotopomer effects during N_2O production from nitrite without influences associated with consumption of N_2O. *P. chlororaphis* cultures showed N_2O production over time (Fig. 2a) and no correlation between concentration and site preference (Fig. 2b). A Rayleigh model (Mariotti *et al.*, 1988) indicated no fractionation in site preference during production. Furthermore, site preferences for N_2O production via nitrite reduction by *P. chlororaphis* and *N. europaea* were virtually identical (Fig. 1, pathway C and B, respectively; Table 1). This suggests that the two organisms share similar mechanisms for nitrous oxide production; and that irrespective of whether or not a nitrifier or denitrifier is involved there is a unique site preference for N_2O produced by nitrite reduction.

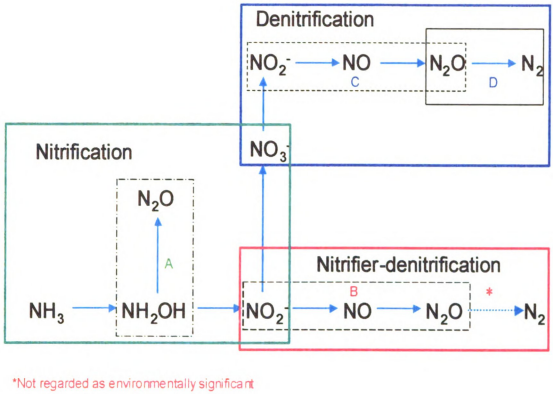

Figure 1. Reactions resulting in N_2O production and reduction. **A,** Production of N_2O by nitrifying bacteria by oxidation of hydroxylamine and **B,** reduction of nitrite during nitrifier-denitrification **C,** N_2O production during denitrification and **D,** its subsequent reduction.

Table 1 **Average Site Preference for reactions leading to N_2O production.**

Pathway	Culture	Ave. Site Preference
Reaction A (Fig. 1)	*N. europaea*	-2.3 +/- 1.9
Reaction B (Fig. 1)	*N. europaea*	-8.3 +/- 3.6
Reaction C (Fig. 1)	*P. chlororaphis*	-8.1 +/- 3.4

My next objective was to determine if consumption of N_2O during denitrification (Fig. 1, reaction D) influences site preference. This was done by monitoring site

preference during N_2O reduction in anaerobic soil mesocosms (agricultural, successional field and deciduous forest soils). Following procedures of Bergsma *et al.* (2002), I verified that N_2O was not being produced in the mesocosms. Briefly, prior to initiating the experiment, the mesocosms were purged with N_2 and, after two weeks, the headspace was sampled for N_2O; N_2O was not detected indicating that production of N_2O was not significant. An N_2O headspace was then established in all mesocosms and N_2O was subsequently consumed over time (Fig. 2c, e).

In the process of generating isotopomer results, fractionation factors (ε) for $\delta^{15}N$ and $\delta^{18}O$ of N_2O were established using a Rayleigh model (Table 2) (Marriotti *et al.*, 1988). Traditional approaches have applied fractionation factors to apportion N_2O produced from either nitrification or denitrification (Perez *et al.*, 2001). However, the results show that fractionation factors for oxygen and nitrogen vary markedly between replicate soil treatments (CAT 1,2,3) and are, therefore, not conservative tracers of denitrification (Table 2). Fractionation was lowest (Table 2) in the mesocosm (CAT 1) with the highest water filled pore space (saturated vs. 91%). This is consistent with the observation that diffusion limits the expression of fractionation, resulting here from differences in the magnitude of water filled pore space (Brandes and Devol, 1997). Since variation in fractionation factors limits their application for N_2O source apportionment, site preference is an important alternative.

