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Low hyporheic denitrification in headwater streams revealed by nutrient injections and *in situ* gas measurements

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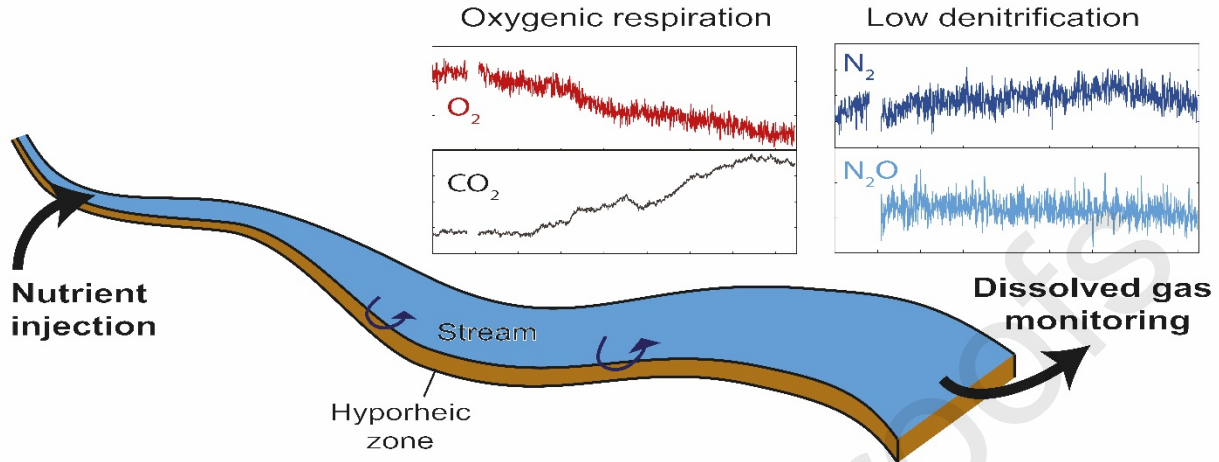
Key words

Denitrification, nitrate, headwater stream, hyporheic zone, membrane inlet mass spectrometry (MIMS)

Highlights

- Continuous gas measurements allow assessing *in situ* biological activity.
- Headwater streams show negligible instream denitrification activity.
- Laboratory assessed denitrifying potential does not ensure *in situ* denitrification.

Graphical abstract



Abstract

Stream networks can retain or remove nutrient pollution, including nitrate from agricultural and urban runoff. However, assessing the location and timing of nutrient uptake remains challenging because of the hydrological and biogeochemical complexity of dynamic stream ecosystems. We used a novel approach to continuously characterize the biological activity in a stream with *in situ* measurement of dissolved gases by membrane inlet mass spectrometry (MIMS). In a headwater stream in western France, we compared *in situ* measurements of O_2 , CO_2 , N_2 , and N_2O (the main gases associated with respiration, including denitrification) with more traditional laboratory incubations of collected sediment. The *in situ* measurements showed near-zero denitrification in the stream and the hyporheic zone. However, the laboratory incubations showed a low but present denitrification potential. This demonstrates how denitrification potential is not necessarily expressed in field hydrological and geochemical conditions. *In situ* measurements are thus crucial to quantify expressed rates of nutrient removal. Broader application of *in situ* gas measurement based on technologies such as MIMS could enhance our understanding of the spatiotemporal distribution of stream and hyporheic processes and overall nutrient retention at stream network scales.

1 Introduction

Headwater streams constitute the largest terrestrial-aquatic interface, and therefore receive globally significant loads of anthropogenic pollutants (Abbott et al., 2019; Bishop et al., 2008; Hannah et al., 2022). As water passes through stream networks, biogeochemical processes can attenuate some of these pollutants through removal or retention (Ebeling et al., 2021; Pinay et al., 2018; Raymond et al., 2016). For some pollutants such as excess nitrogen and phosphorus, the retention and removal in the stream network can amount to a 50% or more decrease in concentration and flux through the stream network (Gruber and Galloway, 2008; Houlton et al., 2019; Severe et al., 2023). Inadequate understanding of where and when this pollutant removal occurs is a major challenge to solving eutrophication globally (Kolbe et al., 2019; Krause et al., 2022; Le Moal et al., 2019).

For nitrate - one of the most abundant forms of anthropogenic nitrogen - denitrification can transform the solute into N_2O and N_2 , removing the reactive nitrogen from the aquatic environment and returning it to the atmosphere (Harvey et al., 2013; Knowles, 1982). Denitrification requires four conditions to occur: hypoxia, an electron donor, denitrifying microorganisms, and the nitrate itself (Abbott et al., 2016; McClain et al., 2003; Oldham et al., 2013). In a stream network, concentration of O_2 is generally high due to constant gas exchange with the atmosphere (Knapp et al., 2015), but low O_2 concentrations can occur in areas that are not in direct contact with the atmosphere, typically the stream bottom sediments, which form the hyporheic zone (Birgand et al., 2007). To a lesser extent, denitrification may also occur in suspended particles in the water column, which sometimes create anoxic micro-zones (Nakajima, 1979).

At the interface between the aquifer and the stream, the hyporheic zone mixes waters with contrasted chemistry and slows down the downstream transport of water, which favors biological reactions (Brunke and Gonser, 1997; Kim et al., 1992; Krause et al., 2011; Triska et al., 1993; Zarnetske et al., 2011). Biological activity is mainly located in the shallowest part of the hyporheic zone, called the "shallow benthic bio layer" (Battin et al., 2003; Briggs et al., 2015; Gomez-Velez et al., 2015; Gonzalez-Pinzon et al., 2012; O'Connor and Harvey, 2008). In streams with gravel, sand and silt beds, the thickness of this reactive benthic layer is often only a few centimeters thick (Arnon et al., 2013; Harvey et al., 2013; Knapp et al., 2017).

