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Evaluation of the N_2 flux approach for measuring sediment denitrification

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Abstract

Direct gas chromatographic measurement of denitrification rates via N_2 fluxes from aquatic sediments can avoid some of the artifacts and complexities associated with indirect approaches and tracer techniques. However, measurement protocols have typically been determined based upon initial results or previous studies. We present a process-level study and simulation model for evaluating and optimizing N_2 gas flux approaches in closed chamber incubations. Experimental manipulations and simulations of both artificial and natural sediments were used to conduct sensitivity analyses of key design parameters in N_2 flux measurements. Experimental results indicated that depletion of labile organic matter during the long incubations required by common protocols (for diffusive off-gassing of porewater N_2) may result in underestimates of denitrification rates in some systems. Simulations showed that the required incubation time was primarily a function of sediment thickness. The best approach found to minimize incubation time and reduce errors was to select the minimum sediment thickness necessary to include the entire depth distribution of nitrification–denitrification for a particular sediment system. Attempts to increase measurement sensitivity and shorten incubation times by reducing the headspace thickness to 1–2 cm generally cause denitrification to be underestimated by 3–13% for gas headspaces, and up to 80% for water headspaces. However, errors were negligible with gas and water headspace thicknesses of 10 cm and 15 cm, respectively. Anaerobic cores to control for non-denitrification N_2 fluxes shortened incubation time, but introduced artifacts in sediments with extensive macrofaunal irrigation.

Keywords: denitrification; sediments; methodology; simulation; models; diffusion

1. Introduction

Denitrification, the microbial reduction of nitrate (NO_3^-) to nitrogen gas (N_2), plays an important role in the N cycle, removing N from pools available for phototrophic uptake (for general reviews, see Seitzinger, 1988; Cornwell et al., 1999). Aquatic sediments have emerged as major sites of activity, accounting for as much as 85% of the global denitrification flux (calculated from: Bowden, 1986; Christensen et al., 1987; Middelburg et al., 1996). Accurate denitrification measures are essential to balance N budgets and to understand the effects of anthropogenic N enrichment (Valiela and Teal, 1979). However, measuring denitrification presents special problems, since N_2 production rates are small relative to background N_2 concentrations, while the other substrates and products of denitrification play roles in a variety of co-occurring microbial processes (Wijler and Delwiche, 1954).

In marine sediments, denitrification has been inferred from porewater NO_3^- profiles (Vanderborght and Billen, 1975) or deviations from the expected stoichiometries of N and P regeneration (Nixon et al., 1975; Joye et al.,

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1996; Giblin et al., 1997). Studies of the role of denitrification in aquatic sediments were aided by the adaptation of $^{15}NO_3^-$ tracer and acetylene block techniques developed in the soil sciences (Yoshinari and Knowles, 1976; van Kessel, 1977; Sørensen, 1978). However, these techniques were restricted to the measurement of denitrification of allochthonous water-column NO_3^-, while the denitrification of NO_3^- produced autochthonously by in situ remineralization and nitrification remained poorly understood (Jenkins and Kemp, 1984). A significant refinement of the ^{15}N isotope techniques (isotope pairing) measures both autochthonously and allochthonously-supported denitrification, but requires isotope ratio mass spectrometry, which is not available to many workers (Nielsen, 1992; Nielsen and Glud, 1996). However, techniques for direct measurement of sediment N_2 production by gas chromatography can permit simple and direct measurements of sediment denitrification using widely available instrumentation (Seitzinger et al., 1980; for reviews see: Seitzinger et al., 1993; van Luijn et al., 1996).

N_2 flux measurements are most easily made with intact sediment cores incubated in closed chambers under an N_2-free headspace, allowing the relatively small denitrification fluxes to be measured against this low background by gas chromatography. Total denitrification (supported by both autochthonous and allochthonous nitrate) can be measured in incubations with headspace water at in situ NO_3^- levels, whereas autochthonously-supported denitrification is measured with NO_3^--free headspaces. The technique is inexpensive and relatively simple to implement, and is preferable to the acetylene block technique for the assessment of sediment N cycling.

However, despite their advantages, the common N_2 flux protocols suffer from artifacts caused by the non-equilibrium conditions imposed by closed chambers and low-N_2 headspaces. In the field, sediment porewater N_2 pools are near equilibrium with atmospheric N_2, but under an N_2-free headspace, they support an initially large diffusive N_2 efflux that must be distinguished from denitrification-derived N_2 production. The underlying denitrification fluxes can be measured once the initially high porewater N_2 pools are depleted through diffusive "off-gassing". However, this long off-gassing preincubation (9–28 d: references in Table 1), may cause changes in important sediment characteristics such as macrofaunal irrigation, labile organic matter pools, and metabolite concentrations. The preincubation time can be shortened by instead controlling for diffusive porewater N_2 off-gassing by parallel chambers where coupled nitrification–denitrification is anaerobically suppressed (Nowicki, 1994). However, the potential anaerobic alteration of macrofaunal irrigation (and therefore of N_2 off-gassing rates) is still of concern (Kristensen and Blackburn, 1987).

Another potential artifact is the inhibition of sediment N_2 fluxes when N_2 accumulation in the chamber headspace causes sediment N_2 accumulation, reducing sediment–water fluxes (Conen and Smith, 2000). Conversely, the accumulation of regenerated NH_4^+ or NO_3^- in the headspace may stimulate denitrification rates (Seitzinger et al., 1993). Accumulations of metabolic products can be mitigated by periodic headspace renewal. However, this approach may introduce N_2 contamination or disturb sediment–water N_2 gradients. Incubations with ambient N_2 concentrations or continuous flows of headspace water address many of these problems, but the required precise gas chromatographic or mass spectrometric measurements make implementation more difficult (Devol, 1991; Kana et al., 1998; Risgaard-Petersen et al., 1998).

The importance of the errors introduced into denitrification measurements by the dynamics of N_2 production and diffusion can be assessed through modeling. Some workers have modeled diffusive N_2 fluxes in closed chambers in order to put constraints on sediment off-gassing times for specific systems (Nowicki, 1994; van Luijn et al., 1996), but a general treatment of the problem as it relates to shortening incubation times and reducing errors has not been attempted. Models of diffusion and denitrification in closed flux chambers differ in important respects from the diagenetic models developed by Berner (1980), and applied to porewater NH_4^+ and NO_3^- profiles in marine sediments (Vanderborght and Billen, 1975; Soetaert et al., 1996). For the purposes of studying the experimental artifacts associated with closed chamber N_2 measurements, organic particle fluxes to the sediments from the water column may be omitted, and the model needs to consider the production and diffusion of N_2 alone. Further, unlike the steady state, open systems modeled in the diagenetic equations, N_2 concentration profiles in sediments within closed flux chambers are not in equilibrium. Models have been developed for field chamber measurements of soil nitrous oxide fluxes (Matthias et al., 1978; Jury et al., 1982; Healy et al., 1996). However, these models of open-bottomed field chambers, unsaturated soils, and ambient headspace gas concentrations, cannot be directly applied to closed chamber incubations of aquatic sediments with low-N_2 headspaces.

