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

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16 **Key words**

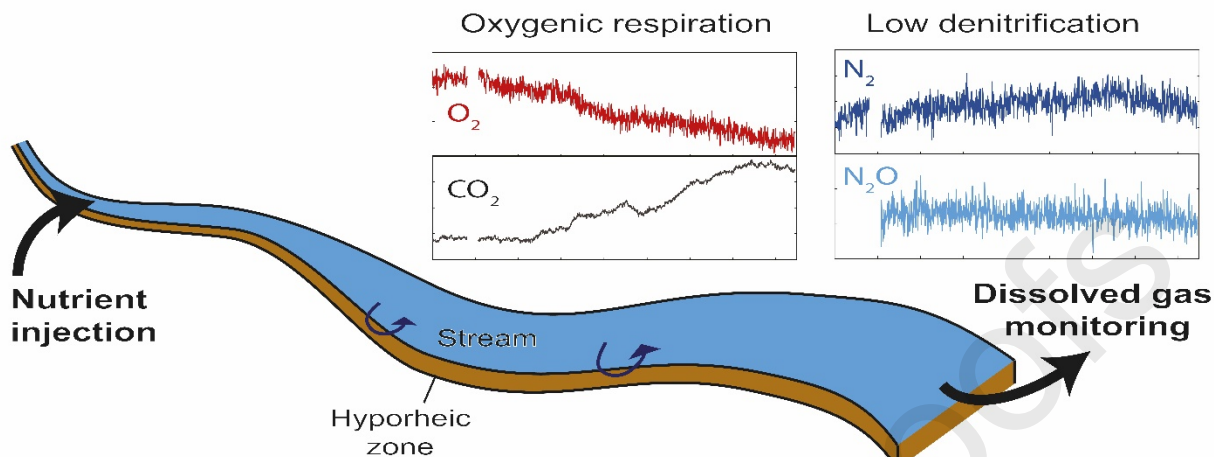
17 Denitrification, nitrate, headwater stream, hyporheic zone, membrane inlet mass
18 spectrometry (MIMS)

19 **Highlights**

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

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Graphical abstract



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25

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Abstract

27 Stream networks can retain or remove nutrient pollution, including nitrate from agricultural
 28 and urban runoff. However, assessing the location and timing of nutrient uptake remains
 29 challenging because of the hydrological and biogeochemical complexity of dynamic stream
 30 ecosystems. We used a novel approach to continuously characterize the biological activity in a
 31 stream with *in situ* measurement of dissolved gases by membrane inlet mass spectrometry
 32 (MIMS). In a headwater stream in western France, we compared *in situ* measurements of O₂, CO₂,
 33 N₂, and N₂O (the main gases associated with respiration, including denitrification) with more
 34 traditional laboratory incubations of collected sediment. The *in situ* measurements showed near-
 35 zero denitrification in the stream and the hyporheic zone. However, the laboratory incubations
 36 showed a low but present denitrification potential. This demonstrates how denitrification potential
 37 is not necessarily expressed in field hydrological and geochemical conditions. *In situ*
 38 measurements are thus crucial to quantify expressed rates of nutrient removal. Broader application
 39 of *in situ* gas measurement based on technologies such as MIMS could enhance our understanding
 40 of the spatiotemporal distribution of stream and hyporheic processes and overall nutrient retention
 41 at stream network scales.

42 1 Introduction

43 Headwater streams constitute the largest terrestrial-aquatic interface, and therefore receive
44 globally significant loads of anthropogenic pollutants (Abbott et al., 2019; Bishop et al., 2008;
45 Hannah et al., 2022). As water passes through stream networks, biogeochemical processes can
46 attenuate some of these pollutants through removal or retention (Ebeling et al., 2021; Pinay et al.,
47 2018; Raymond et al., 2016). For some pollutants such as excess nitrogen and phosphorus, the
48 retention and removal in the stream network can amount to a 50% or more decrease in
49 concentration and flux through the stream network (Gruber and Galloway, 2008; Houlton et al.,
50 2019; Severe et al., 2023). Inadequate understanding of where and when this pollutant removal
51 occurs is a major challenge to solving eutrophication globally (Kolbe et al., 2019; Krause et al.,
52 2022; Le Moal et al., 2019).