The biological activity of the hyporheic zone can be measured at different scales (Krause et al., 2022; Lee-Cullin et al., 2018). Laboratory incubations measure the potential or semi-potential activity of stream bottom sediments (Malone et al., 2018). They are often performed under optimal conditions to assess the amount of extant enzymes for denitrification (Garnier et al., 2010; Lefebvre et al., 2006). The effective activity under actual stream conditions is therefore likely to be lower than the potential activity measured in the laboratory. The biological activity of the hyporheic zone can also be studied *in situ* using mesocosms installed in the stream (Turlan et al., 2007), pore water sampling within the hyporheic zone (Briggs et al., 2013; Harvey et al., 2013; Valett et al., 1996; Zhu et al., 2020), and microelectrodes inserted directly into the sediment (O'Connor et al., 2012). These measurements quantify the biological activity in specific areas, but they are rarely representative of the impact of the entire hyporheic zone on the overall chemical composition of the stream (Harvey et al., 2013; Knapp et al., 2017; Lee-Cullin et al., 2018). The

overall impact of the denitrification occurring in the sediment and in other anoxic zones can be quantified directly by tracer injection experiments at the scale of entire stream reaches (Drummond et al., 2016; Knapp et al., 2017; Mulholland et al., 2002; Valett et al., 1996). The conventional method is to measure the breakthrough curves of injected tracers (Drummond et al., 2012; Gootman et al., 2020). This method frequently causes overestimation of biological activity due to experimental or instrumental truncation of breakthrough curves and associated underestimation of the recovered tracer mass (Drummond et al., 2012). It is therefore of interest to explore alternative methods for measuring actual denitrification at the stream scale, such as nitrogen isotope labeling (Bohlke et al., 2004) or measurement of natural variations in N_2 and Argon (Ar) (Laursen and Seitzinger, 2002; McCutchan et al., 2003).

Here, we developed and tested a novel *in situ* characterization of biological activity based on consumption and production of gases by biogeochemical reactions. We assess the biological activity of the stream and the hyporheic zone by coupling a 6-hour nutrient injection with continuous measurements of gases consumed or produced during oxygenic respiration and denitrification. We injected carbon and nitrogen during several hours and monitored the response of the stream by measuring the downstream evolution of O_2 , CO_2 , N_2 and N_2O concentrations using continuous flow membrane inlet mass spectrometry (MIMS). We compared these *in situ* observations with measurements from laboratory incubations of sediments collected in the streambed to assess how closely measurements of denitrification potential are expressed *in situ*.

2 Material and methods

2.1 Study site

The experiment was conducted in a first-order stream (Le Petit Hermitage) flowing through a forested area in the upstream part of the Pleine-Fougères catchment (Brittany, Western France) (Figure 1). The catchment belongs to the European Long-Term Socio-Ecological research site (LTSER) Zone Atelier Armorique (Thomas et al., 2019). At several locations within the catchment, the potential denitrification activity in sediments was measured under optimal conditions in a previous study by Lefebvre et al. (2006).

The stream has its source in the forest, where there is no direct agricultural nitrogen input, resulting in a nitrate concentration below 1 mg L^{-1} and a nitrate isotopic signature indicative of natural N fixation (Severe et al., 2023). The low natural concentration permits a significant increase of the nitrate concentration during the experiment with a small amount of nitrogen injected. This improves the quality of the signal and reduces the impact of the injection on the environment.

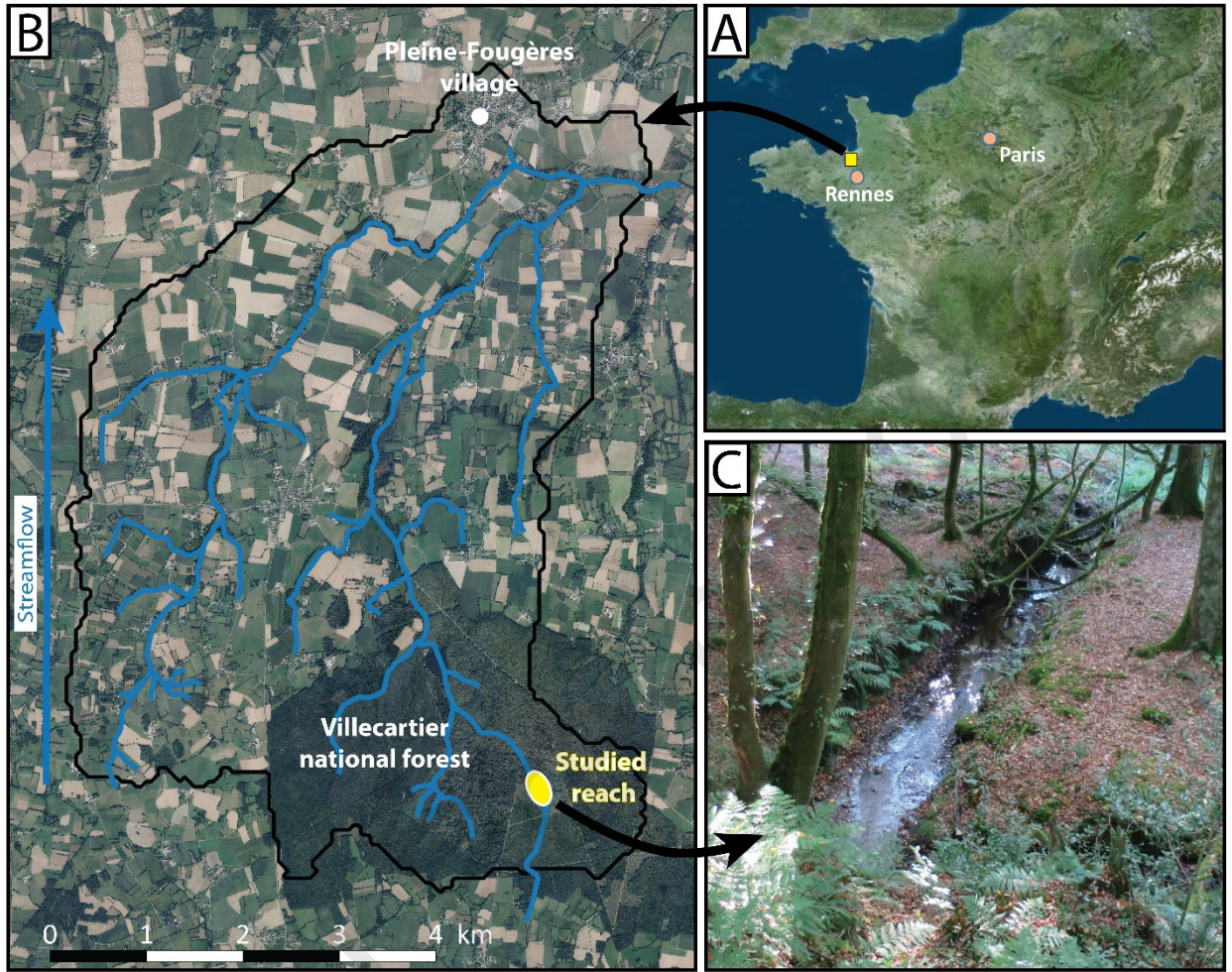


Figure 1. The experiment was conducted on a first-order stream flowing through a forest free of anthropogenic nitrogen inputs. The baseline nitrate concentration was equal to 0.9 mg/L.

