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Coupled nitrification and N₂ gas production as a cryptic process in oxic riverbeds

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The coupling between nitrification and N₂ gas production to recycle ammonia back to the atmosphere is a key step in the nitrogen cycle that has been researched widely. An assumption for such research is that the products of nitrification (nitrite or nitrate) mix freely in the environment before reduction to N₂ gas. Here we show, in oxic riverbeds, that the pattern of N₂ gas production from ammonia deviates by ~3- to 16-fold from that predicted for denitrification or anammox involving nitrite or nitrate as free porewater intermediates. Rather, the patterns match that for a coupling through a cryptic pool, isolated from the porewater. A cryptic pool challenges our understanding of a key step in the nitrogen cycle and masks our ability to distinguish between sources of N₂ gas that 20 years' research has sought to identify. Our reasoning suggests a new pathway or a new type of coupling between known pathways in the nitrogen cycle.

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Nitrogen is a key bio-element for life on Earth, integral to proteins and the very DNA that tells life what to do. A vast reservoir of nitrogen resides in the atmosphere as N₂ gas, unavailable to the majority of life until being fixed by either biological or anthropogenic nitrogen fixation. Life’s organically-bound nitrogen in turn decays to ammonia following excretion or death. To complete the cycle, first nitrogen must be oxidised to nitrite or nitrate which can then be reduced back to atmospheric N₂ gas. This process of ammonia oxidation—known as nitrification—typically occurs in two stages carried out by specialised aerobic chemooautotrophic ammonia- and nitrite-oxidising microbes, for example, in soils, sediments, freshwater, or marine ecosystems (Eqs. 1 and 2, respectively):

\[
2\text{NH}_4^+ + 3\text{O}_2 \rightarrow 2\text{NO}_2^- + 2\text{H}_2\text{O} + 4\text{H}^+ \quad \Delta G^\circ = -270 \text{ kJ (per NH}_4^+) \quad (1)
\]

\[
2\text{NO}_2^- + \text{O}_2 \rightarrow 2\text{NO}_3^- \quad \Delta G^\circ = -79 \text{ kJ (per NO}_3^-) \quad (2)
\]

Nitrite and nitrate can then be reduced to N₂ gas either alone, in a phylogenetically widespread form of microbial anaerobic respiration termed denitrification (Eq. 3a, b) or, in combination with ammonia, in a phylogenetically narrow respiratory pathway termed anaerobic ammonia oxidation, namely anammox (Eq. 4).

\[
2\text{NO}_3^- + 10\text{e}^- + 12\text{H}^+ \rightarrow 2\text{N}_2 + 6\text{H}_2\text{O} \quad \Delta G^\circ = -360 \text{ kJ (per NO}_3^-) \quad (3a)
\]

\[
2\text{NO}_2^- + 6\text{e}^- + 8\text{H}^+ \rightarrow 2\text{N}_2 + 4\text{H}_2\text{O} \quad \Delta G^\circ = -282 \text{ kJ (per NO}_3^-) \quad (3b)
\]

In addition, smaller amounts of N can be returned to the atmosphere as nitrous oxide (N₂O) but we do not consider those further here. Combinations of Eqs. (1) to (4) recycle ammonia back into atmospheric N₂ gas and this coupling between aerobic nitrification and anaerobic N₂ gas production is a key concept in the nitrogen cycle, controlling ecosystem production and the abundance of life on Earth.

Besides the now accepted reactions described in Eqs. (1) to (4), Broda’s original thermodynamic predictions that drove the quest for anammox also included the potential for complete aerobic ammonia oxidation to N₂ gas—that, to the best of our knowledge—has yet to be observed in nature:

\[
4\text{NH}_4^+ + 3\text{O}_2 \rightarrow 2\text{N}_2 + 6\text{H}_2\text{O} + 4\text{H}^+ \quad \Delta G^\circ = -316 \text{ kJ (per NH}_4^+) \quad (5)
\]

