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Characterization of the key pathways of dissimilatory nitrate reduction and their response to complex organic substrates in hyporheic sediments

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Abstract

Laboratory incubations with river-bed sediment collected from riffles and pools were used to quantify potential pathways of dissimilatory nitrate reduction in the hyporheic zone of a groundwater-fed river. Sediments collected from between 5-cm and 86-cm depth in the bed of the River Leith, Cumbria, United Kingdom, were incubated with a suite of ^{15}N-labeled substrates ($^{15}NO_3^-$, $^{15}NH_4^+$, and $^{14}NO_3^-$) to quantify nitrate reduction via denitrification, dissimilatory nitrate reduction to ammonium (DNRA), and anaerobic ammonium oxidation (anammox). Denitrification was the dominant pathway of dissimilatory nitrate reduction in the hyporheic sediments, although recovery of ^{15}N from the ammonium pool indicated that DNRA was also active. The potential for anammox was confirmed by the production of $^{29}N_2$ during the $^{15}NH_4^+$ and $^{14}NO_3^-$ incubation, but it was much smaller than denitrification. Potential rates of denitrification were highest in shallow sediments and decayed exponentially with depth thereafter. There were clear differences in denitrification activity between riffle and pool sediments. After the production of ^{15}N-N_2 had stabilized, we added a spike of bacteriological peptone to determine the effect of complex organic substrates on denitrification potential. The potential rate of denitrification increased uniformly at all sediment depths but the total amount of denitrification fueled by the organic substrates decreased markedly with depth, from 90% in the shallow sediments to 30% in the deepest sediments. In addition, a considerable fraction of the $^{15}NO_3^-$ could not be accounted for, which suggested that up to 87% of it had been assimilated in the deepest sediments.

The hyporheic zone is a key area of biogeochemical activity between the surface water and groundwater of streams and rivers (Boulton et al. 2010). Here exchanges of dissolved and particulate organic matter, oxygen, and nutrients drive the cycling of the key biotic macronutrients (C, H, N, P, S), in turn, sustaining the productivity of the sediment strata in this 'dynamic ecotone' at or beneath the river bed (Gilbert et al. 1990). The contribution that biogeochemical activity within the hyporheic zone makes to the river as a whole is, in turn, governed by the balance between surface and subsurface flow and the intensity of subsurface processes (Findlay 1995).

One particular biogeochemical process that has received a great deal of attention is denitrification: the biological conversion of bioavailable nitrate ($NO_3^- + NO_2^-$ or NO_x^-) into nitrous oxide (N_2O) and, predominantly, inert, dinitrogen gas (N_2). Denitrification is most commonly coupled to the oxidation of organic carbon and is dependent upon the absence of oxygen, although denitrification can occur within anoxic microsites in seemingly well-oxygenated sediments (Triska et al. 1993; Baker et al. 2000). In excess, nitrogen can contribute to eutrophication of aquatic ecosystems; therefore, its removal in the hyporheic zone has clear ecological significance (Mulholland et al. 2009). The hyporheic zone, however, can function as either a nitrogen source or sink depending upon the form of nitrogen present, availability of reactive substrates, and prevailing oxygen status of the subsurface sediments (Storey et al. 2004). Much research has been devoted to understanding patterns of nitrate production and consumption within hyporheic zones across a variety of river systems (Holmes et al. 1996; Duff and Triska 2000; Fischer et al. 2005). Recently, though, the focus has shifted to the more applied perspective of the potential role of the hyporheic zone in attenuating anthropogenically derived nitrate at the interface of groundwater and surface-water exchange (Mulholland et al. 2008, 2009; Rivett et al. 2008).

Despite the increased interest in nitrogen removal by hyporheic sediments, most research has focused on measuring denitrification in the upper few centimeters of the river bed, rather than at depth (Stelzer et al. 2011). This may be due to the assumption that subsurface metabolism can be limited by the availability of organic carbon and denitrification will be concentrated toward the surface where both electron donors and acceptors are likely to be most abundant (Holmes et al. 1996; Baker et al. 2000), but may also reflect the logistical challenges of recovering sediment at depth, especially in armored river beds (Storey et al. 2004). A few studies, however, have demonstrated considerable potential for hyporheic denitrification at 25 cm below the river-bed surface (Lefebvre et al. 2004; Fischer

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et al. 2005; Stelzer et al. 2011). More interestingly perhaps, from a depth-gradient perspective, is the differing biogeochemical response of sediment strata to simple organic substrates, suggesting that the fate of nitrate and dissolved organic carbon within the hyporheic zone could depend upon where in the subsurface the final point of processing occurs (Pfenning and McMahon 1997; Sobczak et al. 2003). Despite this progress, we still know comparatively little about 'denitrification' or other pathways of nitrate reduction at depth in the hyporheic zone.

Our knowledge about the biogeochemistry of nitrogen at depth in the river bed across different habitat types is also poorly constrained. For example, differences in the structure of the river bed below riffles and pools creates differences in groundwater–surface mixing and, therefore, supply of nutrients, carbon, and oxygen to the subsurface. However, mechanistic understanding of how river-bed structure influences processes such as denitrification is lacking. For example, do the fine sediments typical of a depositional environment such as a pool promote subsurface denitrification by reducing surface-water infiltration and, therefore, oxygen delivery (Lefebvre et al. 2006)? Or, does the increased hydrological exchange associated with more turbulent habitats such as riffles promote denitrification by supplying labile organic carbon to greater depths within the river bed (Fischer et al. 2005)?

The relative dearth of knowledge about pathways of dissimilatory nitrate reduction other than denitrification in the hyporheic zone is due, in part, to the widespread use of the acetylene block technique (Yoshinari et al. 1977) to quantify denitrification (Duff and Triska 2000). Although the acetylene block assay gives a relative measure of denitrification potential, it cannot capture N_2 production via anaerobic ammonium oxidation (anammox), which is known to play a significant role in N_2 production in estuarine and marine sediments (Dalsgaard et al. 2005). The addition of acetylene to aquatic sediments is also known to interfere with nitrification, methanogenesis, and sulphate reduction (*see* Seitzinger et al. [1993] for a fuller discussion). In addition, acetylene block cannot be used to directly measure any dissimilatory reduction of nitrate to ammonium (DNRA) or assimilatory reduction of nitrate (Duff and Triska 2000). Even though the latter pathways of nitrate reduction have rarely been measured directly in freshwater sediments, and the existing evidence is conflicting (Burgin and Hamilton 2007), they have been dismissed as being of little importance in subsurface sediments (Rivett et al. 2008).

Here our aim was to use ^{15}N to quantify the potential for denitrification, anammox, and DNRA, in relation to the availability of organic carbon, in sediments recovered from up to 1-m depth in the bed of the River Leith, Cumbria, United Kingdom. We looked at how the pathways of nitrate reduction responded to the addition of complex organic substrates. We hypothesized that any potential for greater DNRA activity relative to denitrification would be confined to sediments with a greater availability of organic carbon, whereas the opposite would be true for anammox.

Methods

Field sites and recovery of sediment cores—The field site comprises a 250-m reach of the River Leith, flowing through Cliburn, Cumbria, United Kingdom (54°39.62′N, 2°37.22′W). Here, the River Leith meanders through a narrow floodplain, which is predominantly of agricultural land use. A series of riffle and pool sequences characterize the channel. The bed consists of a mixture of sand, gravel, and cobbles overlying unconsolidated sands and silts, which are underlain by Permo–Triassic sandstone bedrock. Groundwater contributions to the River Leith can be significant; however, Käser et al. (2009) demonstrated the potential for surface-water downwelling at this site. Nitrate concentrations within the hyporheic zone of the River Leith are highly variable, ranging from below detection to 650 μmol L^{-1}, but are usually higher than that observed in surface water at the site (average surface-water concentration = 250 μmol L^{-1}; Krause et al. 2009). As part of a larger parent project, an initial network of piezometers was installed in June 2009 with a percussion drill along the reach (n = 48, maximum depth below the river bed = 1 m). We used eight independent sediment cores recovered from along the thalweg during the drilling process to characterize the potential pathways of nitrate reduction as a function of depth and stream habitat (riffle n = 4, pool n = 4, *see* Fig. 1). At the time of sample collection the river was under base-flow conditions.