In the present study, we experimentally investigated the effects of incubation time, sediment thickness, and periodic headspace water renewal on denitrification rates measured by the N_2 flux method. We also tested and experimentally validated a simulation model of N_2 production and diffusion in closed chambers with low N_2-headspaces. The model was used to conduct sensitivity analyses in order to optimize the key parameters of N_2 flux measurement protocols and potentially expand the utility of this method. In our analysis, we emphasize the importance of minimizing

Table 1
Experimental procedures for determining sediment denitrification rates by measurement of N_2 gas fluxes in closed flux chambers

Core diam.	Sediment depth	Headspace thickness		Off-gassing preincubation time[a]	Measurement incubation time (total)[b]	Chamber closure time[c]	Controls (off-gassing N_2 flux)	Reference
		Air	Water					
cm	cm	cm	cm	days	days	days		
Low-N_2 gas headspace[d]								
8.0	18–20	5–7	0	0	4 h	4 h	Formalin	Kaplan et al., 1979
7.8	4	1.3	3.2	13	30	1–2	None	Seitzinger et al., 1980
7.8	5	1.3	6.4	11	5–15	1–3	Irradiated/anaerobic	Seitzinger et al., 1984
7.8	7	1.5	6.4	10	14	1	None	Seitzinger and Nixon, 1985
6.8	5–6	6.5	5.1	28	28–56	3–7	Irradiated	Gardner et al., 1987
6.8	7	2.0	8.5	10	4–8	1–2	Anaerobic	Seitzinger, 1987
7.2	7	1.4	4.1	9–12	4–15	1–3	Formalin/anaerobic	Yoon and Brenner, 1992
6.8	6	2.0	21.3	10	4–8	1	Anaerobic	Seitzinger et al., 1993
8.0	5	1.4	15.9	2	5	3–5	Anaerobic	Nowicki, 1994; Nowicki et al., 1997
6.8	6	2.0	17.0	10	4–8	1	None	Seitzinger, 1994
6.8	7	1.8	4.1	9	10–15	2–3	Formalin/$HgCl_2$	Zimmerman and Benner, 1994
5.6	2–4	4.1	4–6	10	20	1	Irradiated/N-Serve	van Luijn et al., 1996
8.0	20	1.4	15.9	2	4–5	4–5	Anaerobic	Nowicki et al., 1999
5.6	2–4	4.1	4–6	10	30	1	Anaerobic	van Luijn et al., 1999
8	5 (soil)	1.0–1.4	0.0	0.08	7	1	Modeled	Watts and Seitzinger, 2000
8.8	8–12	6.6–10	6.6–10	3	4	7	Anaerobic	Hamersley and Howes, 2003; This study
6.8	6	6	0.0	0.5	0.042	0.042	None	Mander et al., 2003
Water-only headspace at ambient N_2[e]								
23[f]	nr	0.0	4–8	na	0.2–1.5	0.2–1.5	na	Devol, 1987, 1991; Devol and Christensen, 1993
15.6	30	0.0	15.9	na	0.17–0.5	0.17–0.5	na	Lamontagne and Valiela, 1995
56.0	9	0.0	1.0	na	≥ 2	1	na	Nowicki et al., 1999
33.9	nr	0.0	10.0	na	1.46	1.46	na	An and Joye, 2001
30	20.5	0.0	3.0	na	0.13–0.17	0.13–0.17	na	Lamontagne et al., 2002
21.5–30.5	nr	0.0	18.7–29.5	na	2–3	2–3	Calculated N_2 solubility	Tomaszek and Czerwieniec, 2003

nr = not reported, na = not applicable.
[a] In some studies, the precise off-gassing preincubation and incubation times were not explicit, and were estimated from the information given.
[b] Total measurement incubation time after off-gassing incubation.
[c] Duration of flux chamber closure between headspace renewal.
[d] Laboratory flux chambers except Kaplan et al., 1979.
[e] In situ flux chambers except Lamontagne and Valiela, 1995.
[f] Square dimensions.

incubation time by optimizing sediment depth and flux chamber dimensions, and evaluate the effectiveness of various protocols for determining denitrification N_2 fluxes.

2. Methods

2.1. Experimental measurements of denitrification

We conducted experiments to examine the effects on measured denitrification rates of (a) long incubation times (during which depletion of labile organic matter pools may lower rates or the accumulation of NH_4^+ or NO_3^- in overlying water may stimulate rates), and (b) the thickness of sediment cores. We measured coupled nitrification–denitrification rates as N_2 flux in closed aerobic flux chambers ($n = 2$) containing intact sediment cores, with parallel nitrate-free anaerobic incubations ($n = 2$) to control for diffusive N_2 off-gassing or N_2 contamination (Nowicki, 1994). Experiments were performed with sandy (porosity 0.53 mL cm^{-3}; C content 0.55 mol C cm^{-3}) and muddy (porosity 0.81 mL cm^{-3}; C content 1.2 mol C cm^{-3}) sediments from the unvegetated tidal channel (<1.3 m depth) of Mashapaquit Marsh, Cape Cod, Massachusetts, USA (method and study site described in Hamersley and Howes, 2003). For all experiments, we collected sediment cores (8–12 cm thick) directly with glass cylinders (8.8 cm diameter × 25 cm). The overlying water was replaced with 7–10 cm of filtered (0.22 μm) half-strength seawater, leaving a 7–10-cm gas headspace. We performed incubations with nitrate-free water (<2 μM) in order to separate sediment from water-column-supported denitrification (our sediments do not export NO_3^-: Hamersley and Howes, 2003). An externally driven magnetic stir bar (60 rpm) was fitted into the cylinder at the gas–water interface. The top and bottom of the flux chamber were sealed with large two butyl stoppers compressed onto the cylinder by a press.

The headspace water and gas were initially flushed with either 80% He/20% O_2 (aerobic chambers) or 100% He (anaerobic chambers) (1 L min^{-1} for 30 min). Aerobic and anaerobic chambers were then subjected to the same experimental procedures. The chambers were pressurized (1.3 atm) so that sample withdrawal or sediment O_2 consumption would not cause negative pressure-driven leaks, and held completely immersed in a water bath (at field temperatures) to minimize diffusion of atmospheric gas into the chamber. A valved port for water sampling was kept filled with water to minimize atmospheric contamination, and water withdrawn for sampling was replaced with He-sparged water. Gas samples (5 mL) were withdrawn through the top stopper with a He-flushed gas-tight locking syringe and a non-coring septum needle, and injected off-column for analysis on a Shimadzu GC-14A gas chromatograph equipped with a thermal conductivity detector and calibrated with certified standards (N_2, O_2, and CO_2). The flux chambers remained sealed throughout the incubation; gas measurements were made daily for the first week and less frequently during the second week, with simultaneous measurement of headspace pressure (Cole-Parmer digital gauge).