In estuarine or coastal sea sediments, combinations of recognised aerobic and anaerobic metabolisms (Eqs. 1 to 4) buffer the flux of terrestrial nitrogen out to sea and are considered to be physically divided between theoxic and anoxic sediment layers—albeit by only a few tenths of millimetres. In rivers, nitrate and nitrite borne from aerobic nitrification (Eqs. 1 and 2), in either the surrounding catchment soils or the riverbed itself, can be transported over large distances (1–100 km) before some 47 Tg N per year is removed from the fluvial network as N₂ gas. Regardless of the setting, the important point to appreciate here is that the products of aerobic nitrification (e.g., nitrate and nitrite) are assumed to be free to mix with any existing nitrate and nitrite in the surrounding porewater before they are subsequently metabolised, anaerobically, to N₂ gas. That is, there is—in effect—only one pool of nitrate and nitrite awaiting reduction to N₂ gas regardless of their origins. Indeed, this concept of free mixing between substrates lies at the very heart of the common ¹⁵N isotope pairing techniques used to disentangle and quantify the recycling of nitrogen in sediments that are major sources of N₂ gas on Earth.

Most research into the coupling between aerobic nitrification and anaerobic N₂ gas production in sediments has studied the two separately using eitheroxic or anoxic incubations, respectively, but now work including oxygen is increasing. Previously we demonstrated thatoxic (~30% to 100% of air-saturation for oxygen) gravel and sandy riverbed sediments harbour a coupling between aerobic nitrification and, seemingly, anaerobic N₂ gas production with that production being attributed to a combination of denitrification and anammox. We now show that the pattern of N₂ gas production from ammonia in these oxic riverbeds violates the prevailing concept that coupled nitrification and N₂ gas production is a two-step process with free nitrite or nitrate as intermediates. Not only does this challenge our understanding of a key coupling in the nitrogen cycle but it also masks our ability to distinguish between denitrification and anammox as sources of N₂ gas. Indeed, it may actually suggest a new pathway or at least a new type of coupling between known pathways in the nitrogen cycle.

### Results and discussion

N₂ gas production is independent from porewater nitrite or nitrate. Following on from our original work on nitrification and putative anaerobic N₂ gas production in oxic riverbeds, we wanted to explore further how these two processes are coupled. We began by collecting sediment from four rivers—two each of predominantly gravel and sand and then extended our sampling to a total of twelve rivers (Supplementary Figure 1 and Supplementary Table 1). We added ¹⁵N-ammonia to oxic sediment microcosms (see Methods) to trace the coupling between nitrification and N₂ gas production both with and without the inhibitor of aerobic nitrification, allylthiourea (~80 µM ATU in the porewater, Treatments 1 & 2, Table 1 and Methods) that does not inhibit denitrification or anammox. As before, we measured...
the immediate production of 15N-N2 gas that was stopped by inhibiting the first step (Eq. 1) of aerobic 15N-ammonia oxidation with ATU (Fig. 1a, Table 1). The coupling between aerobic ammonia oxidation and N2 gas production was clearly strong, however it was not complete. For example, across the twelve rivers, approximately 60% (Fig. 1b) of the oxidised 15N-ammonia tracer was recovered from the porewater as 15NO3−, i.e., as either 15N-nitrite (Eq. 1) or the final product of nitrification, 15N-nitrate (Eq. 2) e.g., 15NO3− is the sum of 15NO2− and 15N2O.

The presence of 15N-ammonia and 15N-NO3− together in the porewater generates two 15N-labelled substrate pools. The fraction of the pool labelled with 15N is termed FA for ammonia (NH3) and FN for NO3− (Eqs. 10 and 11 in Methods). Theoretically, combinations of Eqs. (1) to (4) can draw on these two substrate pools (FA and FN) to produce both the single-15N-labelled, 29N2 gas (e.g., 14N, 15N) and the double-15N-labelled, 30N2 gas (e.g., 15N, 15N) which we illustrate schematically in Fig. 2a. Note that denitrification can draw on NO3− as either NO2− or NO− but anammox is solely fuelled by NO2−. The published and accepted mathematical framework21 (See derivation of equations in Supplementary Note 1) tells us that the fraction of 15N-labelling in each of the substrate pools (FA and FN) must influence the ratio of 29N2 to 30N2 (here termed R) and the overall fraction of 15N in the N2 gas produced e.g., the overall blend of 29N2, 29N2 and 30N2 (here termed FNO2)21,22. While complex, the accepted framework also tells us that so long as we know what fraction of each component part (FA, FN and FNO2) is labelled with 15N, then we can still calculate how the N2 gas is produced e.g., by anammox or denitrification and understand the nature of this key coupling in the nitrogen cycle21,22.