53 For nitrate - one of the most abundant forms of anthropogenic nitrogen - denitrification
54 can transform the solute into N_2O and N_2 , removing the reactive nitrogen from the aquatic
55 environment and returning it to the atmosphere (Harvey et al., 2013; Knowles, 1982).
56 Denitrification requires four conditions to occur: hypoxia, an electron donor, denitrifying
57 microorganisms, and the nitrate itself (Abbott et al., 2016; McClain et al., 2003; Oldham et al.,
58 2013). In a stream network, concentration of O_2 is generally high due to constant gas exchange
59 with the atmosphere (Knapp et al., 2015), but low O_2 concentrations can occur in areas that are not
60 in direct contact with the atmosphere, typically the stream bottom sediments, which form the
61 hyporheic zone (Birgand et al., 2007). To a lesser extent, denitrification may also occur in
62 suspended particles in the water column, which sometimes create anoxic micro-zones (Nakajima,
63 1979).

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

72 The biological activity of the hyporheic zone can be measured at different scales (Krause
73 et al., 2022; Lee-Cullin et al., 2018). Laboratory incubations measure the potential or semi-
74 potential activity of stream bottom sediments (Malone et al., 2018). They are often performed
75 under optimal conditions to assess the amount of extant enzymes for denitrification (Garnier et al.,
76 2010; Lefebvre et al., 2006). The effective activity under actual stream conditions is therefore
77 likely to be lower than the potential activity measured in the laboratory. The biological activity of
78 the hyporheic zone can also be studied *in situ* using mesocosms installed in the stream (Turlan et
79 al., 2007), pore water sampling within the hyporheic zone (Briggs et al., 2013; Harvey et al., 2013;
80 Valett et al., 1996; Zhu et al., 2020), and microelectrodes inserted directly into the sediment
81 (O'Connor et al., 2012). These measurements quantify the biological activity in specific areas, but
82 they are rarely representative of the impact of the entire hyporheic zone on the overall chemical
83 composition of the stream (Harvey et al., 2013; Knapp et al., 2017; Lee-Cullin et al., 2018). The

84 overall impact of the denitrification occurring in the sediment and in other anoxic zones can be
85 quantified directly by tracer injection experiments at the scale of entire stream reaches (Drummond
86 et al., 2016; Knapp et al., 2017; Mulholland et al., 2002; Valett et al., 1996). The conventional
87 method is to measure the breakthrough curves of injected tracers (Drummond et al., 2012;
88 Gootman et al., 2020). This method frequently causes overestimation of biological activity due to
89 experimental or instrumental truncation of breakthrough curves and associated underestimation of
90 the recovered tracer mass (Drummond et al., 2012). It is therefore of interest to explore alternative
91 methods for measuring actual denitrification at the stream scale, such as nitrogen isotope labeling
92 (Bohlke et al., 2004) or measurement of natural variations in N₂ and Argon (Ar) (Laursen and
93 Seitzinger, 2002; McCutchan et al., 2003).