Table 2 **Fractionation factors for N_2O reduction (Reaction D) in soil mesocosms.**

Soil mesocosm treatment type*	$\varepsilon^{15}N$	$\varepsilon^{18}O$
CAT1	-2.9	-0.4
CAT2	-6.8	-13.9
CAT3	-5.3	-8.6
HCS	-5.9	-13.6
DF	-14.6	-38.7

*CAT, conventional agricultural (replicates1, 2, 3); HCS, historically cultivated successional; DF, deciduous forest

In agricultural and historically cultivated successional soils, no change in site preference with reduction in N_2O concentration was observed (Fig. 2d,f). I demonstrated that: (1) site preference can distinguish N_2O produced by denitrification (Fig. 1 reaction C) and nitrifier-denitrification (Fig. 1, reaction B) from that produced during nitrification (Fig. 1, reaction A) (Table 1); and (2) reduction of N_2O to N_2 does not obscure site preference imparted by denitrification in agricultural soils (Fig. 1d). Thus, site preference provides a robust indicator of the process producing N_2O.

Unlike other mesocosms, those composed of deciduous forest soils showed a change in site preference with a decrease in N_2O concentration (Fig. 2f). The associated fractionation factor (slope) was large (-20.4 ‰) (Fig. 2f) and I believe differences in the fractionation for site preference between these soils and agricultural soils reflect differences in microbial community structure. Buckley and Schmidt (2001) showed that microbial community structure differed between fields that were cultivated and those with a history of conventional tillage. Our data reflect differences in process associated with these differences in microbial communities.