We tested the validity of this accepted mathematical framework by changing the fraction of porewater NO3− labelled with 15N (FN) and looking for how this influenced the ratio of 29N2 to 30N2 produced (R). First we directly decreased FN by adding 14N-nitrite to dilute the 15N-nitrite accumulating in the porewater from the oxidation of 15N-ammonia (Treatments 3 and 4, Table 1). Surprisingly, diluting FN had no discernible effect on the values for R produced in the two sets of incubations (Fig. 3b. 2.32, 95% CI 2.01 to 2.64 versus 2.43, 95% CI 2.12 to 2.74, see Table 2 and Supplementary Table 2 for 29N2 and 30N2 production). We then repeated our incubations with just 15NH4+ (with and without ATU, Treatments 1 and 2) across twelve rivers and measured a similar value for R of 1.8 (95% CI, 1.41 to 2.20, Fig. 3c) at an even lower value for FA (see Table 1). Note, we might have expected R to increase steeply as an inverse function of FN (Supplementary Figure 3). We can predict what values for R we might have expected if our N2 gas had been produced by either denitrification or anammox fuelled by porewater nitrite and/or ammonia, respectively (Fig. 2a) and compare them to our
measured $R$ values to highlight the disparity between the two (Fig. 3b, c and Table 2):

Predicted $R$ for denitrification,

$$\text{Predicted } R \text{ for denitrification} = \frac{2 \times F_N \times (1 - F_N)}{F_N^2} \quad (6)$$

Predicted $R$ for anammox,

$$\text{Predicted } R \text{ for anammox} = \left( \frac{1}{F_N} - 1 \right) + \left( \frac{1}{F_A} - 1 \right) \quad (7)$$

Our measured $R$ values were too low to be explained by either denitrification or anammox fuelled by porewater $N$-fuelled by porewater $N$ and/or $A$, and even a mixture of these two processes couldn’t produce such low values for $R$ on average. This consistent disparity between our measured and predicted $R$, according to the accepted model, along with the constancy in $R$, despite differences in $F_N$ (Table 2), strongly implies that porewater $N$ had little influence on the $N$-labelling of the porewater $N$ and/or $A$.
N₂ gas produced from the oxidation of ¹⁵N-ammonia. Further, in an analogous set of incubations where we added ¹⁵N-nitrite instead of ¹⁵N-ammonia, we measured no consistent production of ¹⁵N-N₂ gas (Treatments 5 & 6 Table 1 and Methods). Hence, nitrogen for N₂ formation was not drawn primarily from the porewater NO⁻₃ pool (Fig. 2a). Instead, we propose that any N₂ producing pathways draw from a cryptic nitrogen pool (Fig. 2b) with ¹⁵N-labelled fraction, F_{Ncry}, instead of the familiar porewater pool with ¹⁵N-labelled fraction, F_{Npw}. Indeed, if we invoke a cryptic pool by making the ¹⁵N-labelling of FN the same as ¹⁵N-ammonia in the porewater Fₙ, in Eqs. (6) and (7) and thereby force denitrification and/or anammox to draw on that F_{Ncry} pool, then the predicted R values come closer to our measured R values (R cryptic, Fig. 3c and Table 2).