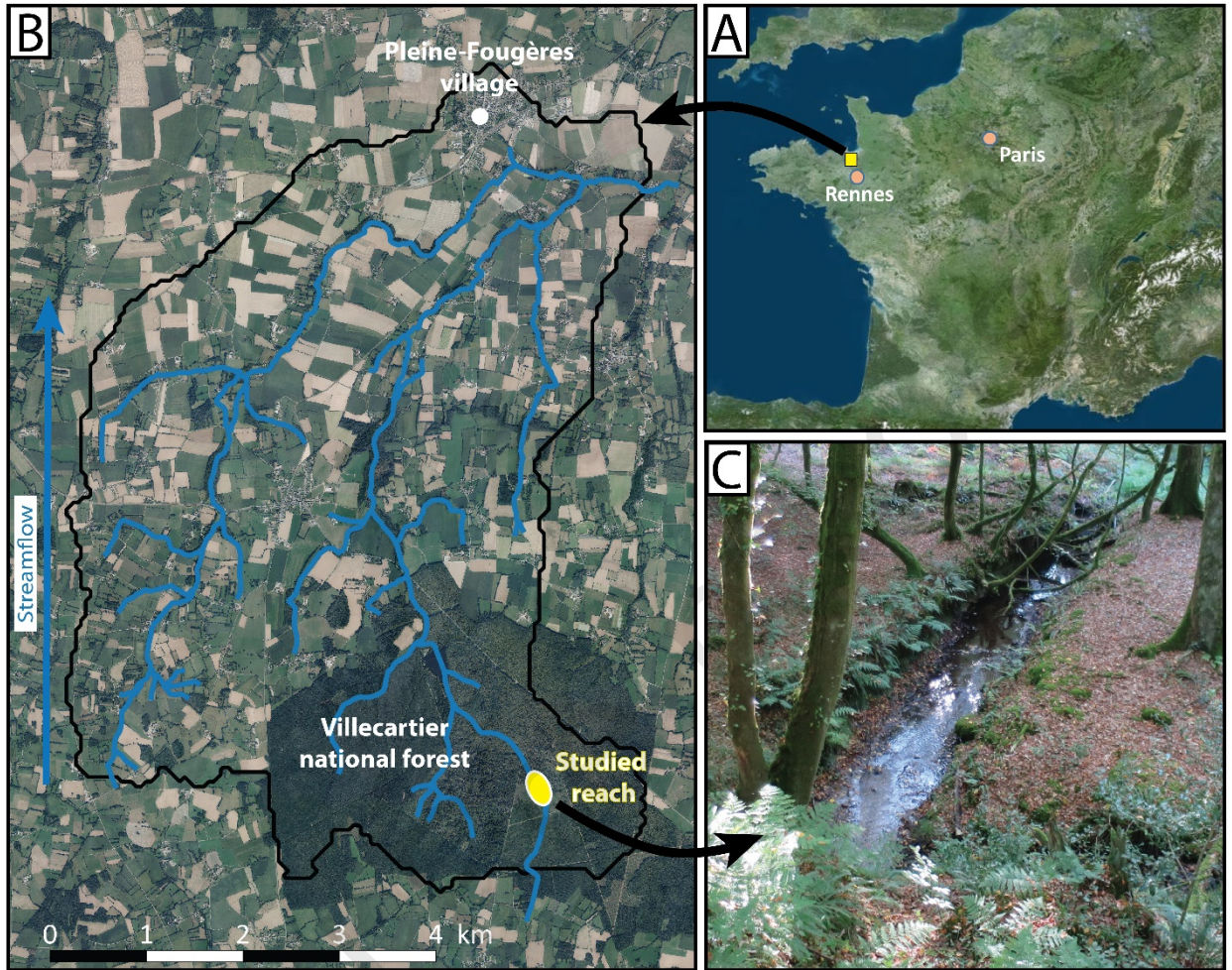
94 Here, we developed and tested a novel *in situ* characterization of biological activity based
95 on consumption and production of gases by biogeochemical reactions. We assess the biological
96 activity of the stream and the hyporheic zone by coupling a 6-hour nutrient injection with
97 continuous measurements of gases consumed or produced during oxygenic respiration and
98 denitrification. We injected carbon and nitrogen during several hours and monitored the response
99 of the stream by measuring the downstream evolution of O₂, CO₂, N₂ and N₂O concentrations
100 using continuous flow membrane inlet mass spectrometry (MIMS). We compared these *in situ*
101 observations with measurements from laboratory incubations of sediments collected in the
102 streambed to assess how closely measurements of denitrification potential are expressed *in situ*.

103 **2 Material and methods**

104 **2.1 Study site**

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

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



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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.