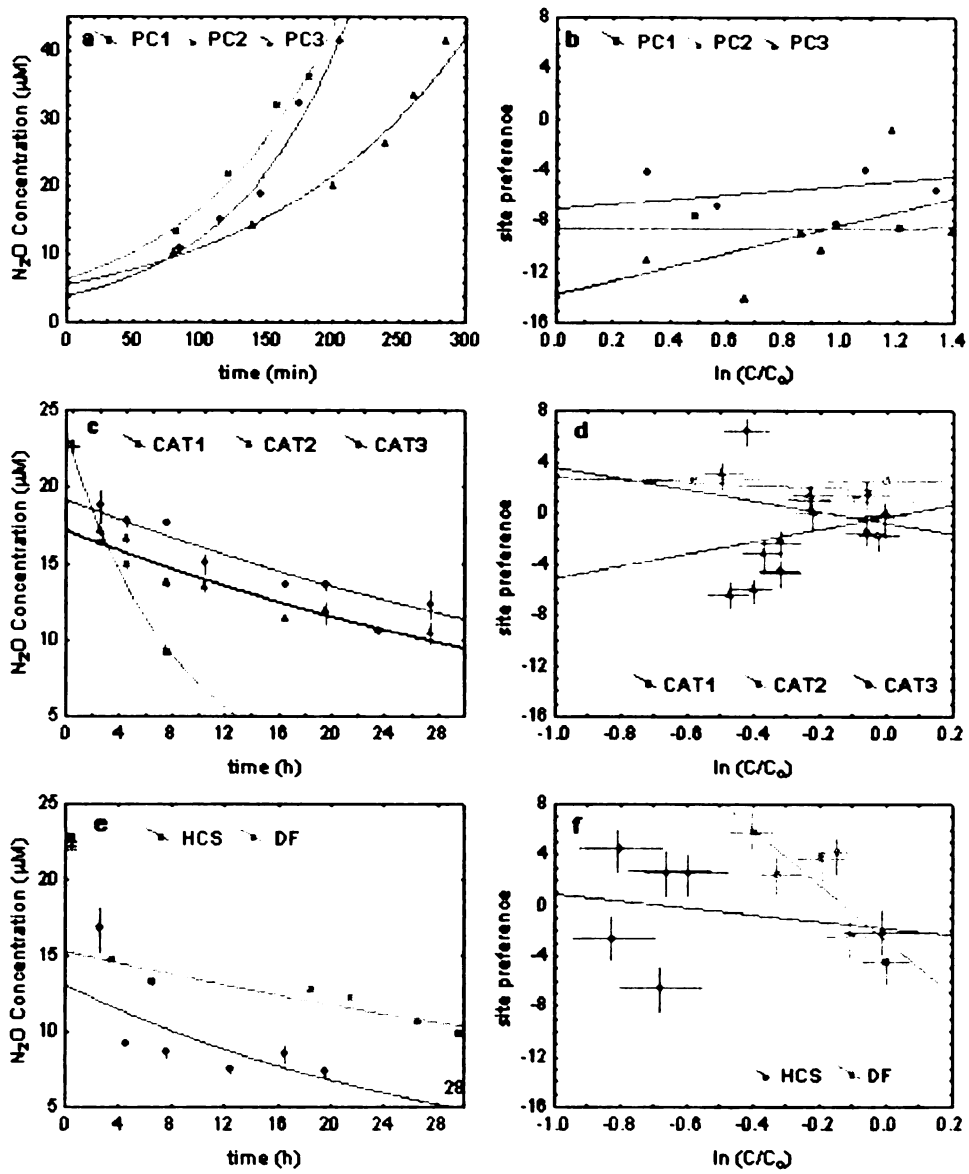

Figure 2. N_2O concentration and isotopomer site preference for denitrification experiments. **a,** Production of N_2O, and **b,** site preference expressed relative to the natural log of the ratio of the observed concentration, C, to that of the initial concentration, C_o, in triplicate cultures of *P. chlororaphis*. **c,** Consumption of N_2O and **d,** site preference in soil mesocosms from three replicate plots with a history of conventional agricultural treatment (CAT1, CAT2 and CAT3). **e,** Consumption of N_2O and **f.** site preference in soil mesocosms from a historically cultivated successional field (HCS) and a deciduous forest (DF). Regression equations for site preference vs. ln (C/C_o) are as follows: **b,** PC1, y = -7.0+ 1.8x; PC2, y = -8.5+ 0.01x; PC3, y = -13.7+ 5.4x **d,** CAT1, y = -0.4 - 2.4x; CAT2, y = 1.9 - 0.6x; CAT3, y = -0.2 + 3.9x **f,** HCS, y = 4.4 - 1.2*x; DF; y = 3.1- 20.4*x.

Toshida and Toyoda (2000) were the first to demonstrate the use of isotopomers for N_2O source apportionment by identifying the origins of N_2O in the troposphere. Our research has focused on individual N_2O-producing microbial reactions. Our data indicate that denitrification imparts the same site preference on N_2O whether it is performed by a nitrifier (nitrifier-denitrification) or traditional denitrifier (denitrification) (Table 1). Site preference imparted by denitrification was distinct from that associated with hydroxylamine oxidation (Sutka *et al.*, 2003) (Table 1). Furthermore, N_2O reduction during denitrification does not alter the site preference imposed on N_2O during nitrite reduction in agricultural and historically cultivated successional soil mesocosms (Fig. 2d,f). Our results and those of Sutka *et al.*, (2003) suggest that site preference distinguishes nitrification (Fig. 1, reaction A) from denitrification (Fig. 1, reactions B,C) in agricultural soils. In contrast to the agricultural soil mesocosms, changes in site preference during N_2O reduction in deciduous forest soil mesocosms (Fig. 2f) may reflect a difference soil microbial community structure attributed to land-use history (Buckley and Schmidt, 2001).

Approximately 80% of the current annual increase in atmospheric N_2O derives from agricultural soils (Veldkamp et al., 1998). Unlike other ecosystems, nitrification or denitrification can be directly managed in agricultural systems. Previously, without the means to distinguish the relative importance of nitrification from denitrification, we have not been able to manage soil microbial processes to mitigate N_2O flux. The ability to apportion sources of N_2O in agricultural soils with isotopomer site preference now provides this possibility and, thus, has profound implications for the management of global N_2O emissions.

APPENDICES

APPENDIX A

Methods

Site Description and Soil Collection

Approximately 4 kg of soil was collected by taking 20 cores from the upper 25 cm of soil from each of three different treatment plots from the replicated series of cropped and unmanaged ecosystems at the Kellogg Biological Station/Long Term Ecological Research main site (http://lter.kbs.msu.edu) in southwest Michigan. Treatments included conventional agricultural treatment (CAT), historically cultivated successional field (HCS) and a deciduous forest (DF). Conventional agricultural tillage treatment crop yields are equivalent to average yields for the USDA Central Region. A 4 mm sieve was used to homogenize soil. Aliquots were covered and allowed to air dry for ca. three weeks.