The effect of incubation time on denitrification rates was determined during 2-week incubations of sediments collected over annual cycles (Hamersley and Howes, 2003). Further experiments were made to determine the efficacy of two procedures designed to reduce the accumulation of NH_4^+ in the headspace. In one experiment (headspace water renewal), the chambers were opened daily and the headspace water was replaced (under Ar) with fresh half-strength seawater, and then resparged with either He/O_2 or He as before. Controls received the same treatment, except that the original headspace water was replaced in the chamber. In a second experiment (zeolite NH_4^+ adsorption), NH_4^+ concentrations were reduced without opening the chambers or removing headspace water in order to reduce the potential for N_2 contamination or disturbance of sediments. Mesh bags of zeolite pellets (6 mesh) were suspended in the headspace water of flux chambers to adsorb dissolved NH_4^+, whereas bags in control chambers contained only polyethylene disks. Headspace water samples collected daily were analyzed immediately for NH_4^+ by a colorimetric indophenol method (Scheiner, 1976). In a final experiment to evaluate the distribution of denitrification with depth in the sediment, denitrification rates were assessed in incubations with sediment cores of four thicknesses (2, 4, 6, and 10 cm).

2.2. Experimental calculations of N_2 flux

Gas flux rates across the sediment–water interface were calculated from the slopes of linear regressions of mass against time, ($n = 3$–5 measurements) (Table 2; Eq. (1)). Cumulative headspace mass (N_i; Eq. (2)) was determined from the headspace gas concentration (C_i) (Ideal Gas Law; Broeker and Peng, 1982), adjusted by the cumulative gas mass withdrawn during sampling (W_{i-1}: Eq. (3)) and the cumulative mass lost via leaks from the chamber (L_i: Eq. (4)). Leaks were calculated from the difference between the expected pressure ($P_{(e)i}$) and the measured pressure (P_i). The expected pressure was calculated from gas sample withdrawals and net changes in the partial pressures of O_2 and CO_2 (Eq. (5)). The volume (V) of the headspace was approximated as $V = V_{gas} + 0.012V_{water}$ (since 1 mL water contains dissolved N_2 equal to ca. 1.2% of 1 mL gas; Weiss, 1970). Denitrification N_2 fluxes were determined from the difference between N_2 fluxes in aerobic and

Table 2
Experimental calculation of denitrification N_2 flux

Equations:

$$F = A^{-1}\frac{\delta N}{\delta t} \quad (1)$$

$$N_i = \frac{P_i V C_i}{RT} + W_{i-1} + L_i \quad (2)$$

$$W_i = \sum_{t=0}^{i} \frac{S_i P_i C_i}{RT} \quad (3)$$

$$L_i = \sum_{t=0}^{i} \left(P_{(e)i} - P_i\right)\frac{V\left(C_i + C_{i-1}\right)}{2RT} \quad (4)$$

$$P_{(e)i} = P_0 - \frac{\sum_{t=0}^{i} S_i P_i}{V} - \left(P_{(O_2)0} - P_{(O_2)i}\right) - \left(P_{(CO_2)0} - P_{(CO_2)i}\right) \quad (5)$$

$$F_d = F_o - F_a \quad (6)$$

Symbols:

Symbol	Description	Units
A	Area of core	m^{-2}
C	Concentration of N_2 in headspace	mL mL^{-1}
F_d	Denitrification N_2 flux from sediments	mmol N m^{-2} d^{-1}
F_a	N_2 flux (anaerobic)	mmol N m^{-2} d^{-1}
F_o	N_2 flux (aerobic)	mmol N m^{-2} d^{-1}
i	Timepoint (numbered sequentially from 0)	none
L	Cumulative N_2 lost through leaks	mmol N
N	N_2 mass in headspace	mmol N
P	Pressure of headspace	atm
$P_{(e)}$	Expected pressure calculated from known losses	atm
$P_{(CO_2)}$	Partial pressure of CO_2 in headspace	atm
$P_{(O_2)}$	Partial pressure of O_2 in headspace	atm
R	Gas constant	atm cm^3 $mmol^{-1}$ $°K^{-1}$
S	Total volume of gas withdrawn for assay	cm^3
T	Temperature	°K
t	Time	d
V	Total volume of gas phase of headspace	cm^3
W	Cumulative N_2 mass withdrawn in gas sampling	mmol N

anaerobic chambers (Table 2, Eq. (6)). The N_2 fluxes in nitrate-free anaerobic chambers where denitrification was inhibited were used to control for the off-gassing diffusion of porewater N_2 into the low-N_2 headspace.

2.3. Simulation modeling of diffusion-driven N_2 fluxes

A simulation model was developed to allow optimization of experimental protocols, to minimize the preincubation time required for sediment off-gassing, and to minimize declines in sediment N_2 efflux resulting from N_2 accumulation in the chamber headspace and sediment during incubation. N_2 fluxes were simulated through multiple 1 cm thick sediment layers with an overlying headspace layer. Sensitivity analysis showed that finer divisions of the sediment column had no effect on modeled rates. Based on experimental evidence, denitrification was assigned to the top 2 cm of sediment alone (see Sections 3.2 and 4.4). No denitrification was ascribed to the filter-sterilized (0.22 μm) headspace water. The model functions as if the gas and water headspaces are in equilibrium, with the partial pressure of N_2 in water calculated from the Bunsen coefficient (Weiss, 1970). The net flux into sediment layer j (F_j) was calculated from Fick's Law (Berner, 1980) using the equation:

$$F_j = \phi D_a \frac{\left(C_{j-1} - C_j\right) + \left(C_{j+1} - C_j\right)}{d}, \quad (7)$$

where ϕ is the porosity of the sediments, D_a is the apparent diffusion coefficient, C_j is the concentration of N_2 in sediment layer j, and d is the thickness of each sediment layer. Fluxes into the headspace (F_H) were calculated from:

$$F_H = \phi D_a \frac{\left(C_0 - C_h \alpha_A\right)}{d/2} \quad (8)$$

where C_0 is the N_2 concentration in the surface sediment layer, C_h is the N_2 concentration of the headspace, and α_A is the Bunsen solubility coefficient (calculated for specific temperatures, salinities, and pressures). The model simulated an incubation time course, with an iteration length of 1 min (chosen after sensitivity analysis). At each iteration, a new N_2 concentration was calculated for each layer (sediment layer j or headspace):

$$C_i = C_{i-1} + \frac{\left(F_i + Dn_i\right)}{\phi V} \quad (9)$$

where for each layer, C_i, F_i, and Dn_i are the N_2 concentration, the N_2 flux, and the denitrification N_2 production, respectively, at time point i, and V is the volume.