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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:

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

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

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

136 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 (°C)	12.3 – 12.9
Nitrate concentration (mg L ⁻¹)	0.9

137 2.2 Injections of nutrients

138 An injection of potassium nitrate (KNO₃) and anhydrous sodium acetate
139 (NaCH₃COO) was performed continuously for 6 hours. Carbon (C) and nitrogen (N) were injected
140 in equal amount. Sodium bromide (NaBr) was co-injected as a conservative tracer. The injection
141 solution was prepared by dissolving the three molecules in a 360 L can filled with stream water.
142 The concentration of the injection solution (Table 2) was calculated to achieve an average NO₃⁻
143 concentration of 6 mg L⁻¹ during the experiment, while the background NO₃⁻ concentration was
144 0.9 mg L⁻¹. The solution was injected into the stream using a peristaltic pump. The average
145 injection rate was close to 1 L min⁻¹ but varied somewhat over time due to instrumental drift.
146 However, since the three injected molecules (acetate, nitrate, bromide) were dissolved in the same
147 solution, the proportion of each molecule remained constant during the injection. Injecting a
148 conservative tracer together with the nutrients allowed comparison of upstream and downstream
149 concentrations despite the instability of the injection rate. The injection lasted 6 hours, allowing
150 the water to flow through the hyporheic zone and return to the stream since the water residence

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

153 Table 2. Nutrient concentrations during the injection. The target anion concentration in the stream is given
154 for an injection rate of 1 L min⁻¹. The baseline anion concentration corresponds to the concentration measured
155 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

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157 **2.3 *In-situ* gas measurements**

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

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

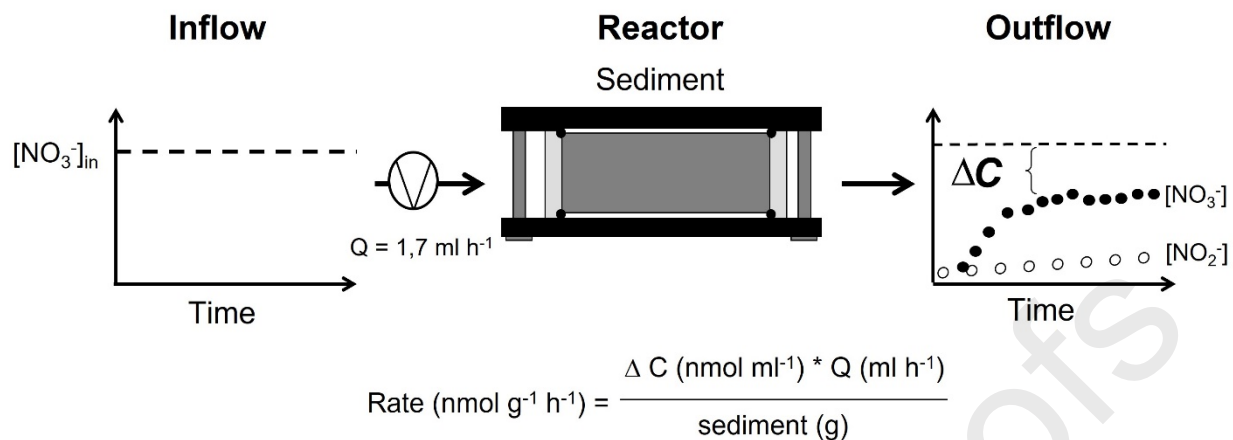
175 Water vapor pressure, Ar partial pressure and temperature were also measured by the mass
176 spectrometer and allowed to normalize the measurements. The values given in partial pressure by
177 the spectrometer were then converted to dissolved gas concentrations by external calibration with
178 gas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