Mesocosm Construction

One hundred grams of dry soil was added to mesocosms (1 L glass Mason jars bearing lids fitted with butyl rubber septa) and packed to a volume of ca. 80 ml. Water was added to achieve ca. 85% water filled pore space (Bergsma *et al.*, 2002). However, heterogeneities in the soil resulted in differences in water filled pore space. We verified that N_2O production was not occurring by following the methods of Bergsma *et al.* (2002). Sealed jars were amended with 500 µl of pure N_2O at atmospheric pressure, delivered with a gas tight syringe (Hamilton).

Sample Collection and Analysis

500-µL headspace samples were taken and stored in vacutainers previously purged with pure N_2 and brought to atmospheric pressure. The gas samples were analyzed on a multi-collector Micromass Isoprime Mass Spectrometer interfaced with a continuous flow

Trace Gas Inlet System for separation and purification of N_2O. This mass spectrometer has been constructed with collectors to simultaneously measure the 5 masses of interest for N_2O isotopomers; 30, 31, 44, 45 and 46. The $\delta^{15}N$, $\delta^{18}O$ and $\delta^{15}N^{\alpha}$ values are obtained from the ratio of the 45:44, 46:44 and 31:30 ion beam ratios, respectively. Corrections are applied for the contribution of ^{17}O to masses 31 and 45 and for the minor rearrangement of ^{15}N between the α and β positions within the ion source (Brand 1995, Toyoda and Yoshida, 1999; Breninkmeijer and Röckmann, 2000; Sutka et al., 2002). The value of $\delta^{15}N^{\beta}$ is calculated given that $\delta^{15}N$ is the average of $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ Toyoda and Yoshida, 1999; Breninkmeijer and Röckmann, 2000).

***P. chlororaphis* Cultures**

Pseudomonas chlororaphis was cultured from a frozen stock (ATCC 43928) provided by J.M. Tiedje, Michigan State University and maintained on Tryptic Soy Broth (TSB; Difco) amended with 5 mM KNO_3. Concentrated cell suspensions made from starter cultures provided sufficient concentrations of N_2O for mass spectrometric analysis. These suspensions were derived by inoculating ten 50 mL serum bottles containing 20 mL of TSB medium with 0.1 mL of starter culture. After 48 hours of incubation at 22 °C cells were concentrated (centrifugation: 8,000 *g* for 10 min at 5 °C). The cell pellet was washed twice with 20 mL of 0.1 M pH 7.5 phosphate buffer containing 171 μM $CaCl_2$, and 78.7 μM $MgCl_2$ and resuspended in 20 mL of sterile TSB. For each replicate, a 25 mL butyl stoppered serum test tube (Bellco) was prepared with 3 mL of the concentrated cell suspension and 1 mL of a 0.01 M KNO_3 stock solution. The tubes were stoppered with a N_2 headspace and incubated at 22 °C. Nitrous oxide was sampled from the headspace approximately every 20 minutes using a 100 or 500 μL gas tight syringe. The

gas sample was immediately injected into a Trace Gas system (Micromass) interfaced to an Isoprime (Micromass) mass spectrometer.