The apparent diffusion coefficient (D_a) was determined by curve-fitting to experimental measures of N_2 fluxes in denitrification-free anaerobic sediments and combusted sand, and is a composite of molecular diffusion, sediment porosity and tortuosity, and advection by headspace turbulence (Boynton et al., 1981; Güss, 1998). The initial concentration of N_2 in the porewater was determined from the equilibrium headspace mass of N_2 in anaerobic, nitrate-free chambers (no denitrification), divided by the water volume of the sediment. Since the results of the model are independent

of the area of the sediments incubated, the term "headspace thickness" is used to describe the vertical component of headspace volume (Matthias et al., 1978). In systems with a water headspace significantly larger than the gas headspace, the effective gas headspace thickness (for comparison to our model results) can be approximated by adding ca. 1.2% of the water headspace thickness to the gas headspace thickness.

The time required for sediment off-gassing and the effect of diffusion processes on measured denitrification rates were calculated with the calibrated model for varying conditions of sediment thickness, porosity, and gas headspace thickness. For simulations of off-gassing preincubations, initial sediment N_2 concentration was set to simulated field N_2 concentrations (steady state, specified denitrification rate, infinite headspace at atmospheric N_2 levels). For simulations of measurement incubations (of preincubated sediments with reduced N_2 pools), the practice of most investigators was followed (initial headspace $N_2 = 0$ atm and sediment N_2 pools $= 0.01$ atm, i.e. Seitzinger, 1988) in order to separate off-gassing from other effects. The results were found to be only weakly dependent on temperature and salinity (which only affect the Bunsen coefficient, α_A), so conditions were held at 20 °C and 32 ppt for all simulations.

3. Results

In incubations of sediments with no nitrification–denitrification (mineral sand and in anaerobic natural sediments), N_2 efflux was initially high because of the large N_2 concentration gradient between sediment and the low-N_2 headspace (Fig. 1a). As sediment N_2 pools became depleted and headspace N_2 levels rose, the efflux slowed and stopped as the system reached equilibrium. In denitrifying sediments (aerobic chambers), the N_2 flux continued after the initial period of rapid N_2 off-gassing, due to the continuous input of new N_2 from denitrification (Fig. 1a). In the aerobic chamber, N_2 fluxes did not approach the actual denitrification rate until day 10 (Fig. 1b). However, by using nitrate-free anaerobic chambers to control for the background off-gassing flux, the actual denitrification rate could be determined by day 3 (Fig. 1b, difference).

3.1. Effect of long incubation times

In the tidal channel sediments of this study, O_2 consumption and denitrification rates usually declined when sediments were incubated for long (>2-week) periods (Fig. 2). In these metabolically active sediments, the O_2 consumption rate in the second week of incubation averaged 19% lower than that measured during the initial 4 days (Fig. 2a) (1 tailed t-test; $n = 39$; $P < 0.001$; range + 24% to −60%). O_2 consumption fell furthest in

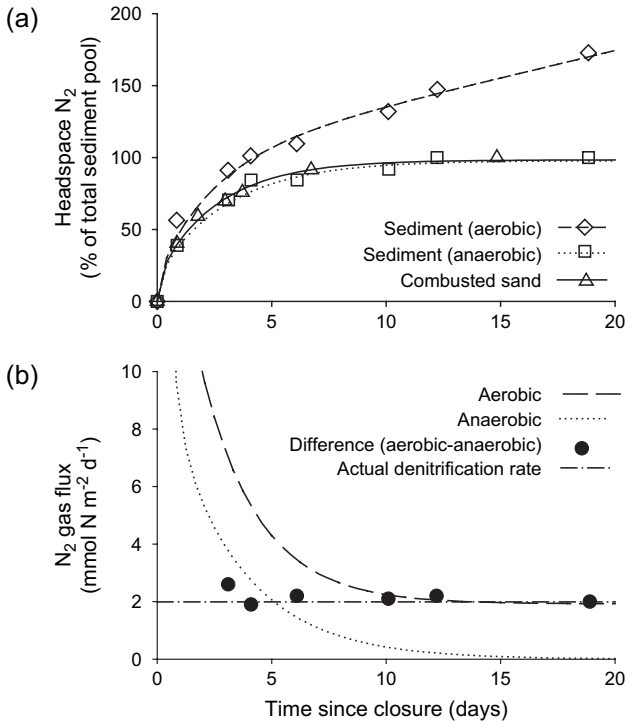

Fig. 1. (a) N_2 flux into low-N_2 headspace from combusted (carbon-free) mineral sand, and tidal channel sediments incubated under aerobic and anaerobic conditions. Modeled N_2 flux plotted as lines; points represent experimental data. N_2 effluxes from anaerobic sediment (nitrate-free incubation) and combusted sand are due to diffusion from sediment pools alone. Excess evolution of N_2 from aerobic sediment (>100%) is due to coupled nitrification–denitrification. All inputs to model are measured parameters except the apparent diffusion coefficient (D_a), selected to fit curve to points. (b) Coupled nitrification–denitrification calculated as the difference in N_2 fluxes between aerobic versus anaerobic incubations (points). Modeled N_2 flux from sediments (slope of curves in (a)) plotted as lines. Denitrification rate calculated using anaerobic controls (points) approaches actual rate faster than the aerobic chamber N_2 flux (dashed line). The first rate measured (0.9 d) is off scale.

sediments with high initial rates, with the relationship most pronounced in sandy sediments (Pearson product moment correlation coefficient $R = -0.82$; $n = 21$; $P < 0.001$). Similarly, the denitrification rate fell by an average of 20% during the second week of incubation when initial denitrification rates were higher than 2.5 mmol N m^{-2} d^{-1} (1 tailed t-test, $n = 21$; $P = 0.013$), with a maximum decline of 88% (Fig. 2b). In sandy sediments, the decline in denitrification was directly related to the initial denitrification rate ($R = -0.82$; $n = 13$; $P < 0.001$).

Procedures designed to reduce headspace NH_4^+ accumulation had no discernable effect on denitrification or O_2 consumption rates. Daily headspace renewal reduced mean NH_4^+ concentrations nearly 5-fold, from 260 μM to 58 μM, but they remained 10-fold higher than in situ (field) porewater concentrations (mean 6 μM) (Table 3). Nevertheless, mean denitrification and O_2 consumption rates with these treatments did not significantly differ from controls (2-tailed