Sediment characteristics and preparation of anoxic sediment slurries—Following recovery of the sediment cores from the river bed, the cores were described, subdivided into four 5-cm depth bands (the shallowest band was from 5 cm to 10 cm below the river-bed surface, the deepest 5-cm band was collected from between 60 and 86 cm depending on core length, *see* Table 1), transferred into zip-lock bags, and stored cool in a portable refrigerator. In total, 31 independent sediment samples were retrieved: four depth bands from seven sites and three depth bands from one site, giving true replication of depth in either a pool or riffle (n = 31, Table 1; note we lost the deepest depth band for one of the riffles during recovery of the core). On return to the laboratory, gravels were removed from the samples by passing wet sediment through a 2-mm sieve by gentle shaking. The removal of gravels was necessary to allow homogenous subsamples of $\sim$ 3 g of wet sediment to be transferred into gas-tight vials (12 mL Exetainer, Labco) in order to perform the various manipulations described below. Though the full magnitude of any one nitrate reduction pathway may have been reduced by the removal of gravels, any potential interplay would have been preserved. Sediment was stored in the capped gas-tight vials in the refrigerator for $<$ 48 h prior to start of the incubations.

The absolute particle-size distribution of the sieved samples was determined by laser diffraction (LS 13 320 Beckman Coulter Counter) after digestion with 30% hydrogen peroxide to remove organic matter. Particles $>$ 2 mm were sorted by sieving air-dried material and weighing the separated fractions. The organic carbon

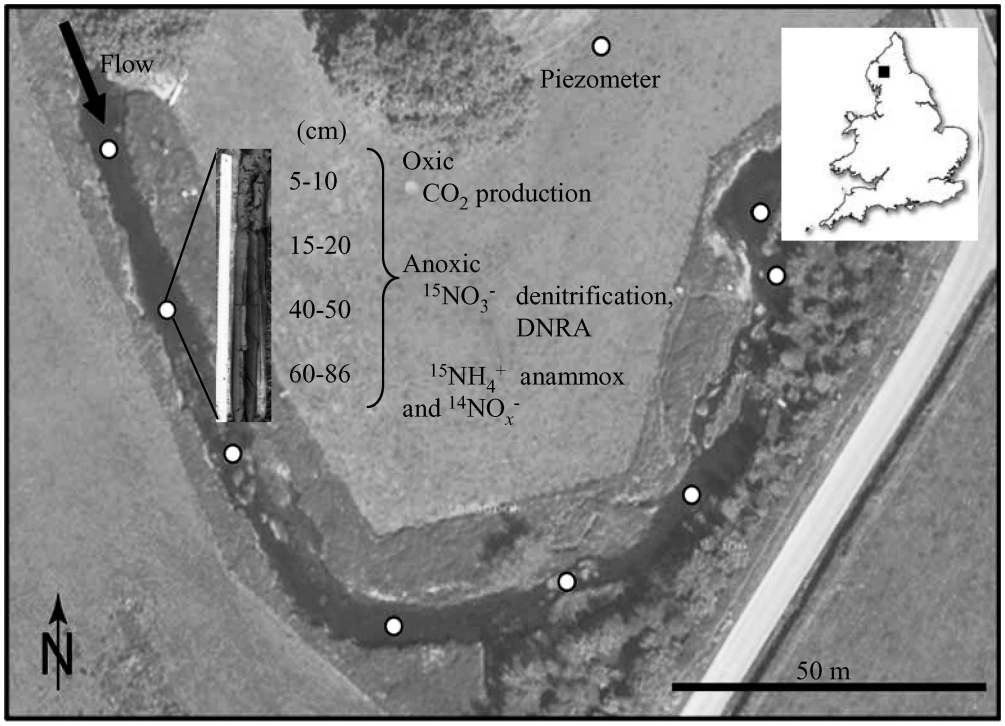

Fig. 1. Aerial photograph of the study reach showing points from which sediment cores were recovered (circles). A photograph of a typical sediment core (total length 1 m), a summary of the various treatments applied to each 5-cm sediment slice (center) and a map of the United Kingdom showing the location of the River Leith (inset) are also shown.

content of the < 2-mm sediments was quantified using elemental analysis (Thermo Finnigan Flash elemental analyzer [EA]) after treatment with 1 mol L^{-1} HCl to remove carbonates (Hedges and Stern 1984). The limit of detection (LOD) for carbon on the EA was 1.5 μg C, which equates to 0.02% organic carbon in our sediment samples of ~ 8 mg. Following all of the incubations and any subsequent measurements, detailed below, all of the vials were opened and the sediment dried to a constant weight at 80°C.

Measuring sediment metabolisms—Measuring denitrification, anammox, and DNRA by the application of ^{15}N substrates: For screening the sediments for the potential pathways of dissimilatory NO_x^- reduction, the samples of prepared sediment in the gas-tight vials and synthetic river water (see below) were transferred to an anoxic hood (CV204, Belle Technology). The river water from the site was high in $^{14}NO_3^-$ and because this could potentially interfere with the anammox assay (*see* below), we took the precaution of using synthetic river water (Smart and Barko 1985). The synthetic river water was bubbled vigorously with oxygen-free nitrogen (British Oxygen Company) for 20 min using the gassing line in the anoxic hood and a 3-mL aliquot of this water was added to the sediment in each of the vials, which were then capped. The sediment slurries were then removed from the anoxic hood and left to 'preincubate' on rollers (Spiramix, Thermo-Finnigan) for 24 h to remove any traces $^{14}NO_3^-$ before beginning the ^{15}N experiments (Trimmer et al. 2003; Risgaard-Petersen et al. 2004).

To start the experiments for denitrification and DNRA, the sediment slurries (n = 31, as above) were enriched to

Table 1. Summary of grain size and organic carbon content of recovered sediments. Median grain size was measured in bulk sediment samples, whereas the organic carbon content (mean ± 1 standard deviation) is for the < 2-mm fraction only.

Habitat	Depth (cm)	n	Substrate	Median grain size (μm)	Organic C (%)*
Pool	5–10	4	Sand with gravel	465	0.05±0.01
Pool	15–20	4	Sand with silt	283	0.06±0.10
Pool	45–50	4	Sand with silt	297	0.02±0.01
Pool	60–86	4	Sand with silt	271	0.02±0.01
Riffle	5–10	4	Sand and gravel	1589	0.07±0.05
Riffle	15–20	4	Sand and gravel	1485	0.05±0.03
Riffle	40–50	4	Sand with silt + gravel	439	0.02±0.02
Riffle	64–85†	3	Sand with silt	300	0.03±0.02

* Sediment organic carbon content is at, or slightly above, the analytical limit of detection (0.02%; *see* text).
† One sample was lost during recovery of the sediment core.

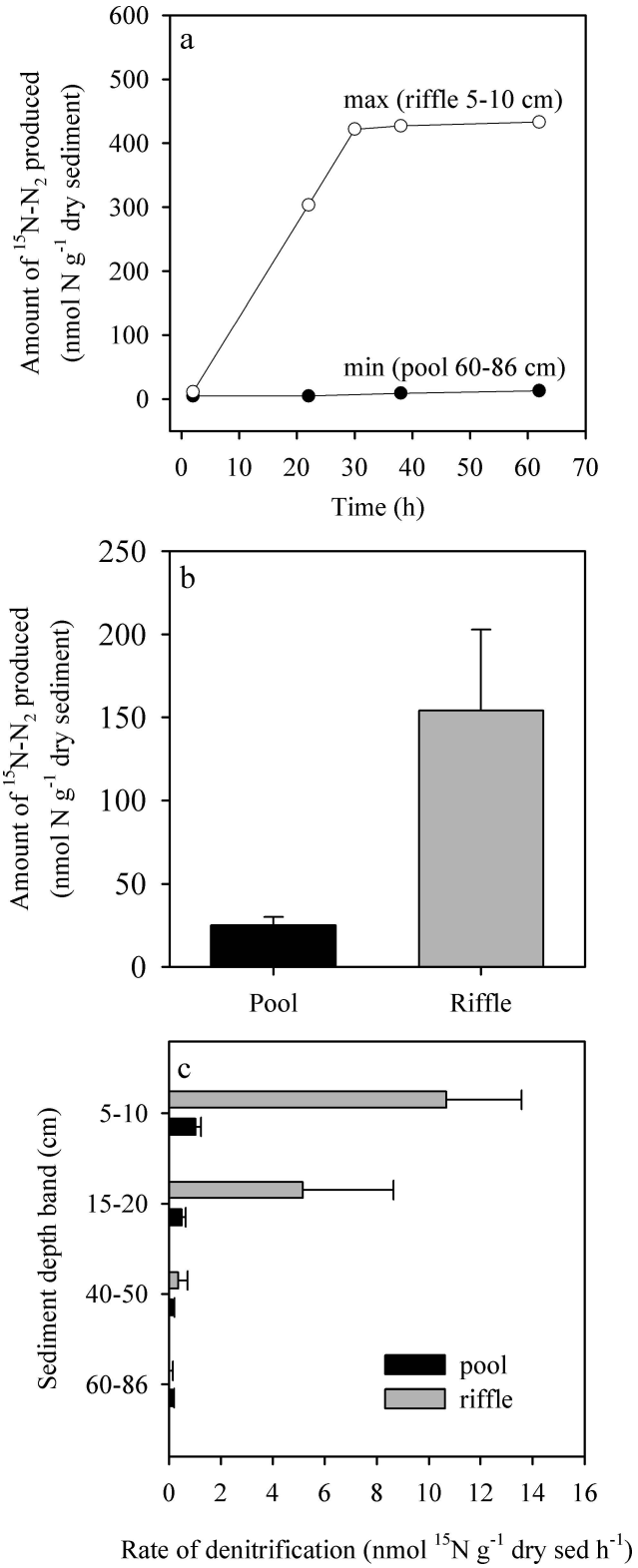

Fig. 2. Denitrification of a 1200-nmol $^{15}NO_3^-$ spike added to hyporheic sediments. (a) Production of ^{15}N-N_2 over time during the incubation of sediments; data are single observations from samples that produced the lowest (min) and highest (max) amounts of ^{15}N-N_2. (b) The amount of ^{15}N-N_2 produced within each vial as a function of habitat, and (c) the potential rate of ^{15}N-N_2 production as a function of depth and habitat. Bars represent mean values, and error bars indicate 1 standard error.