APPENDIX B

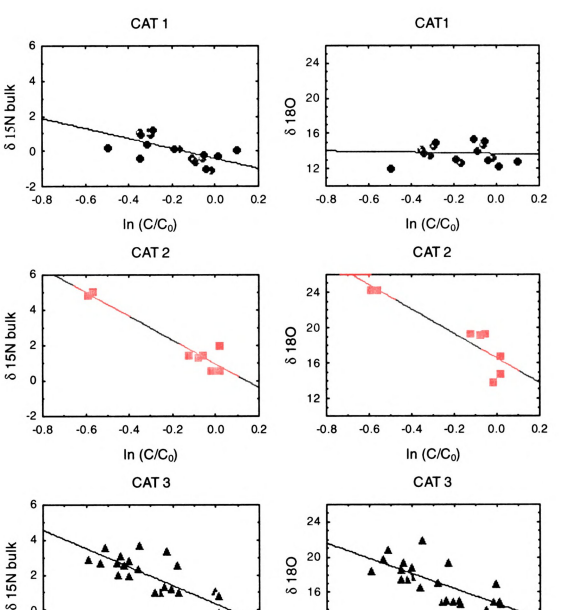

Figure 3 $\delta^{15}N$ and $\delta^{18}O$ vs. ln (C/C_0) for conventional agricultural treatment mesocosms. **a**, $r^2 = 0.4052$; r = -0.6366, p = 0.0045; y = -0.414880127 - 2.8579479*x **b**, $r^2 = 0.0035$; r = -0.0595, p = 0.8147; y = 13.6945294 - 0.36836952*x **c**, $r^2 = 0.9154$; r = -0.9568, p = 0.0002; y = 0.955740909 - 6.75726686*x **d**, $r^2 = 0.8137$; r = -0.9020, p = 0.0022; y = 16.5163472 - 13.8830931*x **e**, $r^2 = 0.6045$; r = -0.7775, p = 0.00001; y = 0.338244567 - 5.28180685*x **f**, $r^2 = 0.5114$; r = -0.7151, p = 0.0001; y = 14.6636102 - 8.63820581*x

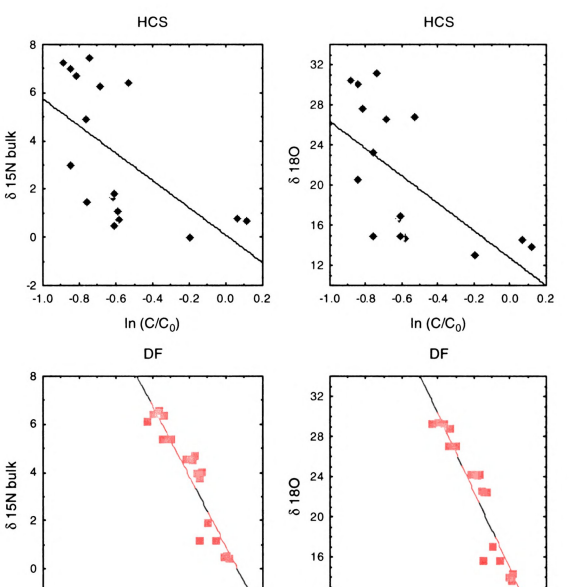

Figure 4 $\delta^{15}N$ and $\delta^{18}O$ vs. ln (C/C_0) for historically cultivated successional (HCS) and deciduous forest (DF) mesocosms. **a**, $r^2 = 0.3562$; r = -0.5969, p = 0.0114; y = 0.0803007579 - 5.67524563*x **b**, $r^2 = 0.3672$; r = -0.6060, p = 0.0099; y = 12.6993928 - 13.6296028*x **c**, $r^2 = 0.8590$; r = -0.9268, p = 0.00000003; y = 0.906057787 - 14.621457*x **d**, $r^2 = 0.8845$; r = -0.9405, p = 0.000000007; y = 14.7188548 - 38.6903078*x