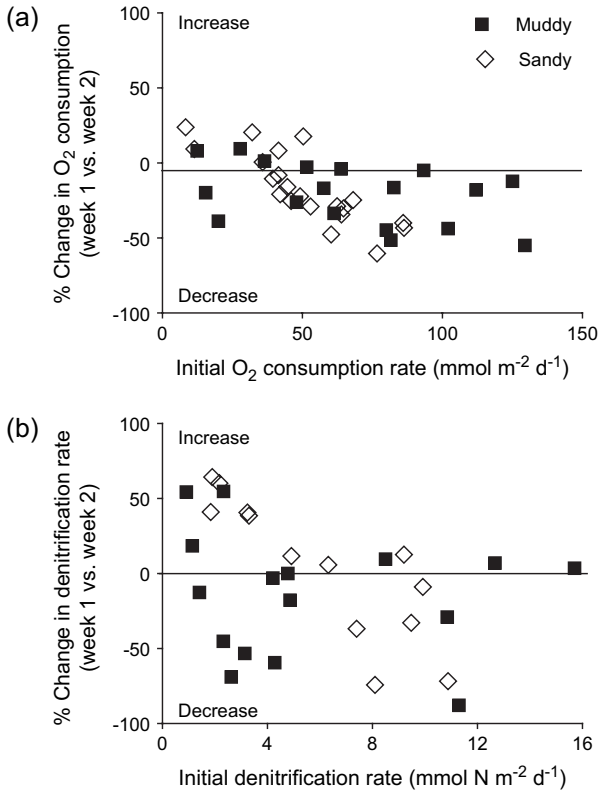

Fig. 2. Change in metabolic rates of tidal channel sediments during long (>2 week) incubations. Rates tend to decline at higher levels of (a) oxygen uptake and (b) denitrification. Each point represents the change in rate from 1 week to the next determined on a single sediment core (with duplicate anaerobic cores used to control for diffusive N_2 fluxes), with each week's rate determination based on 3–5 concentration measurements (R^2 typically > 0.95). Sediments were collected and incubated as part of an earlier study (Hamersley and Howes, 2003).

t-test: $P > 0.18$). Similarly, the zeolite treatment reduced mean NH_4^+ concentrations by 3-fold, from 440 μM to 140–170 μM, but O_2 consumption and denitrification rates remained unaffected (2-tailed *t*-test: $P > 0.18$).

3.2. Depth distribution of denitrification

No significant relationship was found between the thickness of incubated sediment (2, 4, 6, or 10 cm) and O_2 consumption (normalized to sediment carbon content) (linear regression: $R^2 = 0.004$; $n = 8$). Denitrification was similarly poorly correlated with sediment thickness (linear regression: $R^2 = 0.28$; $n = 8$), indicating that both O_2 consumption and denitrification in these sediments were active predominately in the upper 2 cm sediment layer.

3.3. Model verification

The model simulated the high initial N_2 flux both in tidal channel sediments and in combusted sand held in either aerobic and anaerobic flux chambers, as well as the slowing of flux as the porewater N_2 pool became depleted (Fig. 1a). All model parameters were set to values derived from the experiment except the apparent N_2 diffusion coefficient (D_a), which was selected to obtain the best fit of the model to the data. Although the relative importance of the factors contributing to D_a were not determined, for the purposes of this study it was only necessary to determine their composite effect on diffusion, and to show that the D_a so calculated could be applied consistently to the sediment systems in question. D_a was determined to be 0.00012 $cm^2 s^{-1}$ in our natural sediments ($n = 8$), and 0.00017 $cm^2 s^{-1}$ in combusted sand ($n = 4$), about 5 times the molecular diffusion coefficient (Broeker and Peng, 1982). Similar differences between D_a and molecular diffusion coefficients have been observed by other researchers for both artificial and marine sediments, and may be due to interfacial water exchange, irregular sediment surface characteristics, and variable effective sediment porosity (Boynton et al., 1981; Devol and Christensen, 1993; Garban et al., 1995; Güss, 1998). D_a was the same in both aerobic and anaerobic natural sediments, indicating a minor role for macrofaunal irrigation. Oxidized macrofaunal burrows and tubes extending below the visual redox discontinuity of ca. 0.5 cm were rare, since macrofauna in these sediments were primarily amphipods known to irrigate only the top 1 cm of sediment (unpublished data). The higher D_a in the artificial sediment may be due to the absence of the layer of fine organic material at the sediment surface found in our sandy and muddy sediments, which may inhibit interfacial water exchanges.

Table 3
Effects of two experimental manipulations of headspace NH_4^+ concentrations on sediment metabolism

	NH_4^+ (μM)	Denitrification[a] (mmol $m^{-2} d^{-1}$)	O_2 consumption[a] (mmol $m^{-2} d^{-1}$)
Experiment A: headspace water renewal			
Control (unchanged)	260 (41)	3.4 (1.5)	69 (4)
Flushed daily	58 (7)	3.8 (1.9)	70 (7)
Ratio	4.5	0.9	1.0
Experiment B: zeolite adsorption of NH_4^+			
Control (muddy)	440 (10)	2.2 (0.4)	81 (16)
Zeolite (muddy)	140 (40)	2.5 (0.1)	84 (4)
Ratio	3.1	0.9	1.0
Control (sandy)	440 (70)	1.7 (0.5)	106 (2)
Zeolite (sandy)	170 (30)	1.3 (0.8)	100 (36)
Ratio	2.6	1.3	1.1

Values are mean ± SE ($n = 2$).
[a] No significant differences between treatments (2-tailed *t*-test; $P > 0.18$).

4. Discussion

Incubating sediments in chambers with low headspace N_2 levels can facilitate the detection of N_2 fluxes for determination of denitrification rates. However,

biological and physical non-equilibrium conditions during the long preincubations required for off-gassing of the sediment N_2 pool can alter metabolic rates. A simple model can be used to minimize errors by optimizing the key parameters of sediment and headspace gas thickness.

4.1. Off-gassing preincubation times

Although Nowicki (1994) found no change in denitrification rates from days 3–5 to days 7–11 in shallow bay sediments, Gardner et al. (1987) observed declining N_2 flux rates well into the fourth week of incubation in lake sediments. In our sediments, denitrification and O_2 consumption tended to decline during long preincubations similar to those needed to accommodate sediment off-gassing (2 weeks) (Fig. 2). Over time, labile sediment carbon may become depleted, leaving more recalcitrant carbon pools (Kristensen and Blackburn, 1987). Thus, minimizing core holding and incubation times may be most important in sediments with metabolisms supported by carbon pools with high turnover rates.