We measured temperature, discharge, mean velocity, and atmospheric gas exchange rate coefficient (Table 1). Temperature was measured directly within the membrane inlet mass spectrometer (MIMS). Discharge and mean velocity were measured using a slug injection of salt. At the injection site, 200 g NaCl was dissolved in 20 L stream water and injected at once into the stream. At the measurement site, the conductivity was measured continuously by a Hatch probe to obtain the breakthrough curve. The conductivity curve was used to calculate the mean velocity. The conductivity was converted into NaCl concentration using a calibration realized in the laboratory, and the discharge Q was calculated by the following formula:

$$Q = \frac{m_{\text{injected}}}{\int C_{\text{NaCl}}(t) dt} \quad (1)$$

where m_{injected} is the injected salt mass and C_{NaCl} is the salt concentration measured through time.

The gas exchange rate coefficient was measured by coupling a slug injection of NaCl to a slug injection of helium. The canister containing the NaCl solution was saturated with helium by bubbling before being injected into the stream. Helium was measured continuously at the measurement site by MIMS. The method of injection, measurement, and calculation of gas exchange rate coefficient is presented in Vautier et al. (2020a).

Table 1. Characteristics of the studied stream.

Parameter	Measured value
Length of the studied reach (m)	160
Mean velocity (m min^{-1})	3.2
Discharge (L s^{-1})	6
Gas exchange coefficient (d^{-1})	30
Temperature ($^{\circ}\text{C}$)	12.3 – 12.9
Nitrate concentration (mg L^{-1})	0.9

2.2 Injections of nutrients

An injection of potassium nitrate (KNO_3) and anhydrous sodium acetate (NaCH_3COO) was performed continuously for 6 hours. Carbon (C) and nitrogen (N) were injected in equal amount. Sodium bromide (NaBr) was co-injected as a conservative tracer. The injection solution was prepared by dissolving the three molecules in a 360 L can filled with stream water. The concentration of the injection solution (Table 2) was calculated to achieve an average NO_3^- concentration of 6 mg L^{-1} during the experiment, while the background NO_3^- concentration was 0.9 mg L^{-1} . The solution was injected into the stream using a peristaltic pump. The average injection rate was close to 1 L min^{-1} but varied somewhat over time due to instrumental drift. However, since the three injected molecules (acetate, nitrate, bromide) were dissolved in the same solution, the proportion of each molecule remained constant during the injection. Injecting a conservative tracer together with the nutrients allowed comparison of upstream and downstream concentrations despite the instability of the injection rate. The injection lasted 6 hours, allowing the water to flow through the hyporheic zone and return to the stream since the water residence

time in the hyporheic zone of similar streams was estimated between 45 and 246 minutes (Knapp et al., 2017).

Table 2. Nutrient concentrations during the injection. The target anion concentration in the stream is given for an injection rate of 1 L min⁻¹. The baseline anion concentration corresponds to the concentration measured in the stream before the injection.

Dissolved molecule	Chemical formula	Mass in 360 L (g)	Anion of interest	Baseline anion concentration (mg L ⁻¹)	Target concentration (mg L ⁻¹)
Potassium nitrate	KNO ₃	997	NO ₃ ⁻	0.9	6.0
Anhydrous sodium acetate	NaCH ₃ COO	405	CH ₃ COO ⁻	-	-
Sodium bromide	NaBr	38.6	Br ⁻	0.094	0.30

2.3 *In-situ* gas measurements

The originality of this study was to measure *in situ* the reactants and products of biochemical reactions. Oxygenic respiration was followed by measuring CO₂, its final product, and O₂, the reactant. Denitrification was traced by measuring N₂, its final product, and N₂O, an intermediate product. Measurements were performed at the monitoring site, located 160 m downstream the injection site. The gases were measured continuously by continuous flow membrane inlet mass spectrometry (MIMS) installed in a mobile laboratory vehicle, a few meters away from the stream. Stream water was pumped (MP1 Grunfoss pump, 5 L min⁻¹) and brought continuously to the spectrometer by hermetic tubes. Each gas of interest was measured at a time step of approximately one minute by the MIMS. Monitoring the gases in real time avoided the risk of N₂O degradation between sampling and measurement.

The MIMS used in this experiment was modified from the HPR40 system (Hidden Analytical). In this configuration, the membrane (X44® 99) ensuring the inlet of the gases is connected to the vacuum of a Quadrupole Mass Spectrometer (QMS around 10⁻⁵ Torr). Gases are ionized in the QMS by an oxide coated iridium filament allowing the selection of ionization energies (4 – 150 eV) and emission intensities (20 – 5000 µA). After ionization, the quadrupole separates the gases based on their mass to charge ratios. The gases are detected by a Faraday cup or a single channel electron multiplier (SCEM).

Water vapor pressure, Ar partial pressure and temperature were also measured by the mass spectrometer and allowed to normalize the measurements. The values given in partial pressure by the spectrometer were then converted to dissolved gas concentrations by external calibration with gas chromatography measurements (μGC) performed on water samples. Two 500 mL glass bottles intended for μGC analysis were filled every hour at the outlet of the MIMS. To avoid any exchange of gas with the atmosphere, the bottles were immersed in a bucket during the filling. Measurements with μGC were realized within 24 hours following the experiment. The measurement uncertainty of the MIMS is 0.2% for major gases (N_2 , O_2 , CO_2 , Ar) and 2% for low concentration gases (N_2O , He). The measurement uncertainty of the μGC used for calibration is 3%. More details on MIMS measurement, correction and calibration are given in Chatton et al. (2017).