N₂ is produced from ammonia through a cryptic intermediate. We can use both the accepted⁵¹ and a new mathematical framework to more formally justify our proposal for a cryptic intermediate pool or process. First, we define the proportion of N₂ gas coming from anammox relative to denitrification that is conventionally known as ra¹⁵. ra has to lie between 0 and 1 and, in the accepted framework, is expressed as a function of porewater F₉ and Fₙ and R according to²¹ (See Eq. (1) to (14) in Supplementary Note 1):

\[ ra = \frac{(R + 2) \times F_n^R - 2 \times F_n}{(F_N - F_n) \times [(R + 2) \times F_n - 1]} \tag{8} \]

In the accepted framework, however, our measured values for R and porewater F₉ and Fₙ generate nonsensical estimates for ra (e.g., -6.06 to 3.03, not > 0 < 1). Just as for Fig. 3c, we cannot apportion N₂ gas between anammox and denitrification drawing on porewater FN and/or FA – in the conventional sense – to produce our measured R values (Fig. 2a). Next, we define the ¹⁵N-labelling of the N₂ gas produced (FN₂), which, like ra (Eq. 8), also has to lie between 0 and 1 (See Eq. (1) to (14) in Supplementary Note 1).

\[ F_{N2} = F_N - \frac{R \times F_N + 2 \times (F_N - 1)}{2 \times (R + 2 - \frac{1}{F_N})} \tag{9} \]

Unlike ra, which is expressed as a function of both porewater F₉ and Fₙ, only Fₙ is required to parameterise F_{Ncry} (Eq. 9 cf. Eq. 8). That is not to say that F₉ has no influence on F_{N2}, as it be either the F_{Ncry} or F_{Npw} pools—must result from ammonia oxidation drawing on F₉ (Fig. 2).

We can then use solutions to Eqs. (8) and (9) between > 0 < 1 to define a solution space for any combination of F₉, F₉, and realistic values for R (See Supplementary Figure 3 for R as a function of ¹⁵N atom %) that we can visualise as a 3D ribbon (Fig. 4). The height of the ribbon is defined in terms of FN₂ and is depicted here for our average value for F₉ of 0.51 (Table 1 and see Supplementary Fig. 4 for F₉ at 0.1 and 0.9). Overall, the ribbon is very narrow and where FA = FN there are no solutions and this singularity appears as a gap in the ribbon. If F_{Ncry} is isolated and derives solely from the oxidation of FA (Fig. 2b), then F_{Ncry} has to equal FA. Further, if F_{N2} is only dependent on FN (Eq. 9) and this FN is equivalent to F_{Ncry}, then our calculated values for F_{N2}—plotted as functions of our measured values for R and FA (where F_{Ncry} = FA)—should fall near the gap in the ribbon where FN equals FA. This is indeed what we observe and especially for the better parameterised 12 river estimate (Fig. 4). In contrast, if we again force denitrification to be the only source of N₂, and calculate F_{N2} assuming that Fₙ = F_{Npw} (Fig. 2a), then the points fall away from our measured R values. Hence, in the presence of ¹⁵N-ammonia and oxygen, our measured R values only make sense if we assume F_{Ncry} = FA (Fig. 2b i.e., the porewater nitrite pool essentially represents the left-overs of the cryptic transformations during which N₂ is produced.

Internal NO₃⁻ cycling or a novel pathway or organism. We propose that the coupling between ammonia oxidation and N₂ gas production in oxic, permeable riverbed sediments involves a cryptic intermediate pool derived solely from the oxidation of ammonia that remains isolated from the porewater prior to the production of N₂ gas. In one scenario, a cryptic pool, similar to the porewater NO⁻₃ pool, is fed by the oxidation of ammonia to NO⁻₃, or possibly NO (ref. 3,23,24), through nitrification. The pathway from F_{Ncry} to the production of N₂ gas, however, branches off before that NO⁻₃ mixes with the ambient porewater NO⁻₃ (Fig. 2b) and would require internal NO⁻₃ cycling. Internal NO⁻₃ cycling is recognised as a potential source of interference for ¹⁵N isotope tracer studies in the ocean²⁵,²⁶ and is known in the consortia of ammonia oxidisers and anammox bacteria in wastewater CANON reactors (Complete Autotrophic Nitrogen removal Over Nitrite. Figure 2b, reactions 1 & 4) – though the actual mechanism in nature remains unknown.