Clearly, procedures that reduce the incubation time of metabolic measurements will help to avoid artifacts and improve accuracy. N_2 flux simulation provides an approach for optimizing protocols specific to a sediment system to reduce incubation time. For purposes of denitrification rate measurement, it is sufficient that the sediment off-gassing should proceed to the point where the diffusive flux is an acceptably low percentage of the denitrification flux. Longer off-gassing incubations are required for accurate measurement of small denitrification fluxes because the diffusive flux must be allowed to attenuate to a lower level relative to the denitrification flux (Figs. 3a and b). The primary factors controlling off-gassing, and hence the preincubation time required before denitrification rates can be obtained, are the size and dimensionality of the sediment N_2 pool (Fig. 3). Thin sediment cores off-gas quickly both because of their low N_2 content and shorter diffusion path. High porosity (ϕ) sediments contain more water and more N_2 and therefore require longer off-gassing preincubations (Figs. 3c and d). Our model's predicted off-gassing times were similar to

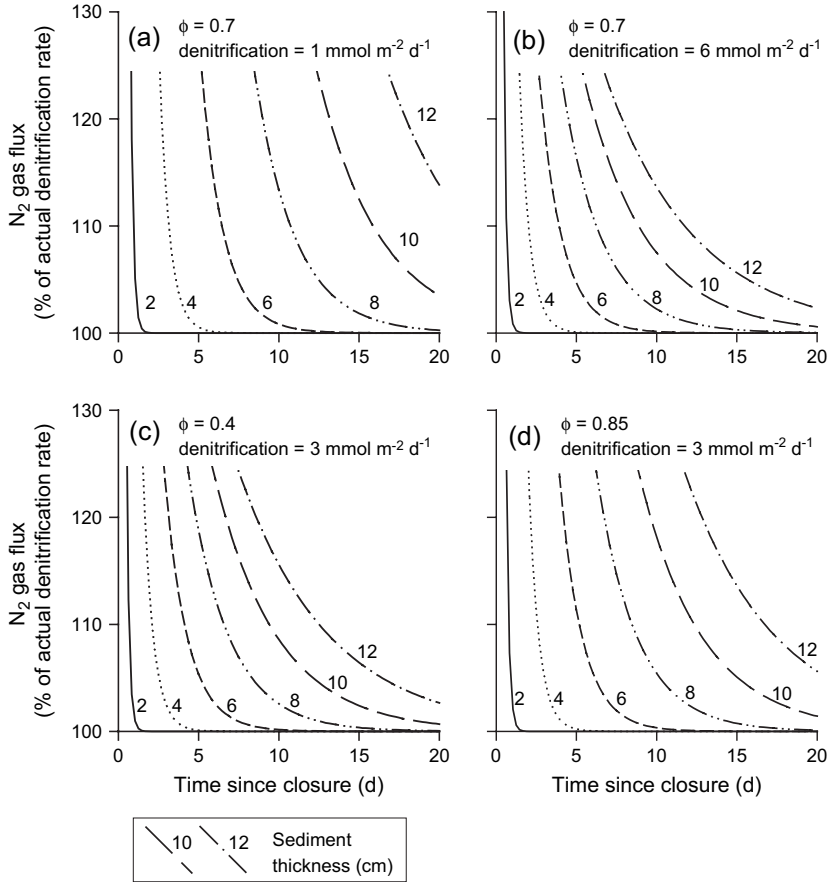

Fig. 3. Modeled off-gassing of porewater N_2 from sediments in aerobic nitrate-free flux chambers. (a) and (b) N_2 flux from sediments of various thicknesses at two denitrification rates. N_2 fluxes in sediments with higher denitrification rates approach actual rate faster since diffusive N_2 fluxes are a smaller proportion of denitrification rate. (c) and (d) N_2 flux from sediments of various thicknesses as a percentage of actual denitrification rate at two sediment porosities representing typical sandy ($\phi = 0.4$) and muddy ($\phi = 0.85$) sediments. Flux decreases over time because of depletion of sediment N_2 pools. Sediments of high porosity and thickness take longer to off-gas because of higher sediment N_2 content.

empirical measurements (i.e. 10 d for a 5–7 cm thick sediment: references in Table 1).

Nitrate-free anaerobic flux chambers can be used to shorten incubation times by controlling for diffusion-driven N_2 fluxes (Nowicki, 1994). In our experiments with anaerobic controls, modeling showed that the denitrification rate could be calculated ca. 5 days earlier than aerobic flux chambers alone would have allowed (Fig. 1b). However, this protocol introduces its own sources of error. In the field, denitrifying sediments are enriched in porewater N_2 above atmospheric equilibrium levels. When the sediments are incubated under a low-N_2 headspace, the N_2 flux out of the sediments is initially equal for both aerobic and anaerobic chambers, since the porewater in both cores is equally enriched in N_2. However, since no further N_2 is generated by coupled nitrification–denitrification within the anaerobic sediments, the difference in N_2 flux rates between the two treatments subsequently increases from zero until it approaches the denitrification N_2 flux (Fig. 4). This delay is longer for thicker sediments because of the greater N_2 storage capacity of the sediments and the longer path for excess N_2 diffusing from the anaerobic sediment core.

4.2. Sediment storage of N_2 resulting from headspace N_2 accumulation

Another approach to reducing incubation time has been to use very small headspaces above sediments, which increase the rate at which headspace N_2 concentrations increase and reduce the interval required to obtain measurable changes. However, the rapid accumulation of N_2 with very small headspaces causes increasing amounts of N_2 to be stored in the sediment, and measured N_2 fluxes to decrease. This effect has been extensively studied for N_2O and other soil fluxes (for examples see Healy et al., 1996; Conen and Smith, 2000), but to our knowledge there is little awareness of the problem as applied to marine denitrification

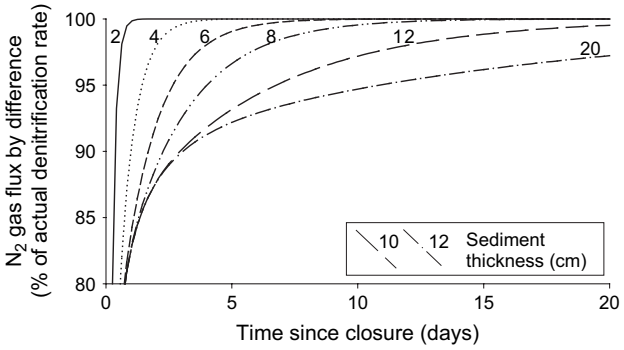

Fig. 4. Modeled denitrification N_2 flux (determined with anaerobic control incubations) from sediments of various thicknesses. Calculated fluxes from thicker sediments are lower because of retention of denitrification-derived N_2 in sediment porewater.

measurements. The same effect holds true for flux measurements of dissolved ions (Devol, 1987; Devol and Christensen, 1993). This underestimation of denitrification increases during periods of chamber closure, but is only of concern with very small headspace thicknesses (1 or 2 cm, see Table 1), where the underestimation can exceed 10% (Fig. 5a and b). The largest underestimations of denitrification rates (up to 13%; Fig. 5b) occur when thick sediments are combined with thin headspaces. Similarly, when using anaerobic chambers to control for diffusive N_2 fluxes, denitrification-derived N_2 in aerobic chambers causes headspace N_2 concentrations to rise faster than in the nitrate-free anaerobic chambers, inhibiting the aerobic flux relative to the anaerobic flux (results not shown).