2.4 Dissolved chemistry

Chemical analysis of dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), and anions (including NO_3^- and Br^-) was performed on samples taken before and during the injection at a timestep of one hour, together with the samples for the analysis of dissolved gases by chromatography. Samples were taken simultaneously at the monitoring site (160 m downstream of the injection site) and close to the injection site (15 m downstream of the injection site to allow the solute to mix in the stream water). The samples were filtered at $0.2\ \mu\text{m}$ directly after sampling, then preserved at 4°C until analysis. The anion concentrations were measured by ion chromatography (ThermoScientific DIONEX DX 120). Dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC) were measured by a carbon analyzer (Shimadzu TOC-VCSH) associated with an autosampler (Shimadzu ASI V). Values are accurate to $\pm 1.2\ \text{mg L}^{-1}$ for nitrate, $\pm 0.6\ \text{ppm}$ for DOC and to $\pm 0.4\ \text{ppm}$ for DIC.

2.5 Nitrate reduction rate determination

Nitrate reduction rates were determined in stream sediments (0 – 2 cm in duplicate) collected at the site adjacent to the nutrient injection site (figure 1). Sediments were placed in flow through reactors, i.e. Plexiglas® rings of 2 cm height and an inner diameter of 42 mm. The sediments were covered with $0.2\ \mu\text{m}$ pore size nitrocellulose filters and glass fiber filters (1.2 mm thick, 47 mm diameter) at each end and O-rings to prevent leakage. The sediments were then supplied with inflow solutions consisting of milliQ water containing KNO_3 (2.5 mM) and acetate (2 mM) via a peristaltic pump (Minipuls, Gilson) with a continuous flow rate of $1.7 \pm 0.1\ \text{ml h}^{-1}$. Anoxic inflow conditions were achieved by bubbling the inflow solutions with N_2 gas for 10 minutes. The reactors experiments were performed at a constant temperature ($20 \pm 0.5^\circ\text{C}$) and in the dark to prevent oxygen production by photosynthesis. The reactor outflow solution was sampled at 4-hour intervals during the day over a period of 48 hours. These outflow samples, 6 per reactor, were analyzed for nitrate and nitrite. Nitrate reduction and nitrite production rates were calculated by the difference in the inorganic nitrogen species in the inflow and outflow solutions (mmol L^{-1}), the flow rate (ml L^{-1}) and the amount of sediment present in the reactors (g dry sediment), as shown in Figure 2. The nitrate reduction rates obtained here were used as a proxy for denitrification rates assuming complete nitrate reduction to N_2O and N_2 and a minor or negligible contribution of other nitrate reducing processes (e.g. DNRA, dissimilatory nitrate reduction to ammonium or Anammox, anaerobic ammonium oxidation). A detailed description of the flow through reactor set up and rate calculations can be found in Laverman et al. (2012).

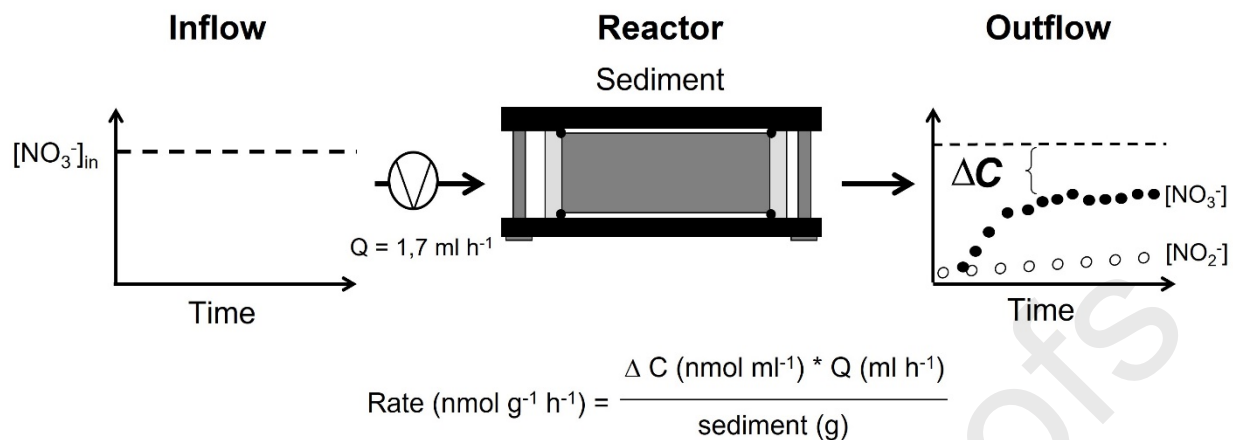


Figure 2. Schematic overview of the flow through reactors allowing the determination of nitrate reduction rates. The reactor contains sediment of a known volume and weight and is supplied with an anoxic inflow solution containing nitrate (NO_3^-) at a known flowrate (Q). The nitrate reduction rates are obtained from the measured concentration difference between inflow and outflow NO_3^- (ΔC) and divided by the amount of sediment (g dry weight) in the reactor cell. The NO_2^- production rates are determined similarly from the output NO_2^- concentrations (no NO_2^- is supplied).

3 Results

3.1 Evolution of the gas content in the stream

O₂, CO₂, N₂, and N₂O concentrations measured during the continuous injection of sodium acetate (NaCH₃COO) and potassium nitrate (KNO₃) are shown in Figure 3. During the injection, the CO₂ concentration increased and the O₂ concentration decreased, attributable to acetate stimulating oxygenic respiration. The increase in CO₂ concentration was more pronounced than the decrease in O₂, relative to baseline concentrations. The higher abundance of O₂ compared to CO₂ in the atmosphere appeared to buffer O₂ variations thanks to equilibration of O₂ concentrations with the atmosphere. The concentrations did not reach a plateau, potentially because of the instability of the injection rate. The N₂ and N₂O concentrations did not vary during the injection. Thus, the injection of potassium nitrate did not significantly stimulate denitrification in the stream.