Alternatively, some aerobic ammonia oxidising bacteria first produce nitrite (reaction 1) that they then reduce to N₂O gas in a process known as nitrifier-denitrification²⁵. Known nitrifier-denitrifier bacteria, however, lack a canonical N₂O-reductase (NOS, nosZ) to reduce N₂O to N₂ gas, so are not currently recognised as complete denitrifiers (reaction 7, Fig. 2b). Nitrosoyan, a soluble red Cu protein isolated from Nitrosomas europaea²⁸, is recognised as a plausible substitute to canonical N₂O-reductase that could enable complete nitrifier-denitrification to N₂ gas³. Our data enable us to test this hypothesis. For example, we know that ¹⁵NO⁻₃ from the initial oxidation of NH₄⁺ exchanges with the porewater (reaction 1, Figs. 1b and 2a) and we would expect, therefore, that ¹⁵NO⁻₃ added to the porewater would be available to any nitrifying-denitrifying bacteria. We have, however, already shown that adding ¹⁵NO⁻₃ to the porewater resulted in no consistent production of N₂ gas (Treatments 5 & 6, Table 1) i.e., N₂ gas production is dependent on the initial oxidation of ¹⁵N-ammonia. This fact, along with the clear discrepancy between the measured and predicted scenarios involving porewater NO⁻₃ (Figs. 3b, 3c & 4) make it hard to reconcile our N₂ gas production with either nitrifier-denitrification or canonical denitrification (reactions 3a, 3b & 7, Fig. 2).

Finally, it is theoretically possible for ammonia to be completely oxidised by oxygen to N₂ gas (equation 5) within a single, unknown organism. Such a reaction offers the simplest explanation for our results, with their strong dependency on aerobic ammonia oxidation and lack of influence from external porewater nitrite. Regardless of the actual pathway that produces the N₂ gas (Fig. 2b), an isolated cryptic intermediate pool has to have the same ¹⁵N-labelling of the ammonia pool (F_{Ncry} = FA). As a consequence of this equality, we can no longer distinguish between sources of N₂ gas, be it a denitrification-like pathway reductively combining N from an oxidised cryptic pool, an anammox-like process drawing on ammonia and cryptic N, or complete ammonia oxidation, as they would all produce ¹⁵N₂ and ³⁶N₂ at the same ratio (Fig. 2b where R is equal for each process).

Our observations challenge the current understanding of a key coupling in the nitrogen cycle in permeable, oxic riverbed sediments that may also apply to other biomes where the oxidation of ammonia is tightly coupled to the production of N₂ gas, such as continental shelf-sediments³⁰,³¹ and groundwater aquifers¹⁷. Whether it transpires that our cryptic coupling is mediated by a novel organism or, as of yet, a masked combination of known players in the nitrogen cycle remains to be resolved.
Methods

Study sites and sediment sampling. We began by collecting sediment samples from four rivers which we subsequently widened to a total of twelve rivers in southern England, UK, between October 2015 and May 2016 (Supplementary Figure 1 and Supplementary Table 1). Among them, the Rivers Lambourn, Darent, Wyllye, Rib, Pant, Stour (1) and Stour (2) have chalk-based, permeable gravel-dominated riverbeds, while the Rivers Marden, Hammer, Medway, Broadstone, Wylye, Rib, Pant, Stour (1) and Stour (2) have chalk-based, permeable gravel-dominated riverbeds, as described elsewhere. At each river, surface sediments (<5 cm) were collected from different locations using Perspex corers (13-cm × 9-cm internal diameter, 827 mL pool (~50%) to enable quantification of N2 gas to the initial aerobic oxidation of ammonia, an additional set of slurries were injected with 100 µl of 14 mM 15NH4Cl, Sigma-Aldrich) to generate the experiments described below.

Aerobic ammonia oxidation in oxic sediment slurries. 15N-NH4+ oxidation experiments were carried out with sediments first from four rivers (the rivers Lambourn, Wyllye, Marden, and Hammer) and then eleven more. In a standard aerobic application of 15N isotope pairing techniques, ambient porewater nitrite, nitrate, and any residual oxygen are removed by pre-incubating the anoxic sediment slurries for 12 h to 24 h before adding any 15N-tracers. Here this was not possible as we were measuring the aerobic oxidation of NH4+ and so to avoid contamination from the high background 14NO3− (14NO3− + 14NO2−), which is typical for these rivers, instead we used nitrite- and nitrate-free synthetic river water (0.12 g/l NaHCO3, 0.04 g/l KHCO3, 0.07 g/l MgSO47H2O, 0.09 g/l CaCl22H2O, pH = 7) to make the sediment slurries as before.