185 **2.4 Dissolved chemistry**

186 Chemical analysis of dissolved organic carbon (DOC), dissolved inorganic carbon
187 (DIC), and anions (including NO_3^- and Br^-) was performed on samples taken before and during the
188 injection at a timestep of one hour, together with the samples for the analysis of dissolved gases
189 by chromatography. Samples were taken simultaneously at the monitoring site (160 m downstream
190 of the injection site) and close to the injection site (15 m downstream of the injection site to allow
191 the solute to mix in the stream water). The samples were filtered at $0.2\ \mu\text{m}$ directly after sampling,
192 then preserved at 4°C until analysis. The anion concentrations were measured by ion
193 chromatography (ThermoScientific DIONEX DX 120). Dissolved organic carbon (DOC) and
194 dissolved inorganic carbon (DIC) were measured by a carbon analyzer (Shimadzu TOC-VCSH)
195 associated with an autosampler (Shimadzu ASI V). Values are accurate to $\pm 1.2\ \text{mg L}^{-1}$ for nitrate,
196 $\pm 0.6\ \text{ppm}$ for DOC and to $\pm 0.4\ \text{ppm}$ for DIC.

197 **2.5 Nitrate reduction rate determination**

198 Nitrate reduction rates were determined in stream sediments (0 – 2 cm in duplicate)
199 collected at the site adjacent to the nutrient injection site (figure 1). Sediments were placed in flow
200 through reactors, i.e. Plexiglas® rings of 2 cm height and an inner diameter of 42 mm. The
201 sediments were covered with $0.2\ \mu\text{m}$ pore size nitrocellulose filters and glass fiber filters (1.2 mm
202 thick, 47 mm diameter) at each end and O-rings to prevent leakage. The sediments were then
203 supplied with inflow solutions consisting of milliQ water containing KNO_3 (2.5 mM) and acetate
204 (2 mM) via a peristaltic pump (Minipuls, Gilson) with a continuous flow rate of $1.7 \pm 0.1\ \text{ml h}^{-1}$.
205 Anoxic inflow conditions were achieved by bubbling the inflow solutions with N_2 gas for 10
206 minutes. The reactors experiments were performed at a constant temperature ($20 \pm 0.5\ ^\circ\text{C}$) and in
207 the dark to prevent oxygen production by photosynthesis. The reactor outflow solution was
208 sampled at 4-hour intervals during the day over a period of 48 hours. These outflow samples, 6 per
209 reactor, were analyzed for nitrate and nitrite. Nitrate reduction and nitrite production rates were
210 calculated by the difference in the inorganic nitrogen species in the inflow and outflow solutions
211 (mmol L^{-1}), the flow rate (ml L^{-1}) and the amount of sediment present in the reactors (g dry
212 sediment), as shown in Figure 2. The nitrate reduction rates obtained here were used as a proxy
213 for denitrification rates assuming complete nitrate reduction to N_2O and N_2 and a minor or
214 negligible contribution of other nitrate reducing processes (e.g. DNRA, dissimilatory nitrate
215 reduction to ammonium or Anammox, anaerobic ammonium oxidation). A detailed description of
216 the flow through reactor set up and rate calculations can be found in Laverman et al. (2012).