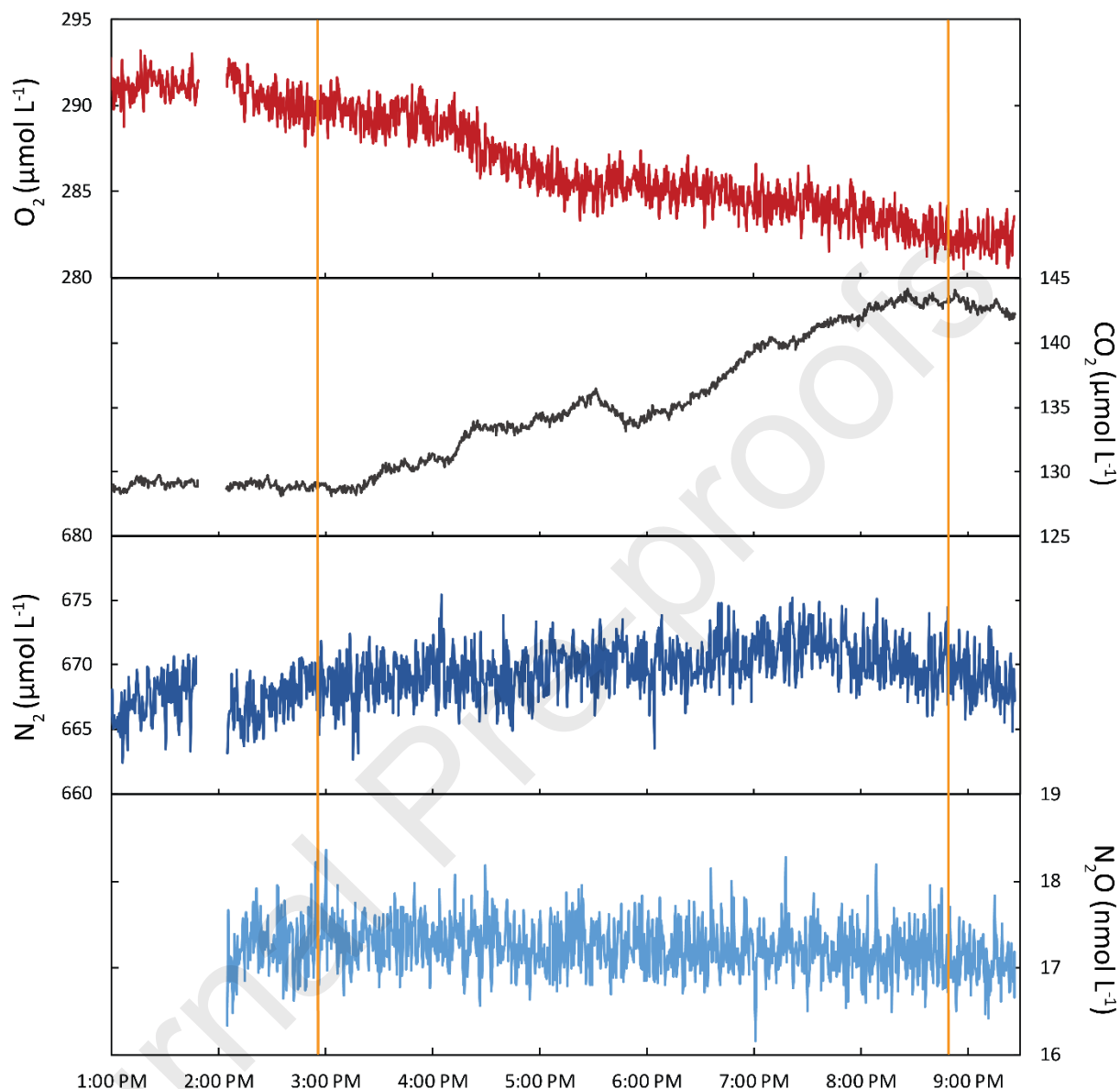


Figure 3. Changes in CO_2 , O_2 , N_2 et N_2O concentrations during the continuous injection of sodium acetate and potassium nitrate. The beginning and the end of the injection are indicated with orange lines. Differences between these upstream and downstream NO_3^- , DOC and DIC concentrations materialize the potential reactions occurring in the studied river reach.

3.2 Carbon and nitrate in the stream

The concentration changes of NO_3^- and dissolved carbon were consistent with the trends observed for gases. Figure 4 shows NO_3^- , DOC, and DIC as a function of the conservative tracer Br^- at the measurement and injection sites. The concentrations did not reach a plateau, neither at the measurement site nor at the injection site, again likely due to the instability of the injection rate. Between upstream and downstream, the dissolved organic carbon (DOC) concentration decreased while the dissolved inorganic carbon (DIC) increased, in comparison with the

conservative tracer Br^- . This is consistent with a degradation of organic carbon into inorganic carbon along the stream reach, in this case with the consumption of injected acetate by oxygenic respiration. In contrast, the ratio between NO_3^- and Br^- concentration remained the same between upstream and downstream, even if the concentrations of both elements were lower downstream due to dispersion. This is consistent with the absence of NO_3^- degradation along the reach.