Oxic slurries were prepared by adding approximately 3 g sediment (~0.75 ml of porewater) and 2.7 ml air-saturated synthetic river water into 12 ml gas-tight vials (Exetainer, Labco), leaving an approximate 6 ml headspace of air which is equivalent to ~58 µmol O2 per prepared vial. We know from previous incubations with similar sediments from 28 rivers37 respiration rates to be ~187 nmol O2 g−1 h−1, on average (±64.3, 95%, C.I.), that would consume ~12% of the total oxygen during a 12 h incubation. In addition, we also checked oxygen over time using a microelectrode (50 µm, Uniensi) in parallel sets of scaled-up slurries (120 mL with the same ratio of sediment to water to headspace) for two rivers and found comparatively little consumption as before39 and see example in Supplementary Figure 2.

To trace the oxidation of ammonia to N2 gas, the prepared oxic slurry vials were then sealed and injected with 100 µl of 14 mM 15NH4Cl stock-solutions (98 atom% 15N, Sigma-Aldrich) to generate final porewater concentrations of ~390 µM 15NH4+. This high 15N concentration ensured sufficient labelling of the ammonia mono-oxygenase inhibitor allylthiourea (ATU), to give final porewater concentrations of ~390 µM 15NH4+ and ~80 µM ATU. While we have shown previously that 80 µM ATU inhibits aerobic ammonia oxidation in gravel and sandy riverbed sediments35, higher concentrations maybe required in other settings38. All of the oxic slurry vials were then incubated on a shaker (120 rpm, Stuart SSL1) for up to 12 h (Table 1, Treatments 1 and 2) in a temperature-controlled room at 12 °C. Incubations amended with just 15NH4+ were terminated at 0 h, 0.5 h, 1 h, 3 h, 4.5 h, 6 h, 9 h, and 12 h while those amended with both 15NH4+ and ATU were terminated at 0 h, 3 h, 6 h, and 12 h by injecting 100 µl of formaldehyde (38%, w/v) through the vial septa. All vials were then stored upside down prior to quantification of 29N2 and 30N2 by mass-spectrometry and R is then simply 29N2/30N2 (see below).

In addition to measuring the production of 29N2 and 30N2 gases (R), the fraction of 15N in the inorganic nitrogen porewater pools (Fp for ammonia and FN

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Fig. 4 Orientations of the solution space ribbon with both measured and predicted values for R. Here we present all data in just one solution space for the average fraction of 15N in the ammonia pool (Fp) of 0.51 and combinations of Eq. (8) (Fp,2) and 9 (ra) both yielding values between 0 < r < 1. R is the ratio of 29N2 to 30N2 and Fp and Fp,2 the fraction of 15N in the NO3− and N2 gas pools, respectively. To plot Fp,2 for each of our measured values of R we have to assume that Fp equals Fp measured in the porewater. In the solution space, there are no solutions where Fp = Fp (i.e., 0.51) and this singularity appears as a gap in the ribbon. Despite measurable changes in porewater Fp, the average values for both the 4-river and 12-river study appear near to each other and the gap where Fp = Fp. Note that the better parameterised 12-river average touches the gap and by inference, Fp ≈ Fp,2 (Fig. 2b). Denitrfication fuelled by porewater NO3− predicts values away from our measured values for R. Note, for the single predicted denitrification R values we use the median Fp values below.
for NO$_3^-$ e.g., NO$_3^-$ plus NO$_2^-$) needed to be quantified too (see Eqs. 6 to 9). To avoid any potential interference from formaldehyde, on the analysis of the inorganic nitrogen species, a parallel set of $^{15}$NH$_4^+$ amended slurries was prepared solely for nutrient analyses. At each time point (as above for $N_2$ gas analysis), vials were injected with 20 µL of 1.6 M NaOH to preserve nitrite before being frozen at $-20$ °C. Samples were defrosted and centrifuged at 1200 rpm for 10 min and the collected supernatant analysed (see below).