Many workers have measured N_2 gas fluxes into water headspaces with ambient N_2 levels so that the small denitrification fluxes measurably affect headspace N_2 concentrations (references in Table 1). This procedure avoids the necessity for sediment off-gassing, and can be employed in the field for in situ measurements. However, a number of problems related to the difficulty of measuring the small relative changes in headspace N_2 have been noted, including large errors (Lamontagne and Valiela, 1995) and depletion of headspace O_2 before significant N_2 accumulation can occur (Devol, 1991; Lamontagne and Valiela, 1995; Nowicki et al., 1999). Further, the rapid increase in the partial pressure of N_2 in water-only headspaces results in underestimations of denitrification (resulting from sediment storage) much more severe than those noted with gas headspaces (up to 80%; Fig. 5c). Even with short incubations and/or large headspaces, the errors introduced by common experimental protocols (modeled from data in Table 1) may range from 8 to 40%. Similar calculations support the observation that denitrification rates in in situ benthic flux chambers (1 cm water headspace) were $>50\%$ lower than rates measured in the same sediments with gas headspaces (Nowicki et al., 1999).

During long periods of chamber closure, pressure losses that result from O_2 consumption (while much of the CO_2 produced remains in dissolved forms) as well as from leaks and sample withdrawals, can also significantly affect calculations of headspace N_2 mass (Eqs. (2)–(5)). During incubations of tidal sediment cores (this study), these losses were up to 0.03 atm d^{-1}, which could have resulted in an overestimation of the denitrification flux by 15% over the course of a 5-day incubation if uncorrected. Regular measurements of headspace pressure may therefore be required to ensure accurate measurements of N_2 gas flux.

4.3. Disturbances of N_2 flux by headspace renewal

During long periods of chamber closure, measurements of N_2 flux may be affected by headspace pressure

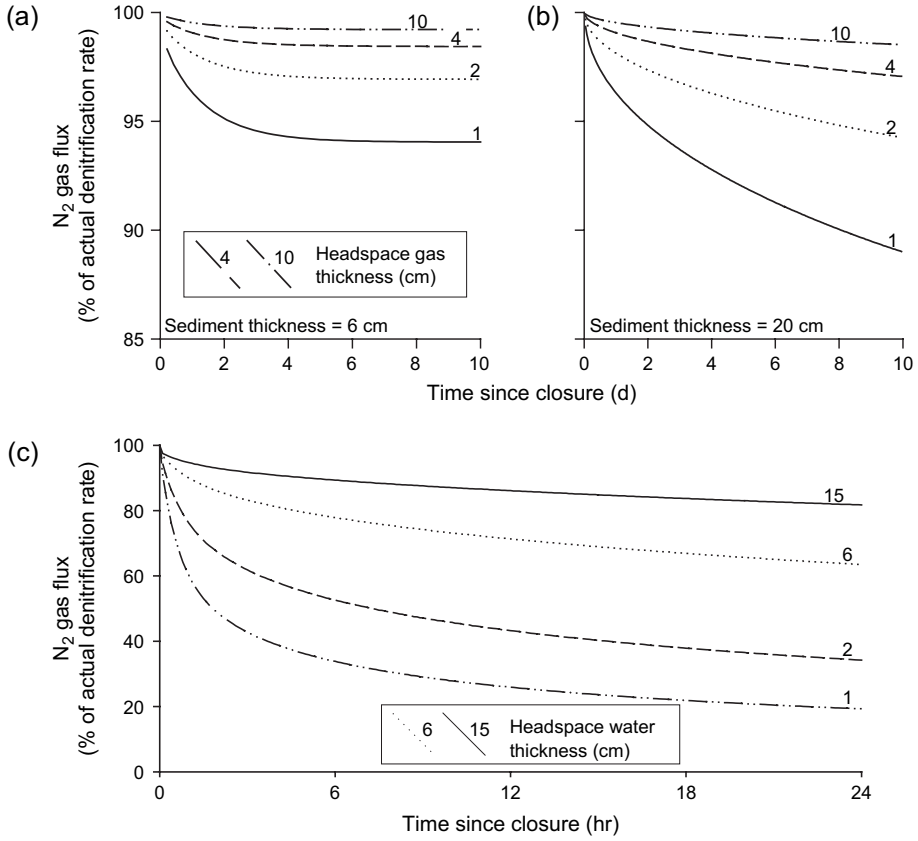

Fig. 5. Modeled underestimation of denitrification rates in N_2 flux measurements resulting from headspace N_2 accumulation and sediment N_2 storage. The effect is largest for small headspaces. At higher sediment porosities and thicknesses, sediment storage of N_2 and inhibition of N_2 flux are greater. (a) and (b) Underestimation of denitrification in an aerobic flux chamber at two sediment thicknesses. (c) Underestimation of denitrification in chambers with water-filled headspaces. Note difference in *y*-axis scale. Sediment thickness has no effect since efflux is against ambient N_2 background and so requires no off-gassing.

changes, depletion of O_2, or accumulation of NH_4^+, NO_3^-, or N_2. To control these errors, many investigators renew the headspace water and gas every 1–2 d to restore the headspace to initial conditions (see Table 1, compare "Chamber closure time" to "Measurement incubation time"). However, this approach requires up to twice as many gas concentration measurements, because an extra initial measurement must be made after each headspace renewal. In addition, headspace renewal introduces the possibility of atmospheric N_2 contamination (Nowicki, 1994; van Luijn et al., 1996), as well as changes in the sediment–water N_2 concentration gradient that can cause perturbations in N_2 fluxes. In simulations, after 2 d of closed chamber incubation, N_2 gas fluxes remained close to true denitrification fluxes (Fig. 6). After the headspace was renewed, a new low-N_2 gas phase was established. The subsequent increase in the concentration gradient across the sediment–water interface caused a sharp spike in N_2 flux, followed by a gradual return to pre-renewal conditions. As expected, the fluctuations were greatest with thin headspaces, because the accumulation of N_2 in the headspace and sediments was also greater. N_2 flux spikes after headspace renewal have been previously reported, but were attributed to contamination by atmospheric N_2 (Nowicki, 1994). In one case they were compensated by waiting for an additional day before beginning measurements, however, the correct approach would be to integrate both the spike and the subsequent inhibition, since neglecting the spike will result in an underestimate of denitrification.

Nevertheless, in the experiments of the present study, neither daily headspace renewal nor zeolite adsorption was successful in reducing NH_4^+ concentrations to in situ levels. Further, neither treatment affected measured denitrification rates compared with maintaining the chamber closed throughout, making their utility questionable (Table 3). Headspace renewal may not reduce headspace NH_4^+ concentrations sufficiently to affect coupled nitrification–denitrification, but instead may only introduce artifacts. On the other hand, long periods of chamber closure increase headspace N_2 accumulation, NO_3^- accumulation, headspace O_2 depletion, and pressure changes. In our experiments, we found that the entire incubation could be done without opening the chamber or renewing the headspace. Careful experimental design can overcome the problems of long chamber