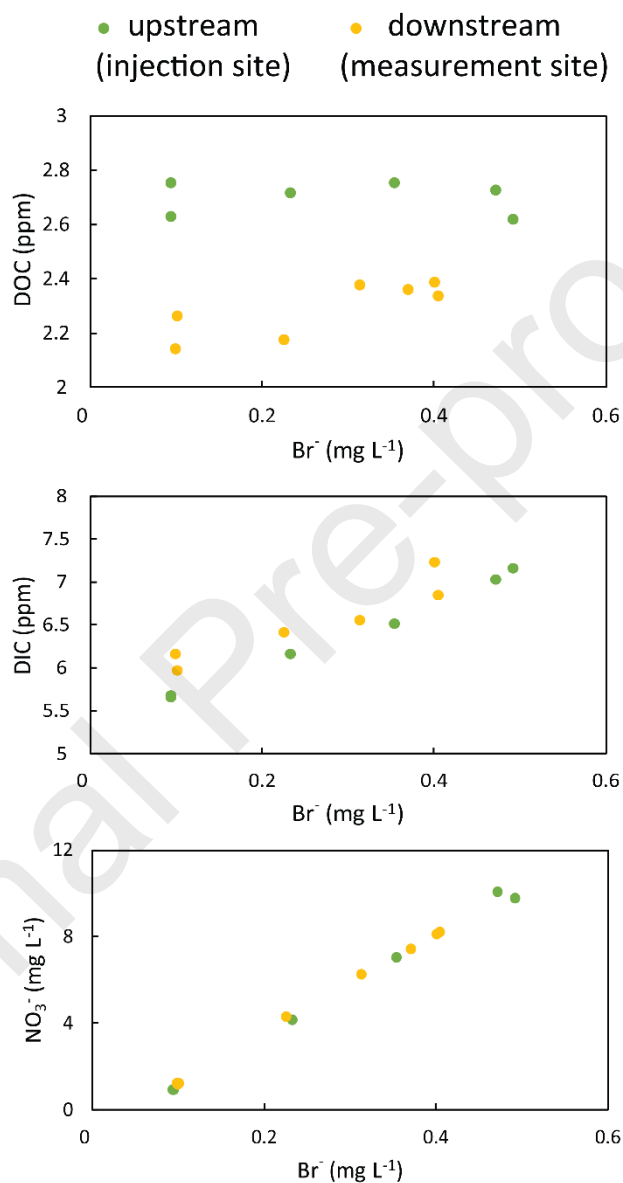


Figure 4. NO_3^- , DOC, and DIC concentrations as a function of conservative tracer concentration Br^- during continuous injection of sodium acetate and potassium nitrate.

3.3 Nitrate reduction rate in the sediment

The nitrate reduction and nitrite production rates determined in flow-through reactors containing stream sediments supplied with nitrate or nitrate and acetate are shown in Figure 5. Average nitrate reduction rates were $12.6 (\pm 3.9)$ nmol gram dry sediment⁻¹ h⁻¹ with nitrate and

10.4 (± 3.2) nmol gram dry sediment⁻¹ h⁻¹ when nitrate and acetate were supplied to the sediments. The rates were averaged over the duplicate reactors and the different sampling points during the incubation (6 sampling points, see section 2.5). Some nitrite production was observed (Figure 5) during nitrate reduction, with somewhat higher nitrite production when nitrate was supplied alone (2.3 ± 1.9) compared to the nitrite production in sediments that were supplied with nitrate and acetate (0.5 ± 0.3). The differences were not significantly different neither for nitrate reduction rates, nor for the nitrite production rates. Ammonium concentrations in the outflow of the reactor were below the detection limit, therefore no ammonium production (release) rates were deduced.

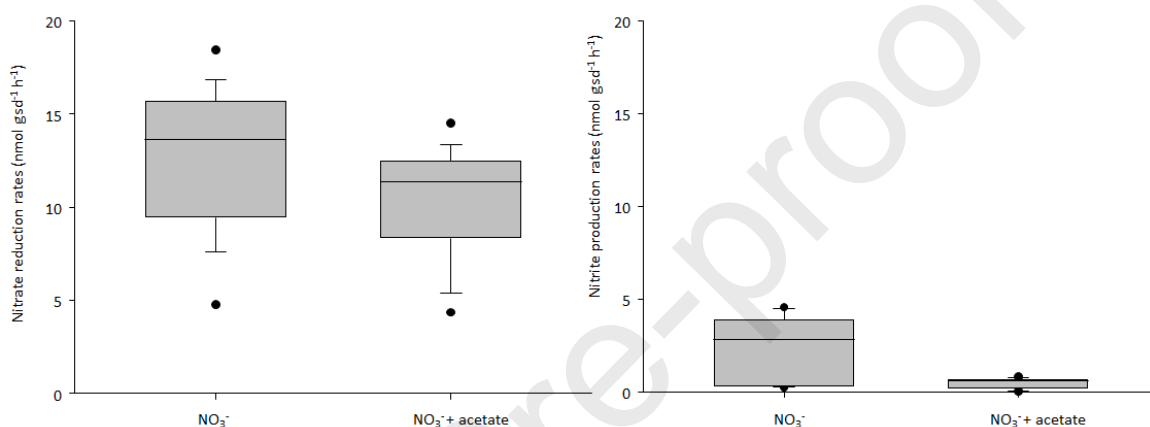


Figure 5. Box plots of nitrate reduction (left) and nitrite production (right) rates in the sediments supplied with nitrate or nitrate and acetate. Boxes encompass the upper and lower quartiles while the line indicates the median, dots are outliers.

4 Discussion

4.1 Assessment of low *in-situ* denitrification

During the 6 hours of nitrate and acetate continuous injection, the clear increase in CO₂ and decrease in O₂ during the injection, combined with a consumption of organic carbon and production of inorganic carbon, is evidence of oxygenic respiration. On the other hand, neither N₂ or N₂O production nor NO₃⁻ consumption was observed. This suggests a very low denitrification activity in the stream and the hyporheic zone.

It could be argued that N₂ atmospheric partial pressure is so high that its potential production through denitrification could be hidden by the chemical equilibrium between stream and atmosphere. However, this effect should not affect the N₂O signal, since N₂O atmospheric partial pressure is very low.

It can be reasonably assumed that the water had time to circulate through the hyporheic zone and flow back to the stream during the experiment. Indeed, the duration of injection (6h) was 1.5 to 8 times longer than the residence time in the hyporheic zone estimated by Knapp et al. (2017) for a streambed composed of gravel, sand and silt (45 to 246 minutes). The hyporheic residence

time evaluated by Gootman et al. (2020) in first and second order streams is even shorter (1.8 seconds to 7.9 minutes). Thus, our results indicate a low denitrification activity not only in the water column but also within the hyporheic zone.

4.2 Comparison between potential and *in-situ* denitrification

The incubation of the stream sediments showed nitrate reducing activity, accompanied with some nitrite production. The nitrate reduction rates were not stimulated by the addition of organic carbon (acetate). These rates were low compared to rates obtained using the same experimental approach in other soils and sediments. In first and fourth order river stream sediments nitrate reduction rates reached 150 and 180 nmol gds h⁻¹ (Laverman et al., 2012; Yan et al., 2013) respectively, rates that are an order of magnitude higher than found in the current study. The low nitrate reduction rates and the lack of effect of the addition of acetate on these rates suggests a small denitrifying bacterial population. Furthermore, the lack of ammonium release from the sediments indicates very low overall activity in the sediment; the degradation of organic matter results in the production and release of ammonium which was below the detection limit. This is also in line with a modest denitrifying population and activity most likely due to low available organic carbon present in the streambed.