350 μmol L^{-1} $^{15}NO_3^-$ by injecting 100 μL of a stock solution (deoxygenated 12 mmol L^{-1} $Na^{15}NO_3$ [99.3 ^{15}N atom%], Sigma-Aldrich) through the butyl septum of each sample vial, with each vial receiving 1200 nmol $^{15}NO_3^-$. This enrichment was representative of the average in situ pore-water concentration for NO_3^- of 286 μmol L^{-1} determined in a previous study (Krause et al. 2009). The vials were then placed back on the rollers and incubated at a constant laboratory temperature of 22°C in the dark. For denitrification, the production of ^{15}N-N_2 gas ($^{29}N_2$ + $^{30}N_2$) was monitored by repeatedly sampling the headspace of each vial over time (at 2 h, 22 h, and 30 h, and then every 24 h thereafter for 8 d, *see* Fig. 2) and measuring the *m/z* ratios for 29 and 30 ($^{29}N_2$ and $^{30}N_2$) by continuous-flow isotope ratio mass spectrometry (Thermo-Finnigan, Delta Matt Plus) as described in Trimmer and Nicholls (2009). After the denitrification experiment had finished, biological activity was stopped by the addition of $ZnCl_2$ (100 μL of 3.7 mol L^{-1}). Changes in natural abundance $^{29}N_2$ and $^{30}N_2$ concentrations were determined in a parallel set of reference sediment slurries (n = 31) prepared in the same way as above, except fixed with $ZnCl_2$ (as above) at the beginning of the incubation and lacking the addition of ^{15}N substrates.

The sediment slurries used to quantify denitrification were then assayed for any DNRA potential, where an increase in $^{15}NH_4^+$ in a sediment slurry at the end of the incubation, relative to the reference sample, was evidence of DNRA activity. Ammonium was extracted from all sediment slurries using KCl (15 mL of 2 mol L^{-1}) where samples were mixed on a shaker table at 100 revolutions min^{-1} for 2 h before centrifuging (1700 × g, 5 min). The supernatant was filtered through a 0.2-μm polypropylene membrane (VWR International) and subsamples were collected and frozen at −20°C for later measurement of ammonium concentration by wet chemistry (*see* nutrient analyses) and $^{15}NH_4^+$ content by mass spectrometry. Samples for $^{15}NH_4^+$ analysis (1 mL) were transferred into gas-tight vials (3 mL Exetainer, Labco) and bubbled with helium (Commercially pure grade, British Oxygen Company) for 20 min to remove any ^{15}N-bearing gases in solution. The $^{15}NH_4^+$ content of the sample was then quantified using hypobromite oxidation as described by Risgaard-Petersen et al. (1995), and subsequent analysis of any ^{15}N-N_2 enrichment in the headspace by mass spectrometry.

The potential for anammox in the sediment was quantified using a parallel set of slurries (n = 62, two sets of 31 slurries, *see* below) and established methodologies (Thamdrup and Dalsgaard 2002; Trimmer et al. 2003; Risgaard-Petersen et al. 2004). Here, the production of $^{29}N_2$ in the presence of $^{15}NH_4^+$ and $^{14}NO_3^-$, relative to its absence in the presence of $^{15}NH_4^+$ alone, would be taken as positive proof of the potential for the anammox reaction. All sediment slurries were enriched to 200 μmol L^{-1} $^{15}NH_4^+$ by injecting 100 μL of a stock solution (deoxygenated 12 mmol L^{-1} $^{15}NH_4Cl$ [99.4

^{15}N atom%], Sigma-Aldrich) through the septum as above, which resulted in ≥ 90% of the pore-water ammonium being labeled with ^{15}N, because the ambient concentration is usually between 4 μmol L^{-1} and 7 μmol L^{-1} (Krause et al. 2009). Half of the slurries ($n = 31$) were additionally spiked with $^{14}NO_3^-$ to a final concentration of 350 μmol L^{-1} (as above). The headspace was then analyzed over time for the production of $^{29}N_2$ and $^{30}N_2$ (as above). In addition, any production of ^{15}N-N_2 in the sole presence of $^{15}NH_4^+$ would be indicative of other anaerobic pathways of ammonium oxidation (e.g., that coupled to the reduction of either manganese or iron oxides [Hulth et al. 1999]).

Aerobic production of CO_2 as an indicator of the bioavailability of organic carbon: We used the aerobic rate of CO_2 production in the sediments to estimate the relative potential bioavailability of organic carbon (i.e., the amount of organic carbon that could be mineralized to CO_2 in the different sediment samples [Dauwe et al. 2001]). Samples of the recovered sediment (< 2-mm fraction, $n = 31$) were simply incubated in the gas-tight vials with a headspace (~ 6 mL) of air and the production of CO_2 measured over time by gas chromatography with flame ionization detection (Agilent Technologies), after catalytic reduction of CO_2 to CH_4, and calibration against certified cylinders of CO_2 (Trimmer et al. 2009). At the start of the incubation, the headspace in each gas-tight vial would have contained ~ 56 μmol O_2 (at 0°C and 101.325 kPa) and, because the maximum amount of CO_2 produced after 10 d was only 2.6 μmol CO_2, respiration could be assumed to have been aerobic throughout the entire incubation. We then summed the total amount of CO_2 produced during the incubation and used this as a lower estimate of the total amount of carbon available to support heterotrophic pathways of nitrate reduction in the parallel anaerobic incubations.

Manipulating sediment metabolism by addition of complex organic substrates: After 2–3 d steady production of ^{15}N-N_2 in the denitrification vials (from above), we spiked the sediment slurries with a complex mixture of organic substrates (100 μL of autoclaved 0.05 g L^{-1} Bacteriological Peptone, L37, Oxoid) and then continued monitoring the headspace (as above) for the production of ^{15}N-N_2 for a further 5 d. The bacteriological peptone solution contains a wide spectrum of polypeptides of differing molecular weights, which we converted to a known total amount of organic carbon using elemental analysis, in-line with continuous-flow isotope ratio mass spectrometry (Thermo-Finnigan, Flash-EA, and Delta Matt Plus). Accordingly, each 100-μL spike contained 920 nmol of carbon. We repeated this exact exercise with the parallel sediment slurries being used to measure the aerobic production of CO_2 and, in addition, note that the KCl extraction and quantification of $^{15}NH_4^+$ for the measurement of DNRA was done at the very end of the incubation after the addition of organic C.

Nutrient analyses—Concentrations of NO_3^-, NO_2^-, and NH_4^+ in KCl extracts were measured using a segmented-flow auto analyzer (Skalar) and standard colorimetric techniques. The LOD and precision for NO_3^- and NO_2^- was 0.2 μmol L^{-1} ± 1%, and for NH_4^+ was 0.5 μmol L^{-1} ± 5%.