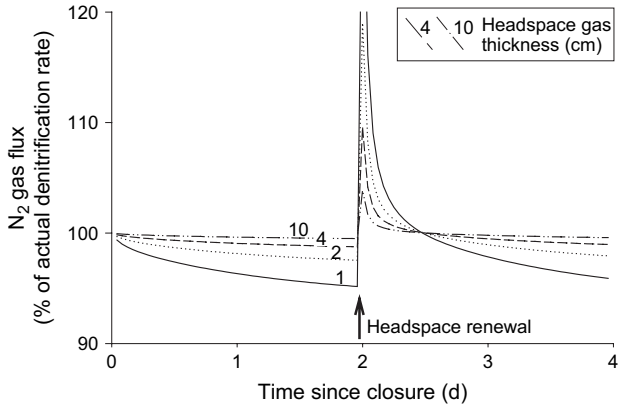

Fig. 6. Modeled N_2 fluxes during headspace renewal. After an initial off-gassing preincubation, the chamber was closed (days 0–2), headspace was renewed (arrow), and chamber was closed again (days 2–4; as in Seitzinger, 1988 and others). A sharp increase in N_2 flux results because the sediment–water concentration gradient increases when headspace gas is replaced by low-N_2 gas. Fluctuations in N_2 flux are larger for small headspaces.

closures, but headspace flushing may still be necessary if NH_4^+ or NO_3^- accumulation is known to stimulate denitrification rates (Seitzinger et al., 1993).

4.4. Sediment thickness and depth distribution of denitrification

Sediment depth is the key experimental parameter that has the greatest effect on off-gassing preincubation times (Figs. 3 and 4) and (along with headspace thickness) the inhibition of N_2 efflux by headspace N_2 accumulation and sediment storage (Fig. 5). However, the chosen thickness must also reflect the depth distribution of denitrification. The depth distribution of denitrification depends on the penetration of O_2 (redox structure of sediment column) and the availability of labile organic C, but sediment thicknesses of 2–20 cm have been used by various researchers (Table 1). From the deep sea to the continental shelves, heterotrophic activity is typically restricted to the top 15 cm of the sediments (Soetaert et al., 1996); however in estuarine and salt marsh sediments, most C respiration takes place in the upper 2 cm (Jørgensen, 1982; Howes et al., 1985). In the present study, denitrification and O_2 consumption rates in 2 cm thick cores were not significantly different from those of thicker cores. Similarly, in model simulations, the best fit to data from our tidal channel sediments was obtained with denitrification distributed across the top 2 cm of sediment (data not shown). Although the minimum thickness of sediment required for an accurate denitrification measurement will vary among sediment systems, the thicknesses typically used (2–7 cm; Table 1) may appropriately depth-integrate sediment processes while shortening off-gassing incubation times.

4.5. Macrofaunal sediment irrigation

The use of anaerobic chambers to control for off-gassing N_2 fluxes assumes that the only difference in N_2 flux between aerobic and nitrate-free anaerobic chambers is N_2 derived from denitrification. However, in sediments with significant bioirrigation, if macrofauna are killed or inactivated by anoxia, the reduction in sediment irrigation will decrease the flux of porewater N_2 (Aller, 1980; Kristensen and Blackburn, 1987). The reduced off-gassing correction will then result in an overestimate of denitrification. We modeled this effect by increasing the apparent diffusion coefficient (D_a) within aerobic sediments relative to anaerobic sediments to simulate the effects of macrofaunal irrigation. When D_a is the same for both the aerobic and the anaerobic core (no macrofaunal irrigation), the calculated denitrification flux initially is lower than the actual denitrification rate (as in Fig. 4). In contrast, when D_a in the aerobic core is increased by a factor of 1.5, simulating macrofaunal sediment irrigation, the initial calculated flux initially exceeds the actual denitrification rate (Fig. 7a). In thin sediments, the larger D_a means

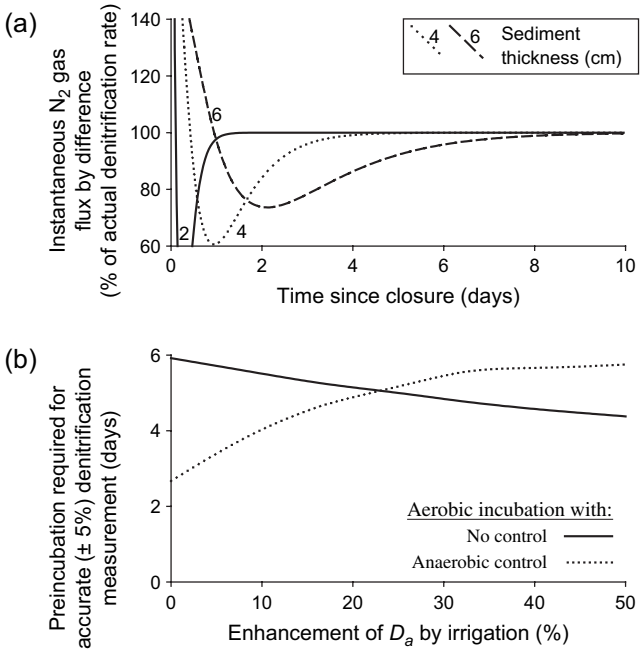

Fig. 7. Modeled effects of macrofaunal irrigation on denitrification N_2 fluxes calculated from parallel aerobic–anaerobic incubations. (a) N_2 flux for a variety of sediment thicknesses when irrigation enhancement of porewater N_2 flux in the aerobic chamber was 1.5 times the anaerobic apparent diffusion coefficient (D_a) (compare to Fig. 4a but note difference in scale). (b) Effect of irrigation-enhanced D_a on the off-gassing preincubation time required for accurate ($\pm 5\%$) denitrification measurement. In this example, the use of an anaerobic control increased the preincubation time required for an accurate denitrification measurement when irrigation-enhancement of D_a was $>20\%$. This figure serves as an example only, since the shape of such a figure is highly dependent on factors like porosity, denitrification rate, and diffusion coefficient.

that the initial porewater N_2 is quickly exhausted, causing N_2 flux rates to rapidly drop below those of the anaerobic core, where diffusive off-gassing of porewater N_2 pools continues. This effect is transient; eventually N_2 pools in the anaerobic core are exhausted as well, and the calculated flux returns toward the actual denitrification value. In thicker sediments with deep macrofaunal irrigation, porewater N_2 pools are not quickly exhausted, even with the higher D_a. The calculated denitrification flux remains elevated above the actual level for many days, and may extend the required off-gassing incubation time beyond that required for aerobic flux chambers alone (Fig. 7b). The effect on measured denitrification rates of macrofaunal enhancement of D_a varies with the degree of enhancement. The precise macrofaunal enhancement of D_a will not likely be known, but with sediments that are deeply bioturbated (such as by polychaetes), the use of anaerobic controls may not have any advantage over uncontrolled incubations.

N_2 gas flux measurements offer a simple, yet accurate way to directly measure denitrification rates in flooded sediments with inexpensive equipment. Although some of the potential errors are small, the sum of multiple sources of error could be significant. However, artifacts and errors can be avoided with proper preparation. Although the results of this study can be generalized to many sediment types, model studies of particular sediment systems may be required in order to determine the appropriate off-gassing incubations and understand potential errors.

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