The rates determined in the intact sediments were comparable to those previously obtained by Lefebvre et al. (2006) in the catchment of the studied stream. In the latter, incubations were carried out under optimal conditions for denitrification: anoxia, glucose, and nitrate in optimal proportions (Smith and Tiedje, 1979), temperature of 20°C, frequent agitation. The potential denitrification enzyme activity (DEA) was measured by the acetylene inhibition technique (Yoshinari and Knowles, 1976). The range of potential DEA ranged between 6 and 39 nmol N₂ g dry sediment h⁻¹, which is comparable to the rates found in the current study.

This low yet measurable potential activity in the laboratory does not translate into a measurable effective denitrification *in situ*. The actual conditions of oxygenation, temperature, and probability of substrate-bacteria encounter do not allow the denitrification potential to be expressed in the stream. Thus, predicting the biological activity of the stream based on laboratory experiments should be taken with caution. The potential activity measured in the lab gives the maximum value of the effective activity, which can be far from being reached *in situ*. In a comparative study between mesocosms installed in the stream and in the laboratory, Turlan et al. (2007) showed that the rate of nitrate consumption measured in the stream was significantly lower and more variable than the rate measured in the laboratory, even if the laboratory experiments were conducted in a setup very similar to the stream, under non-optimal conditions. By combining a study at the whole stream scale and at the scale of specific profiles of the hyporheic zone, Knapp et al. (2017) further showed that the global rate of degradation of the tracer resazurin measured at the stream scale was significantly lower than the rate measured at a smaller scale in the hyporheic zone. These studies are consistent with our results and emphasize the importance of measuring biological activity rates at the scale at which they are used.

4.3 Hypothesis for the low *in situ* denitrification

The CO₂ and O₂ measurements show that the stream has measurable oxygenic respiration activity, but very little denitrification activity. This in line with the fact that O₂ is a better electron

acceptor than NO_3^- so that in the presence of O_2 and NO_3^- , oxygenic respiration is more favorable than denitrification (Knowles, 1982). The strong gas exchange with the atmosphere, which provides permanent re-oxygenation of the stream (Hall et al., 2016; Jahne and Haussecker, 1998), thus explains the low denitrification in the water column. However, the injection lasted 6 h, meaning that the water had time to circulate through the hyporheic zone (Knapp et al., 2017), a zone theoretically preserved from direct contact with the atmosphere (Krause et al., 2011). Our experiment indicates a low impact of hyporheic processes on the chemical composition of the stream.

The low impact of the hyporheic processes has several plausible explanations. A first one would be the low available amount of organic carbon being responsible for the low intrinsic activity of the hyporheic zone. Indeed, sediment organic carbon content has been shown to be a major control of potential denitrification rate (Wu et al., 2021). In our case, during the incubation of stream sediment in the laboratory, the addition of acetate did not increase nitrate reduction rates. This suggests the presence of a small denitrifying bacterial population, probably due to a low carbon content of the sediment as nitrate concentrations in the stream are high and not a limiting factor. The low amount of ammonium released from the sediment also indicates a low level of carbon degradation, and therefore probably a low initial amount of carbon in the sediment. Another explanation for low hyporheic activity would be that biological activity is concentrated in the benthic reactive layer, the surface portion of the hyporheic zone. Yet the thickness of the benthic reactive layer has been estimated being only 2 cm by Knapp et al. (2017). In small stream beds, the first few centimeters of sediment can be unstable (Schipka and Pavan, 2009). A sudden increase in flow driven by a large rainfall event can displace stream bottom sediments, especially if they are loosely joined, such as gravels. Sediment instability has a strong impact on biological activity (Atkinson et al., 2008). It favors the renewal of pore water and thus limits the development of anoxic zones conducive to denitrification, although anoxic micro-niches seem to be able to develop in some oxygenated sediments (Briggs et al., 2015). Finally, the metabolic activity of the streambed is influenced by the strength and direction of the water exchanges between the stream and the hyporheic zone (Wang et al., 2022). The recirculation of stream water into the sediments is particularly limited in gaining streams (Boano et al., 2008; Caruso et al., 2016; Trauth et al., 2014), that are streams fed by resurgent groundwater, which exerts an ascending pressure on the hyporheic zone. The resulting residence time in the hyporheic zone may be too short for substantial denitrification to occur. It is likely that denitrification occurs specifically at critical hydrological moments (eg flood events) (Singh et al., 2022). The impact of hyporheic processes on stream chemistry not only depends on the denitrification potential of the hyporheic zone, but also on the time the water spends in the hyporheic zone (Bech et al., 2023; Trauth et al., 2014).

4.4 Interest of *in-situ* gas measurement for microbial activity assessment

In situ measurements of gaseous reactants and products of biochemical reactions (O_2 , CO_2 , N_2O , N_2) allowed to assess the occurrence of oxygenic respiration as well as the absence of denitrification in the stream and its hyporheic zone. This method is a promising alternative to the classical exploitation of breakthrough curves to assess the biological activity at the scale of a whole stream reach. It offers the opportunity to inject nutrients over a long period of time (6 h in this case), giving the natural system time to react. In the present study, the main advantage of an

injection lasting several hours was to allow the water to recirculate through the hyporheic zone. Such injections could also be applied to wells to assess the biological activity in groundwater (Bochet et al., 2020; Frei et al., 2020).

Continuous flow membrane inlet mass spectrometry (MIMS) allows visualization in real time of the biological response of the entire stream corridor to nutrient injections or in natural conditions. The experiment realized here is a new example of the wide range of applications of this technology (Burlacot et al., 2020; Chatton et al., 2017; Giroud et al., 2023; Klaus et al., 2022; Vautier et al., 2020a, 2020b; Weber et al., 2019). This opens prospects for application to other hydrological systems (e.g. streams with varied substrates, larger rivers, groundwater), which would enable advancement towards a broad characterization of biological activity in terrestrial aquatic ecosystems. Furthermore, this method allows direct assessment of N₂O emissions, thus improving overall estimates of N₂O emissions from terrestrial aquatic ecosystems.