Calculation of rates, stoichiometries, and statistical analyses—The incubations with $^{15}NH_4^+$ (with and without $^{15}NO_3^-$) were used to confirm the presence of the anammox reaction (Thamdrup and Dalsgaard 2002), and to inform the way in which rates of ^{15}N-N_2 production by denitrification or anammox (if found) were calculated in the parallel incubations with $^{15}NO_3^-$. The $^{15}NH_4^+$ and $^{14}NO_3^-$ assay revealed that the potential for anammox in the hyporheic sediments was negligible (*see* Results); therefore, all ^{15}N-N_2 produced during the incubation of sediments amended with $^{15}NO_3^-$ was considered to be the result of denitrification. Production of ^{15}N-N_2 (i.e., $^{29}N_2$ or $^{30}N_2$) was quantified in the headspace of the incubated sediments as excess above reference samples to which no $^{15}NO_3^-$ was added according to Thamdrup and Dalsgaard (2000):

$$p^xN_2\left(\text{nmol } N_2L^{-1}\right) = \left[\left(\frac{^xN_2}{\sum N_2}\right)_{\text{sample}} - \left(\frac{^xN_2}{\sum N_2}\right)_{\text{reference}}\right] \times \sum N_{2\,\text{sample}} \times \alpha^{-1} \quad (1)$$

where p^xN_2 is the amount of excess $^{29}N_2$ or $^{30}N_2$ in the headspace of the incubation vessel; $^xN_2$: ΣN_2 represents the ratio of the $^{29}N_2$ or $^{30}N_2$ mass spectrometer signal to the total N_2 signal ($\Sigma N_2 = {}^{28}N_2 + {}^{29}N_2 + {}^{30}N_2$) for either sample or reference; and α is the calibration factor (signal: nmol N_2 L^{-1}) obtained by measurement of vials containing atmospheric air. The (nano)molar amounts of excess $^{29}N_2$ and $^{30}N_2$ within each vial were then calculated using the concentrations from Eq. 1, headspace and liquid volumes in the vials and the Bunsen solubility coefficient for N_2 (Weiss 1970). Finally the total amount of ^{15}N-N_2 ($\Delta^{15}N$-N_2) produced via the denitrification of $^{15}NO_3^-$ was calculated using the following equation (Nielsen 1992; Thamdrup and Dalsgaard 2000):

$$\Delta^{15}N - N_2\left(\text{nmol N vial}^{-1}\right) = p'^{29}N_2 + 2 \times p'^{30}N_2 \quad (2)$$

where p' $^{29}N_2$ and p' $^{30}N_2$ are values derived from Eq. 1 expressed as nmol of N_2 per vial. Rates of potential denitrification of the $^{15}NO_3^-$ spike were calculated by regressing $\Delta^{15}N$-N_2 against time and normalizing the slope by the respective dry weight of each sediment sample.

The amount of $^{15}NO_3^-$ reduced to ammonium, and subsequently oxidized to N_2 using the hypobromite assay, was determined using the principles of Thamdrup and Dalsgaard (2000), where $^{29}N_2$ and $^{30}N_2$ concentrations above that of the reference sample, and $\Delta^{15}N$-N_2, were indicative of DNRA rather than denitrification (*see* Eqs. 1 and 2). The hypobromite assay was performed at the end of the incubation, after the addition of carbon; therefore, only the total amount of nitrate reduced via the DNRA pathway rather than the rate of reaction, was calculated.

The fate of the $^{15}NO_3^-$ spike added to the hyporheic sediments was determined by comparing the amount of ^{15}N

added to the amount of $^{15}NO_3^-$ that was reduced through denitrification or DNRA. Also included in this ^{15}N budget was the amount of NO_x^- remaining in the sediment sample at the end of the incubation as determined by NO_x^- analysis of the KCl extracts. The budget was balanced with an 'assimilation' term, which was the difference between the amount of $^{15}NO_3^-$ spike added and the ^{15}N accounted for in the NO_x^-, NH_4^+, and N_2 pools, and which is thought to represent the amount of ^{15}N incorporated into microbial biomass during the sediment incubation (Kelso et al. 1999).

Production of CO_2 was quantified relative to the amount of CO_2 present in the gas-tight vials at the start of the sediment incubation. Potential rates of CO_2 production were calculated in an analogous manner to that previously described for N_2, with moles of CO_2 as determined by gas chromatography used rather than $p^{29}N_2$ and $p^{30}N_2$.

Denitrification N_2 equivalents were determined using the stoichiometry proposed by Fenchel and Blackburn (1979):

$$5'CH_2O' + 4NO_3^- \rightarrow 2N_2 + 4HCO_3^- + CO_2 + 3H_2O \quad (3)$$

where 'CH_2O' represents organic matter. For example, the 100-μL spike of bacteriological peptone contained 920 nmol of carbon, which, according to Eq. 3, could support the reduction of 736 nmol of nitrate to form 736 nmol of ^{15}N-N_2 (as N). To calculate the yield of N_2, the number of moles of ^{15}N-N_2 produced following the organic substrate addition was divided by 736 nmol, and the quotient multiplied by 100 to express yield as a percentage. The yield of carbon recovered after the addition of organic substrates was calculated in a similar manner to N_2, except the amount of carbon recovered as CO_2 was compared to the amount of carbon in the bacteriological peptone spike.

The effect of depth, habitat (pool, riffle), and carbon addition on the sediment metabolisms measured was investigated by ANOVA completed in SPSS version 14. Prior to use in the ANOVA, data were log-transformed to improve their distribution, and normality was assessed using the Shapiro–Wilk *W*-test. The level of significance was set at 0.05 for all statistical analyses. Pair-wise comparisons were performed if the interaction term in the ANOVA was statistically significant to elucidate relationships within the data set. Comparison of ^{15}N-N_2 and CO_2 yields following the addition of organic substrates was performed with a paired-sample *t*-test in SPSS. Linear regressions used to calculate rates of ^{15}N-N_2 and CO_2 production were performed on nontransformed data in Microsoft Excel 2003. Regression analysis used to investigate correlations between variables were also performed in Microsoft Excel; however, log-transformed data were used in these tests.

Results

Field-site and sediment characteristics—All of the sediments recovered were comprised of a sand matrix with differing amounts of gravel, silt, or clay depending on the habitat and depth in the river bed from which the samples were collected. Gravels were most common in shallow riffle sediments ($\leq$ 20 cm), where the highest median grain sizes were also recorded (Table 1). The organic carbon content of the $<$ 2-mm fraction of the sediments ranged from $<$ 0.02% to 0.2%. There was no relationship between sediment organic carbon content and stream habitat or river-bed depth (ANOVA: $p = 0.66$ and 0.21 for habitat and depth, respectively, $n = 31$; data not shown but *see* Table 1).

Aerobic production of CO_2 as an indicator of the bioavailability of organic carbon—All of the sediment samples produced CO_2 linearly over time (average $r = 1.00$ over 26 h, $n = 31$; examples shown in Fig. 3a). Maximum rates of production were measured in the top fraction of sediment ($\leq$ 10 cm), with production decreasing thereafter with sediment depth (Fig. 3c and Table 2). The rate of decay in production of CO_2 with depth was the same in both the riffle and pool sediments (Table 3). There was a significant positive correlation between the amount of available organic carbon, indicated by CO_2 production, and the measured organic carbon content of the sediment ($r = 0.58$, $p < 0.001$, $n = 31$; data not shown). The greatest amount of potentially available organic carbon was present at the top of the sediment core, decaying thereafter with depth as for the potential rates (Fig. 3 and Table 2). The amount of bioavailable carbon in the sediments was positively correlated with median grain size ($r = 0.43$, $p = 0.034$, $n = 31$; data not shown), and overall, more available carbon was associated with sediments from riffles compared to sediments from pools (Fig. 3b and Table 2).

Denitrification, DNRA, and anammox—The pattern of ^{15}N-N_2 gas production by denitrification was very similar to that for CO_2. Linear production of ^{15}N-N_2 over time was observed in all samples (average $r = 0.99$, $n = 31$); however, the time scale of linearity varied between sediments (Fig. 2a). Most sediments collected from depths $\geq$ 40 cm in the river bed showed a lag of up to 22 h between $^{15}NO_3^-$ addition and ^{15}N-N_2 production. Conversely, in shallow riffle sediments ($\leq$ 10 cm) the $^{15}NO_3^-$ spike was quantitatively converted to ^{15}N-N_2 within 38 h of addition. The amounts of ^{15}N-N_2 and CO_2 produced within each vial were positively correlated ($r = 0.72$, $p < 0.001$, $n = 31$; data not shown). Maximum denitrification activity was measured in the top sediment layers and overall potential rates of denitrification were greater in the riffles compared to the pools (Fig. 2c and Table 2). Denitrification activity decayed with sediment depth, though the rate of decay was sharper in the riffles than the pools (Fig. 2c and Table 3).

A small proportion of the $^{15}NO_3^-$ spike was recovered as $^{15}NH_4^+$ in the sediment slurries, indicating potential for DNRA. The amount of nitrate reduced via the DNRA pathway varied from below detection ($<$ 1.9 nmol N g^{-1} [dry sediment, sed]) to 20 nmol of N g^{-1} (dry sed; Table 4). There was no relationship between DNRA potential and depth or habitat in the hyporheic sediments (Table 3).