Manipulating the degree of $^{15}$N-labelling in the porewater NO$_2^-$ pool ($F_{SO}$ as $F_{SO}^{15}$). In typical anoxic sediment slurry incubations used to quantify $N_2$ gas production from denitrification and anammox, the fraction of porewater substrate labelled with $^{15}$N ($F_{SO}$ or $F_{SO}^{15}$) influences the ratio of $^{15}$NO$_2^-$ to $^{15}$NO$_3^-$ produced. To characterise the influence of porewater NO$_2^-$ on the coupling between $^{15}$NH$_4^+$ oxidation and $^{15}$N-N$_2$ production in our sediment slurries, we manipulated the fraction of porewater NO$_2^-$ labelled with $^{15}$N. Oxidic sediment slurries from the first four riverbeds were injected (100 µl) with combinations of stock-solutions of 14 mM $^{15}$NH$_4^+$ and 840 µM $^{15}$NO$_2^-$ or just 14 mM $^{15}$NH$_4^+$ and both with or without 2.8 mM ATU. This generated final porewater concentrations of ~390 µM $^{15}$NH$_4^+$, ~24 µM $^{15}$NO$_2^-$ or ~80 µM ATU and the prepared vials were then incubated on a shaker as above (see Table 1, Treatments 3 and 4). As above, oxide slurries vials were sacrificed at different time points for $^{15}$N$_2$ gas analysis and with a parallel set of $^{15}$NH$_4^+$ or $^{15}$NH$_4^+$ plus NO$_2^-$ amended slurries solely for nutrient analyses.

To further test the dependency of $N_2$ gas production on the initial oxidation of $^{15}$N-ammonia, we also performed a set of analogous incubations with sediments from the first four rivers with $^{15}$NO$_2^-$ (Table 1, Treatments 5 and 6). Here everything was the same (amount of sediment, with or without ATU, incubation times, oxygen etc.) except the $^{15}$N-labelling was added with nitrate rather than ammonia (as above) to final concentrations of ~390 µM $^{15}$NH$_4^+$ and ~24 µM $^{15}$NO$_2^-$ (98 atom% $^{15}$N, Sigma-Aldrich). If active, we would have expected $N_2$ gas production from reactions 3b and 4.

Analytical methods. Headspace of the oxide slurry samples were analysed for $^{15}$N-$N_2$ using a continuous-flow isotope ratio mass spectrometer (Sercon 20–22, UK) as described elsewhere. The mass spectrometer has a sensitivity of 0.1% $^{15}$N which here translates to approximately 0.1 mmol $^{15}$N-$N_2$ g$^{-1}$ dry sediment. To determine porewater $F_{SO}$ (NO$_2^-$ or NO$_3^-$ below) the concentration of $^{15}$NH$_4^+$ in the $^{15}$N$_2$-treated slurries was measured, the preserved supernatants were diluted and 3 ml of sample transferred into a new 3 ml gas-tight vial (Exetainer, Labco), the vial capped and a 0.5 ml helium headspace (BOC) added. Samples were injected with 100 µl of sulfamic acid (4 mM in 4 M HCl) and placed on a shaker (120 rpm, Stuart SSL1) overnight to reduce $^{15}$NO$_2^-$ to $^{15}$N$_2$-N$_2$ and the headspaces subsequently analysed for $^{15}$N$_2$ by gas chromatography (NCMS 2, NA Instruments). Then we added 0.125 µl of tin-cups, reweighed, and combusted at 1000 °C in an integrated elemental analyser and a 0.5 ml helium headspace (BOC) added. Samples were injected with 100 µl of 15NH$_4^+$ stock-solution to $N_2$ gas analysis. Before and after the addition of 15NH$_4^+$, the supernatant analysed (see below).

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Author contributions

M.T. and L.O. conceived the study and L.O. performed all of the experiments and B.T. formulated the mathematical framework. L.O. and M.T. analysed the data and M.T. and B.T. drafted the manuscript. All authors commented on and revised the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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