5 Conclusion

Nutrient injections combined with continuous measurements of dissolved gases using mass spectrometry (MIMS) allowed to assess the *in situ* biological activity of a headwater stream and its hyporheic zone. While oxygenic respiration was revealed by O₂ consumption and CO₂ production, the absence of N₂ or N₂O production signed a very low denitrification activity. The conclusions obtained based on gas measurements were confirmed by dissolved organic and inorganic carbon as well as NO₃⁻ concentrations. Low *in situ* denitrification can be explained by low carbon availability in the streambed sediment, by the instability of the streambed, and by the limited infiltration into the hyporheic zone.

Laboratory incubations of sediments carried out in parallel with field measurements revealed a low yet measurable potential denitrification activity in the sediments. This potential activity was not expressed *in situ*. This highlights the difference between potential activity in the laboratory and actual activity *in situ*. Depending on the stream's geochemical and hydrological conditions, *in situ* activity is likely to be lower than potential activity. *In situ* field measurements are crucial to assess the effective denitrification activity.

The originality of the proposed method is to measure the reactants and products of biological reactions (O₂, CO₂, N₂O, N₂) instead of exploiting the breakthrough curves of the injected tracers. This experiment shows that membrane inlet mass spectrometry (MIMS) offers new avenues for assessing *in situ* biological activity, especially denitrification, in terrestrial aquatic ecosystems. Along with the assessment of their biological activity, it further allows a direct measurement of the gases produced by the aquatic ecosystems, especially greenhouse gases such as CO₂ or N₂O. This opens a way to better understand and quantify global emissions of greenhouse gases due to biochemical activity in inland waters.

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Journal Pre-proofs

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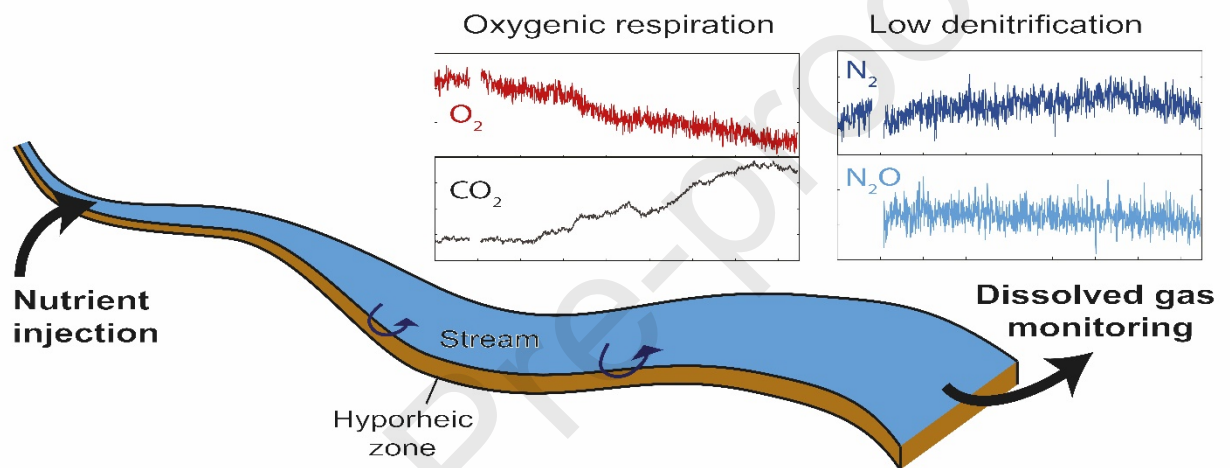
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Abstract

Stream networks can retain or remove nutrient pollution, including nitrate from agricultural and urban runoff. However, assessing the location and timing of nutrient uptake remains challenging because of the hydrological and biogeochemical complexity of dynamic stream ecosystems. We used a novel approach to continuously characterize the biological activity in a stream with *in situ* measurement of dissolved gases by membrane inlet mass spectrometry (MIMS). In a headwater stream in western France, we compared *in situ* measurements of O₂, CO₂,

N₂, and N₂O (the main gases associated with respiration, including denitrification) with more traditional laboratory incubations of collected sediment. The *in situ* measurements showed near-zero denitrification in the stream and the hyporheic zone. However, the laboratory incubations showed a low but present denitrification potential. This demonstrates how denitrification potential is not necessarily expressed in field hydrological and geochemical conditions. *In situ* measurements are thus crucial to quantify expressed rates of nutrient removal. Broader application of *in situ* gas measurement based on technologies such as MIMS could enhance our understanding of the spatiotemporal distribution of stream and hyporheic processes and overall nutrient retention at stream network scales.



Highlights

- Continuous gas measurements allow assessing *in situ* biological activity.
- Headwater streams show negligible instream denitrification activity.
- Laboratory assessed denitrifying potential does not ensure *in situ* denitrification.

Figure 6. The experiment was conducted on a first-order stream flowing through a forest free of anthropogenic nitrogen inputs. The baseline nitrate concentration was equal to 0.9 mg/L.

Figure 2. Schematic overview of the flow through reactors allowing the determination of nitrate reduction rates. The reactor contains sediment of a known volume and weight and is supplied with an anoxic inflow solution containing nitrate (NO₃⁻in) at a known flowrate (Q). The nitrate reduction rates are obtained from the measured concentration difference between inflow and outflow NO₃⁻ (ΔC) and divided by the amount of sediment (g dry weight) in the reactor cell. The NO₂⁻ production rates are determined similarly from the output NO₂⁻ concentrations (no NO₂⁻ is supplied).

Figure 3. Changes in CO₂, O₂, N₂ et N₂O concentrations during the continuous injection of sodium acetate and potassium nitrate. The beginning and the end of the injection are indicated with orange lines. Differences between these upstream and downstream NO₃⁻, DOC and DIC concentrations materialize the potential reactions occurring in the studied river reach.

Figure 4. NO₃⁻, DOC, and DIC concentrations as a function of conservative tracer concentration Br⁻ during continuous injection of sodium acetate and potassium nitrate.

Figure 5. Box plots of nitrate reduction (left) and nitrite production (right) rates in the sediments supplied with nitrate or nitrate and acetate. Boxes encompass the upper and lower quartiles while the line indicates the median, dots are outliers.

CRediT author statement

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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