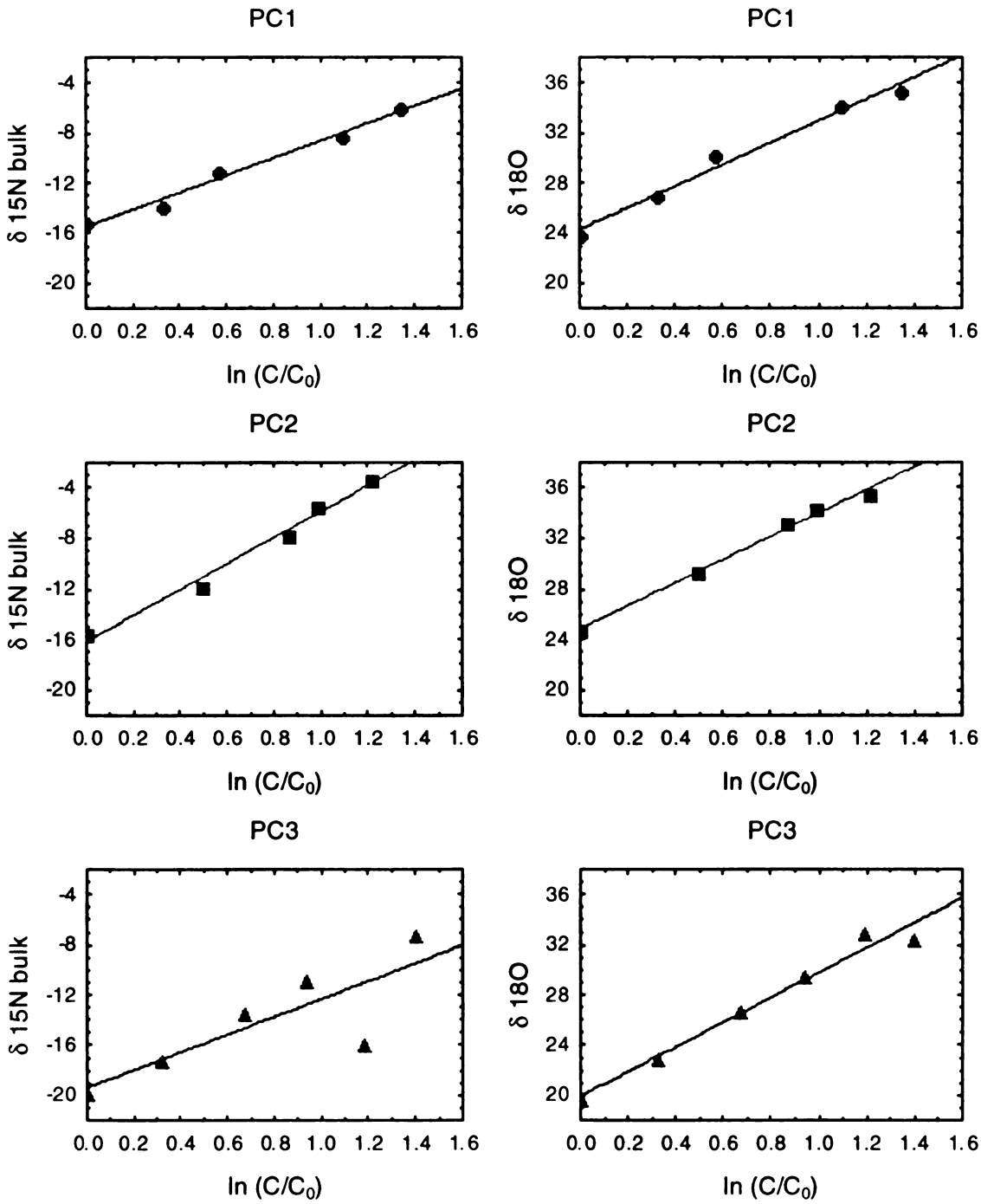

Figure 5 $\delta^{15}N$ and $\delta^{18}O$ vs. ln (C/C_0) for *Pseudomonas chlororaphis* (PC) cultures. **a**, $r^2 = 0.9838$; r = 0.9919, p = 0.0009; y = -15.5253953 + 6.89719754*x **b**, $r^2 = 0.9817$; r = 0.9908, p = 0.0011; y = 24.252223 + 8.71316266*x **c**, $r^2 = 0.9833$; r = 0.9916, p = 0.0009; y = -16.1674224 + 10.2834111*x **d**, $r^2 = 0.9920$; r = 0.9960, p = 0.0003; y = 24.8183474 + 9.17864406*x **e**, $r^2 = 0.6670$; r = 0.8167, p = 0.0473; y = -19.3910815 + 7.08615027*x **f**, $r^2 = 0.9721$; r = 0.9860, p = 0.0003; y = 19.9194665 + 9.89224398*x

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