Screening for anammox potential resulted in only very low levels of $^{29}N_2$ enrichment in the slurries amended with $^{15}NH_4^+$ ($\pm^{14}NO_3^-$). We quantified the LOD of our anammox assay as $\sim$ 0.03 μmol L^{-1} $^{29}N_2$ (= 0.08 nmol $^{29}N_2$ g [dry sed]$^{-1}$ with our sample volumes) using the

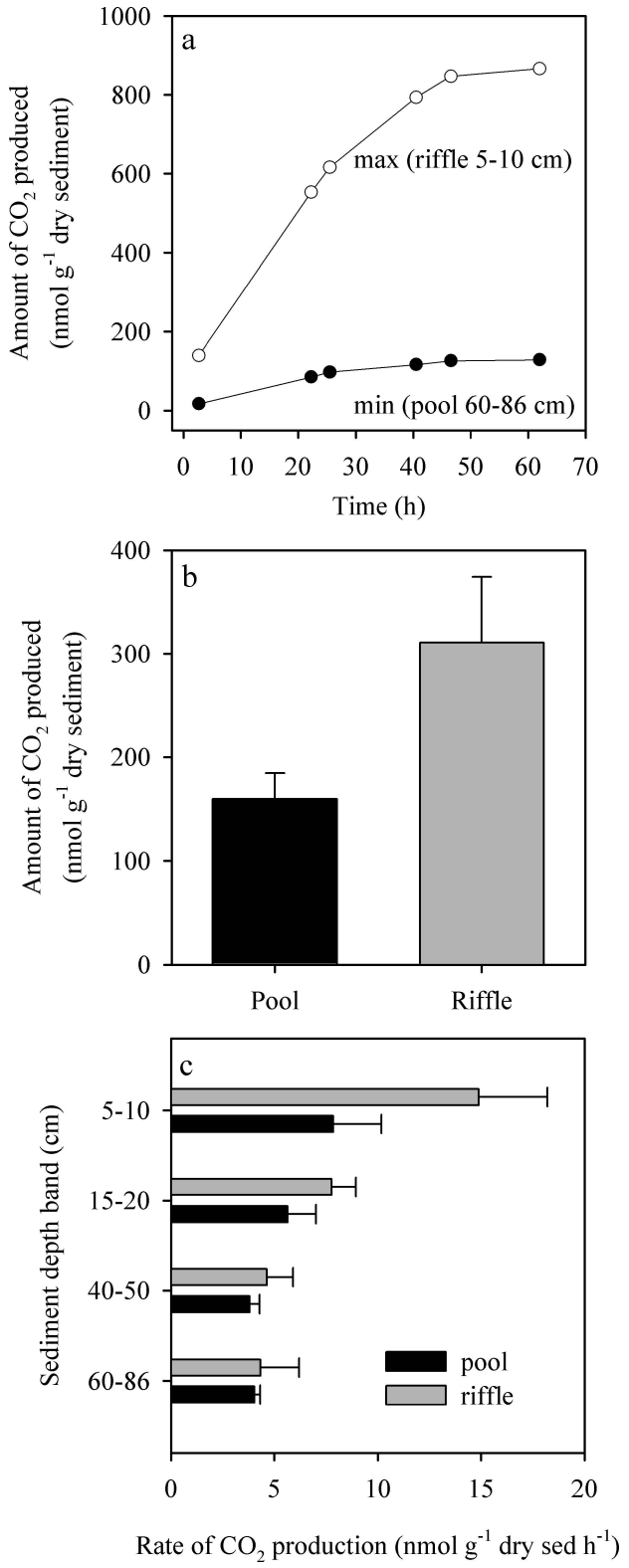

Fig. 3. Aerobic production of CO_2 from hyporheic sediments. (a) Accumulation of CO_2 in the headspace over time; data are single observations from samples that produced the lowest (min) and highest (max) amounts of CO_2. (b) The amount of CO_2 produced within each vial as a function of habitat, and (c) the potential rate of CO_2 production as a function of depth and habitat. Bars represent mean values, error bars indicate 1 standard error.

sensitivity of the mass spectrometer (0.1‰ for N). Using this detection limit, we could measure no significant production of $^{29}N_2$ in the sole presence of $^{15}NH_4^+$ but, although very slight, the enrichment in $^{29}N_2$ with $^{15}NH_4^+$ and $^{14}NO_3^-$ was significant (Table 4). There is, therefore, a very modest potential for the anammox reaction in the sediments of the River Leith, but no evidence to suggest other anaerobic pathways of ammonium oxidation.

Response by sediment metabolisms to the addition of complex organic substrates—Production of ^{15}N-N_2 gas recommenced after the addition of complex organic substrates to the recovered sediments, with a marked increase in the potential rate of denitrification observed (Fig. 4a). Excluding those samples where the $^{15}NO_3^-$ spike was completely reduced to ^{15}N-N_2 early in the incubation ($n = 4$), the denitrification activity of the hyporheic sediments increased by a factor of 2.4 on average ($n = 27$), after the addition of organic substrates. Interestingly, there was no significant interaction between increased rates of denitrification potential and depth, with rates increasingly uniformly at all depths (Table 5 and Fig. 4b).

Although the increase in potential denitrification rate was consistent across all depths following the addition of organic substrates, there was a substantial decrease in the overall yield of ^{15}N-N_2 gas with sediment depth. At ≤ 10 cm in the river bed, between 54% and 125% (92% ± 14%) of the amount of nitrate able to be denitrified by the addition of 920 nmol of carbon was recovered as ^{15}N-N_2, while in deeper sediments (≥ 40 cm) the yield of ^{15}N-N_2 was between 13% and 72% (28% ± 3%; Fig. 4c).

Production of CO_2 within the aerobic sediment incubations continued after the addition of complex organic substrates (Table 5). The increase in CO_2 observed after the addition of organic substrates was not due solely to the input of fresh carbon, because yields of CO_2 in excess of the amount of carbon added in the bacteriological peptone spike were recorded (data not shown). In shallow sediments (≤ 10 cm, $n = 5$), an average of 86% of the amount of carbon added was recovered as CO_2. In contrast, in sediments collected from depths ≥ 40 cm in the river bed, the yield of carbon was only 57% of that added, on average ($n = 14$; Fig. 4c). There were no significant differences between yields of CO_2 and ^{15}N-N_2 in sediment recovered from < 20 cm in the river bed (paired t-test, $t = -0.64$, df $= 4$, $p = 0.56$ for 5–10 cm and $t = 0.92$, df $= 5$, $p = 0.40$ for 15–20 cm, *see* Fig. 4c). In deeper sediments, however, recovery of CO_2 was higher than that of ^{15}N-N_2 (paired t-test, $t = 4.39$, df $= 6$, $p = 0.005$ for 40–50 cm and $t = 3.56$, df $= 6$. $p = 0.012$ for 60–86 cm).

Comparing pathways of nitrate reduction—Denitrification was by far the dominant pathway of dissimilatory nitrate reduction, especially in shallow (≤ 10 cm) riffle sediments where in excess of 1015 nmol $^{15}NO_3^-$, or 85% of the spike, was denitrified on average (Fig. 5). Considerable amounts of $^{15}NO_3^-$ remained either unreacted, or reduced only to $^{15}NO_2^-$, in the aqueous phase of the sediment slurries (Table 4). The amount of ^{15}N recovered from the NO_x^- pool increased with depth in both riffle and pool

Table 2. Summary of organic matter mineralization and denitrification in hyporheic sediments including the rate and amount of CO_2 production under aerobic conditions, and the potential rate and amount of ^{15}N-N_2 production via denitrification. Rates are calculated where linear production of CO_2 or ^{15}N-N_2 was observed (*see* text). Amounts of CO_2 or N_2 produced are the total amount of analyte produced per g of dry sediment at the end of the 62-h incubation. Data are mean ± 1 standard error for rates and nmol. Units for rate measurements are nmol per g of dry sediment per hour, which is shortened to nmol g^{-1} h^{-1} below.

Habitat	Depth (cm)	CO_2 produced (nmol g^{-1})	CO_2 flux (nmol g^{-1} h^{-1})	^{15}N-N_2 recovered (nmol N g^{-1})	Denitrification rate* (nmol N g^{-1} h^{-1})
Pool	5–10	272±63	8±2.3	45±13	1±0.2[a]
Pool	15–20	154±42	6±1.4	27±7	0.5±0.16[a]
Pool	45–50	101±12	4±0.5	13±4	0.1±0.08[a]
Pool	60–86	107±14	4±0.3	15±4	0.2±0.05[a]
Riffle	5–10	624±138	15±3	363±61	11±3[b]
Riffle	15–20	289±48	8±1.2	179±102	5±3.5[c]
Riffle	40–50	161±38	5±1.3	27±20	0.4±0.36[a]
Riffle	64–85	152±72	4±1.9	4±0.2	0.00±0.04[a]

* Superscript denotes where differences between denitrification rates are significant (pair-wise comparisons to investigate simple main effects following significant interaction term in the ANOVA [*see* Table 3], $p<0.05$).

sediments, ranging from < 5% of the $^{15}NO_3^-$ added in shallow sediments (≤ 10 cm) to in excess of 30% of ^{15}N spike at depth. At depths ≥ 40 cm, the amount of ^{15}N residual within the NO_x^- pool of the sediments exceeded the amount of $^{15}NO_3^-$ denitrified. Sampling of the N_2, NO_x^-, and NH_4^+ pools of the slurries recovered between 13% and 100% of the $^{15}NO_3^-$ spike. The amount of $^{15}NO_3^-$ spike unaccounted for in these pools increased with depth and we apportioned this unaccounted-for ^{15}N as that assimilated into microbial biomass (Fig. 5).

Discussion

Flow is a key dynamic in hyporheic biogeochemistry and removing sediment from the river bed would clearly alter the physical–chemical properties of the sediment (Findlay 1995). In addition, our ^{15}N assays were carried out under complete anoxia, which would maximize the potential for any denitrification, relative to the fluctuating oxygen regime of the hyporheic zone (Malcolm et al. 2006). That said, it would be very challenging indeed to irrefutably prove the potential for anammox, any other pathways of anaerobic ammonium oxidation or assimilatory reduction of nitrate genuinely in situ (Thamdrup and Dalsgaard 2002). Sediment slurries have value in exploring the potential fate of numerous substrates under controlled laboratory conditions.

Dominance of nitrate reduction by denitrification—In sediments recovered from the River Leith, denitrification proved to be the only substantial pathway of dissimilatory nitrate reduction, in line with both the predictions and observations for other subsurface environments (Duff and Triska 2000; Rivett et al. 2008). Rates of potential denitrification varied from below detection to 16 nmol N g^{-1} (dry sed) h^{-1}, which is equivalent to ~ 19 nmol N cm^{-3} (wet sed) h^{-1}, and is at the lower end of the range reported for other river-bed sediments (García-Ruiz et al. 1998; Sheibley et al. 2003; Arango et al. 2007). In addition, given the correlation between ^{15}N-N_2 and aerobic CO_2 production, and the uniform response by denitrification at all depths to the addition of complex organic substrates, it appeared to be classic heterotrophic denitrification.

Maximum denitrification activity was measured in the top 5–10 cm of sediment but, even here, the average activity was only 5.9 nmol N g^{-1} (dry sed) h^{-1}. However, it is important to consider that there may have been considerable 'hotspots' of activity on the immediate surface of the river bed not collected by our sampling technique (*see* depth classes in Table 1). For example, Arango et al. (2007) demonstrated that particulate organic matter in among gravels on the river-bed surface, or the epilithic biofilm on the larger stones and cobbles, can support high rates of denitrification. Sediment cores were retrieved as part of a piezometer installation program; therefore, we did not aim to recover the immediate sediment surface. Also, the removal of gravels from sediments used to quantify the potential rates of denitrification could have resulted in an underestimation of the true denitrification potential by excluding microbial communities and bioavailable carbon associated with particles > 2mm in diameter. An exponential relationship through all of our data explains the decay

Table 3. Univariate ANOVA results for the effects of habitat and depth on the amount and potential rate of both CO_2 and ^{15}N-N_2 production, and dissimilatory nitrate reduction to ammonium (DNRA), during the incubation of hyporheic sediments. Data given are *F*-values, total $n = 31$.

Source	Amount CO_2	Rate CO_2	Amount ^{15}N-N_2	Rate ^{15}N-N_2	DNRA
Habitat	8.48*	2.54	14.1**	9.41*	0.068
Depth	9.60**	7.21**	8.43**	11.1**	0.651
Habitat × depth	0.714	0.968	5.95*	4.03*	1.59

* $p \leq 0.05$; ** $p \leq 0.001$.

Table 4. Fate of the 1200-nmol $^{15}NO_3^-$ spike (~ 450 nmol $^{15}NO_3^-$ g^{-1} [dry sed]) added to hyporheic sediments, expressed as nmol of N per g of dry sediment, and confirmation of the anammox reaction measured with $^{15}NH_4^+ + {}^{14}NO_3^-$. The range of measurements for each process is given below. BDL = below detection limit of 1.9 nmol N g^{-1} for the dissimilatory nitrate reduction to ammonium (DNRA) assay or 0.08 nmol $^{29}N_2$ g^{-1} for anammox potentials. Production of $^{29}N_2$ by the anammox reaction was close to the detection limit; however, when the entire data set was considered ($n = 31$) the production was significantly different to zero ($p < 0.05$).

Habitat	Depth (cm)	Fate of $^{15}NO_3^-$ (nmol g^{-1})			Anammox potential (nmol $^{29}N_2$ g^{-1})
		Denitrification	DNRA	NO_x^-	
Pool	5–10	166–410	BDL–7	0–14	BDL–0.2
Pool	15–20	190–286	BDL–9	0–91	BDL–0.2
Pool	45–50	67–108	BDL–18	19–50	0.1–0.8
Pool	60–86	90–128	2–20	34–196	0.1–0.4
Riffle	5–10	378–449	4–9	0–52	BDL–0.2
Riffle	15–20	134–428	2–10	0–87	0.1–0.2
Riffle	40–50	58–140	BDL–11	32–145	0.1–0.8
Riffle	64–85	41–91	BDL–5	16–70	BDL–0.2

in denitrification activity with depth very well (Fig. 2) and suggests that at the river-bed surface, denitrification activity within the sediments could approach ~ 19 nmol N g^{-1} (dry sed) h^{-1}.

Depth within the river bed had a stronger influence on denitrification potential than river habitat (Table 3); however, rates of denitrification potential in riffle sediments were 10 times faster, on average, than those in the pool sediments. Enhanced denitrification within riffles was not constrained to the shallowest sediments. Differences in denitrification potential between riffle and pool sediments were sustained to at least 20 cm in the river bed (Table 2). In contrast, Lefebvre et al. (2006) found that denitrification potential was highest in pool sediments and argued denitrification activity within riffles should be minimal. Lefebvre et al. (2006) contend that riffles promote hydrologic connectivity between the surface and subsurface environment, and as a consequence, denitrification potential within riffles is suppressed by the ingress of dissolved oxygen contained within infiltrating surface water. The relationship between oxygen delivery and denitrification within the hyporheic zone is likely true of many aquatic systems; however, the facultative nature of denitrifiers enables denitrification to proceed in anoxic microsites within oxygenated environments (Triska et al. 1993; Baker et al. 2000). As a result, the supply of electron donors to sustain the denitrification reaction, whether organic carbon or reduced inorganic species, may actually be more important than the nonlocalized presence or absence of oxygen in governing the prevalence of denitrification within the river bed (Holmes et al. 1996; Fischer et al. 2005; Mermillod-Blondin et al. 2005).

All of the recovered sediments were very low in carbon, with a maximum organic carbon content of 0.2%, and 90% of the samples having an organic carbon content of < 0.08%. Hence, sediment-bound organic carbon was unlikely to be the major source of carbon to the hyporheic community of the River Leith and heterotrophy is likely to depend on dissolved organic carbon inputs from surface water, groundwater, or lateral flows (Kaplan and Newbold 2000). The shallow riffle sediments of the River Leith were characterized by a large proportion of gravels in the sand matrix common to all of the recovered sediments. The larger median grain size of these shallow riffle sediments (Table 1) could promote infiltration of surface water into the river bed, especially at the head of the riffle sequences, transporting both oxygen and dissolved and particulate carbon down into the riffle (Sobczak et al. 2003; Storey et al. 2004). The increased amount of bioavailable carbon, measured as aerobically produced CO_2, observed within shallow riffle sediments (Table 2), and positive correlation between median grain size and bioavailable carbon across all samples supports the contention of downwelling surface water inferred above. The implications of this relationship between grain size and carbon availability are two-fold: first, highest amounts of bioavailable carbon are not always associated with fine-grained substrates such as clays or silts (in contrast to Lefebvre et al. [2006], for example), and second, due to the decrease in median grain size with depth in the river bed (Table 1), the supply of organic carbon to the hyporheic sediments of the River Leith most likely comes from above (i.e., surface water or shallow lateral inputs). In sediment collected under base-flow conditions, potential denitrification rates were both higher, and sustained to greater depths, within riffles compared to pools. Therefore, hyporheic denitrification potential in the River Leith appears to be controlled by the supply of electron donors that directly support denitrification, rather than the oxygen status of the sediments.

Nitrate reduction by pathways other than denitrification—Nitrate removal via DNRA within the sediments of the River Leith is of minor importance in comparison to denitrification. The DNRA assay was performed on slurries that had received the spike of bacteriological peptone; therefore, DNRA activity should have been stimulated well above ambient levels. Even after the addition of readily metabolized organic substrates, the maximum amount of $^{15}NO_3^-$ tracer that could be directly attributed to DNRA was 20 nmol g^{-1} (dry sed, 4% of the tracer), and even that is not likely to be of any real ecological significance. It would appear that a combination of low organic-matter content, coupled to high concentrations of nitrate, favored the respiratory metabolism of denitrification over the more fermentative pathway of DNRA, which is often more associated with comparatively

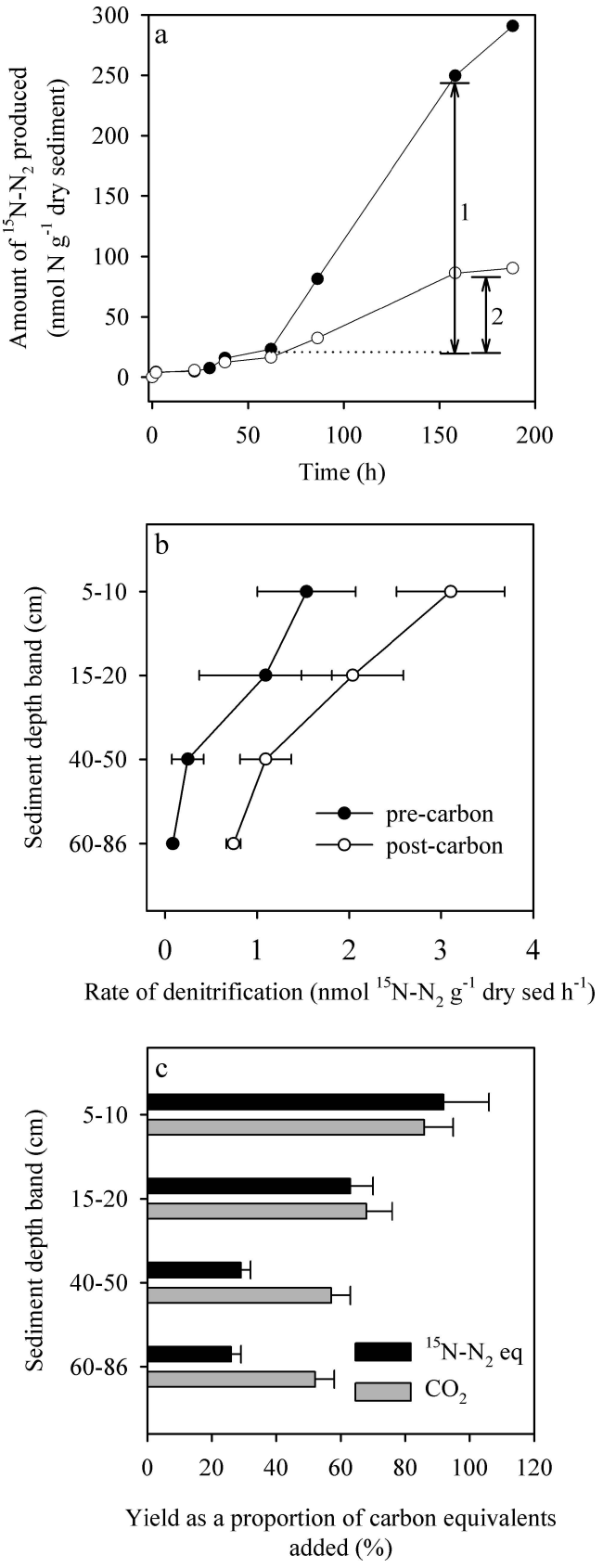

Fig. 4. Effect of the addition of complex organic substrates on ^{15}N-N$_2$ production from hyporheic sediments. (a) Example of ^{15}N-N$_2$ production over time from shallow (1 = riffle, 5–10 cm) and deep (2 = pool, > 60 cm) sediments: organic substrate was added immediately after the measurement at t = 62 h. Data are single observations from two samples. The amount of ^{15}N-N$_2$ produced after the addition of organic substrates is indicated by the vertical arrows. (b) Rates of potential denitrification before and after the addition of organic substrates as a function of depth, and (c) yields of ^{15}N-N$_2$ and CO$_2$ recovered after the organic substrate addition. ^{15}N-N$_2$ yields were calculated by comparing the amount of ^{15}N-N$_2$ produced to the amount of N$_2$ production expected from denitrification fueled by the addition of 920 nmol of carbon. CO$_2$ yields were calculated the as the mole of CO$_2$ recovered relative to 920 nmol. Differences in CO$_2$ and ^{15}N-N$_2$ yields are only significant in the > 40-cm depth bands ($p < 0.05$, *see* text). (b,c) Data are mean values, ± 1 standard error, total $n = 27$.

low ratios of nitrate to organic carbon (Tiedje 1988; Kelso et al. 1999). It is likely, however, that the potential for DNRA was underestimated by our experimental approach because assimilation of DNRA-derived $^{15}NH_4^+$ was not quantified (Kelso et al. 1999). It is also possible that the ^{15}N not recovered from the sediments as N$_2$, NO_x^-, or NH_4^+, from which assimilation was inferred, was actually due to loss of gaseous ^{15}N from the vials. We do not believe that leakage from the gas-tight vials occurred during the incubation because N$_2$ depletion (i.e., dilution of ~ 100% N$_2$ with 79% N$_2$ [air]), would have been apparent in the total N$_2$ mass spectrometer signal (ΣN_2; Eq. 1). The amount of inferred assimilation is not affected by the lack of quantification of ^{15}N label within the N$_2$O pool because gas samples for ^{15}N-N$_2$ analysis were first passed through the reduction column of the EA and, therefore, any gaseous nitrogen oxides would have been reduced to N$_2$.

The anammox metabolism has been shown to be widespread in many estuarine and marine sediments, from both temperate and arctic latitudes; yet, despite the characterization of the candidate bacteria in freshwaters, including sediments, its activity remains poorly characterized (Dalsgaard et al. 2005; Penton et al. 2006; Nicholls and Trimmer 2009). There is clearly a high potential for the reduction of nitrate to supply nitrite for anammox in the River Leith (average concentration of nitrite across the piezometer network in Jul 2009 was 1.5 μmol L^{-1} ± 0.4 SE, $n = 161$; data not shown), yet anammox activity within our sediment slurries was minor. The key dynamic that limits anammox potential within hyporheic sediments might be fluctuating oxygen in the river bed, not simply from above but throughout the three dimensions of the sediment matrix. Recent high-resolution field observations have shown rapid and short-term fluctuations in oxygen concentrations in situ in hyporheic sediments (Precht et al. 2004; Malcolm et al. 2006), conditions not conducive to the obligate anaerobic metabolism of anammox bacteria. In contrast, in the muddy and more cohesive estuarine and marine sediments where anammox is often significant, the delivery of oxygen is governed by diffusion from above and, as a consequence, there will always be a depth in the sediment where oxygen is absent, yet nitrate is available,

Table 5. Univariate ANOVA results for the effects of habitat, depth, and carbon addition on the amount and potential rate of both CO_2 and ^{15}N-N_2 production during the incubation of hyporheic sediments. Data given are *F*-values, total $n = 27$.

Source	Amount CO_2	Rate CO_2	Amount ^{15}N-N_2	Rate ^{15}N-N_2
C addition	20.0**	0.661	107**	15.2**
C addition × habitat	0.018	1.69	0.007	0.097
C addition × depth	0.069	0.030	1.21	0.037
C addition × habitat × depth	0.013	0.147	1.14	0.15

** $p \leq 0.001$.

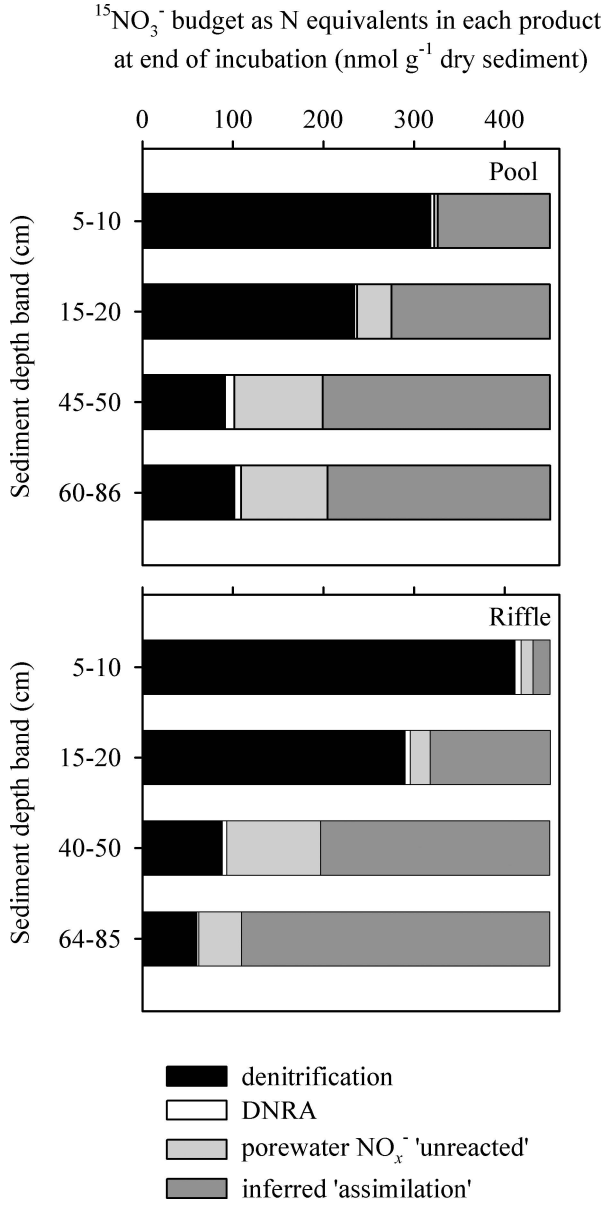

Fig. 5. Fate of the 1200-nmol $^{15}NO_3^-$ spike added to hyporheic sediments recovered from pools and riffles. Anammox is not included because anammox potentials measured via the $^{15}NH_4^+$ and $^{14}NO_3^-$ assay were negligible (*see* Table 4). Data shown are the mean of at least three measurements. Error ranged from < 1% for mean DNRA values to < 15% for the means of denitrification and inferred assimilation.

and, under these relatively more stable redox conditions, anammox is sustained (Dalsgaard et al. 2005).

Availability of carbon in complex organic substrates controls pathways of nitrate reduction—Despite the scarcity of particulate organic carbon in our hyporheic sediments, the total amount of aerobically produced CO_2 suggested sufficient bioavailable C to fuel denitrification. For example, the sediments contained 236 nmol of aerobically 'bioavailable' C g^{-1} (dry sed) on average, which could, in theory, sustain the average rate of denitrification potential (2.3 nmol N g^{-1} [dry sed] h^{-1}) for 81 h. The addition of a spike of bacteriological peptone to the $^{15}NO_3^-$-amended slurries was intended to test the carbon dependence of denitrification within the hyporheic sediments. Concomitant addition of the same amount of organic carbon to sediments where aerobic production of CO_2 was being quantified also provided a basis from which the relative ability of microbial communities to metabolize a complex mixture of organic substrates could be assessed. If we had added a simpler, more labile form of carbon to the incubated sediment (for example, glucose) as per the assay of Tiedje (1988), it is unlikely that substantial differences in carbon processing and, as a consequence, denitrification efficiency, between sediment strata would have been revealed (Findlay et al. 2003).

Not surprisingly, following the addition of bacteriological peptone to the hyporheic sediments, CO_2 evolution within the aerobic slurries increased. In sediments from 5 cm to 20 cm, we measured far greater amounts of CO_2 production than the 920 nmol of carbon added, with this 'excess' decreasing with depth (*see* Fig. 4c; however, highest yields are not shown because the $^{15}NO_3^-$ spike was converted to ^{15}N-N_2 before the organic substrate addition within these sediments). Hence, some forms of organic C may be present in the sediment but remain inaccessible to the microbial community because of co-limitation, by access to amino acids or inorganic elements, for example (Sobczak and Findlay 2002). Alternatively, the addition of bacteriological peptone provided energy that the microbial consortium was able to exploit in order to gain access to previously refractory forms of organic carbon (Bull 1980; Kaplan and Newbold 1995). In sediments recovered from ≥ 40-cm depth into the river bed, lower CO_2 yields (~ 50%) indicated that the access of the microbial community to the complex organic substrates was reduced. As a result, heterotrophic processes in the hyporheic zone will become carbon-limited within the deeper sediment strata as resources available to sustain metabolism diminishes.

The addition of organic substrates to the $^{15}NO_3^-$-amended slurries stimulated denitrification (Fig. 4a) and simply confirmed the dominance of heterotrophic denitrification throughout the sediments. Interestingly, the increase in the potential rate of denitrification was similar at each sediment depth (Table 5). Despite the widespread limitation on denitrification by carbon in these sediments, most markedly at depth, there appears to be a resident ubiquitous denitrifying community of comparable density, which, on exposure to organic substrates, is quite capable of denitrifying any available nitrate. Alternatively, the consistent 'step-up' in the potential rate of denitrification across all depths could be explained by lower densities of more 'efficient' bacteria adapted to scarcer organic substrates.

Despite the consistency in the increase in the potential rate of denitrification after the addition of organic carbon, the yield of ^{15}N-N_2 per mole of carbon added decreased with sediment depth. At 5–10 cm, the total amount of ^{15}N-N_2 produced by denitrification was equivalent to 90% of the carbon added as peptone, but this decreased to 30% in the deepest sediments (Fig. 4c). Peptone is a mix of proteins, polypeptides, and simpler amino acids; therefore, we suggest that the denitrifying community at the top of the hyporheic was able to metabolize the majority of the peptone mix into precursor substrates to fuel respiration. Whereas, in the deepest sediments, the community could only metabolize the simpler and less abundant fraction; hence, their yield of ^{15}N-N_2 dropped. A similar decrease was also seen in the yield of CO_2 with depth after the addition of peptone (Fig. 4c). Together this suggests that, at depth in the hyporheic, access to the complex organic substrates was restricted not only to the denitrifying community, but to the microbial community as a whole.

This hierarchy of access to organic substrates with depth in the river bed could be explained if the delivery of organic matter to the hyporheic is predominantly from above (Kaplan and Newbold 1995, 2000; Sobczak and Findlay 2002). Then the initial hydrolysis of complex organic matter will occur in surficial sediments, with ever-simpler fractions percolating down to the strata below, and in turn, being metabolized by a particular microbial community adapted to the predominant substrate at each depth. Hence, if the denitrifying community is truly adapted to simple organic substrates at depth, then for denitrification to occur at depth within the hyporheic, any inputs of dissolved organic substrates from the groundwater below, or through lateral flows, would need to be comparatively simple (e.g., short chain fatty acids). Alternatively, when a source of complex organic substrates enters the deep hyporheic, for example through scour and deposition associated with storm events (Kaplan and Newbold 2000), the denitrifiers would be reliant upon the initial cleaving of substrates by other members of the microbial consortia.

The ability of the denitrifying community to utilize complex organic substrates also influenced the dominant pathway of nitrate reduction. Findlay et al. (2003) observed a similar dependence of metabolic processes on carbon availability in perfusion core and mesocosm experiments. In the top sediment layers of the River Leith, dissimilatory denitrification dominated, but at depth, where denitrifiers became carbon-limited, more of the ^{15}N remained within the NO_x^- pool and, more importantly, appeared incorporated into microbial biomass (Fig. 5). Kelso et al. (1999) inferred a comparable fraction of assimilatory NO_3^- reduction in slurries similar to ours but provided no insight into the mechanism. Here, it is unlikely that the denitrifiers could have assimilated all of the ^{15}N unaccounted for in the sediment slurries, because the energy requirements for assimilation of NO_3^- are high and the amount of ^{15}N assimilated was approximately three-fold higher than the amount of ^{15}N respired to N_2. Hence, assimilation by microorganisms other than denitrifiers appeared significant at depths $\geq$ 40 cm in the river bed. The increased assimilatory reduction of nitrate to organic nitrogen, rather than dissimilatory reduction of nitrate to N_2, has ecological implications. Incorporation of ^{15}N into microbial biomass does not represent a true sink for nitrogen; thus, at depth in the river bed the nitrate-attenuating function of the hyporheic zone is impaired, which could lead to increased export of nitrate downstream.

Our research has focused on changes in biogeochemical function with depth in the hyporheic zone; however, our conclusions could apply equally to lateral flow paths in the hyporheic. Within gravel bars, for example, downwelling surface water carries a complex mixture of organic substrates into the subsurface, which will be progressively metabolized before upwelling occurs (Holmes et al. 1996; Kaplan and Newbold 2000; Sobczak et al. 2003). As such, the total capacity of hyporheic denitrification will most likely be governed by the availability of complex organic substrates as a whole (carbon, amino acids, trace elements, etc.) to the entire microbial community, rather than just the respective availability of either labile or refractory carbon compounds.

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