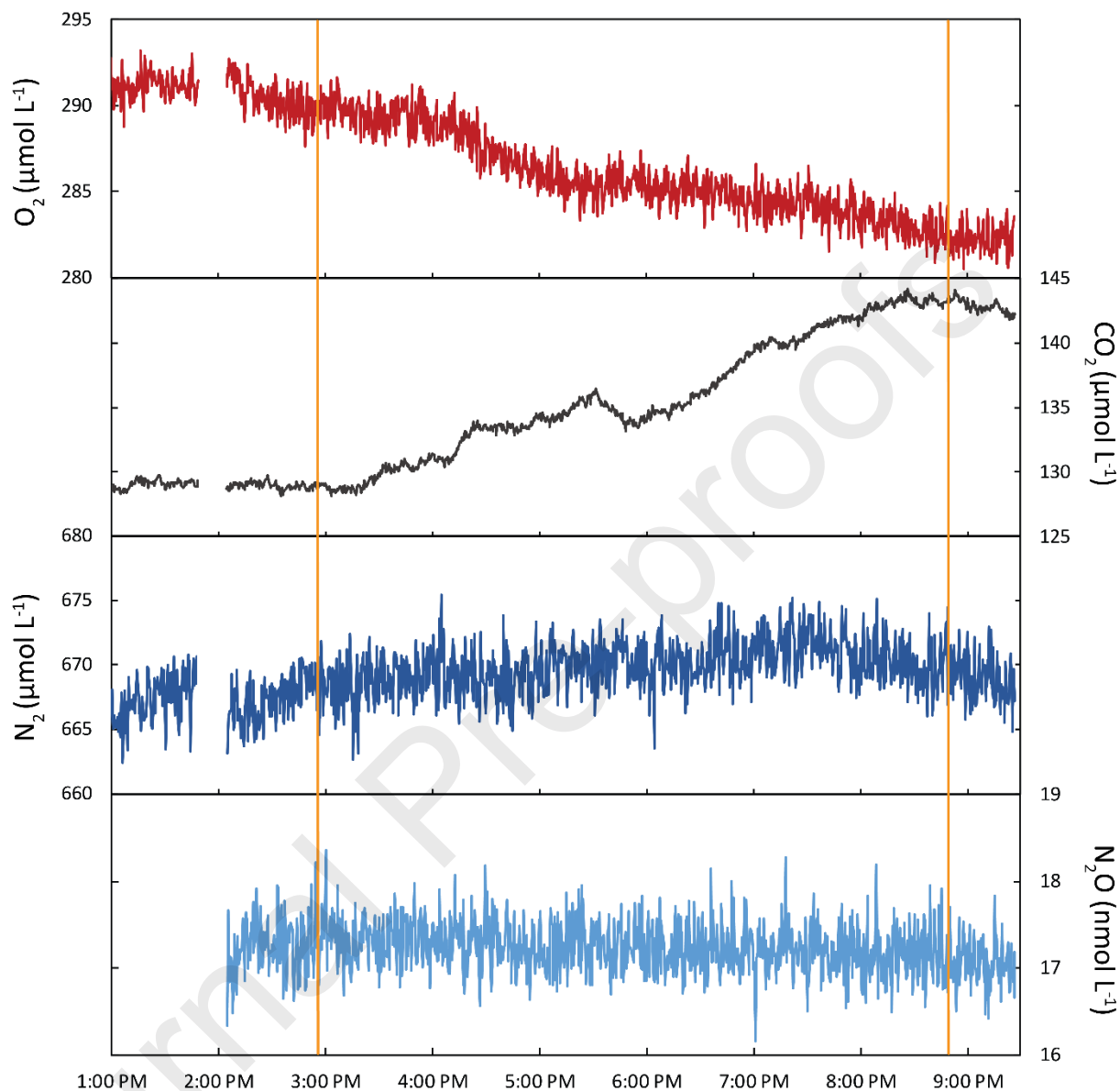
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218 Figure 2. Schematic overview of the flow through reactors allowing the determination of nitrate reduction rates. The
 219 reactor contains sediment of a known volume and weight and is supplied with an anoxic inflow solution containing
 220 nitrate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

223 **3 Results**

224 **3.1 Evolution of the gas content in the stream**

225 O_2 , CO_2 , N_2 , and N_2O concentrations measured during the continuous injection of
226 sodium acetate ($NaCH_3COO$) and potassium nitrate (KNO_3) are shown in Figure 3. During the
227 injection, the CO_2 concentration increased and the O_2 concentration decreased, attributable to
228 acetate stimulating oxygenic respiration. The increase in CO_2 concentration was more pronounced
229 than the decrease in O_2 , relative to baseline concentrations. The higher abundance of O_2 compared
230 to CO_2 in the atmosphere appeared to buffer O_2 variations thanks to equilibration of O_2
231 concentrations with the atmosphere. The concentrations did not reach a plateau, potentially
232 because of the instability of the injection rate. The N_2 and N_2O concentrations did not vary during
233 the injection. Thus, the injection of potassium nitrate did not significantly stimulate denitrification
234 in the stream.



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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.

240 3.2 Carbon and nitrate in the stream

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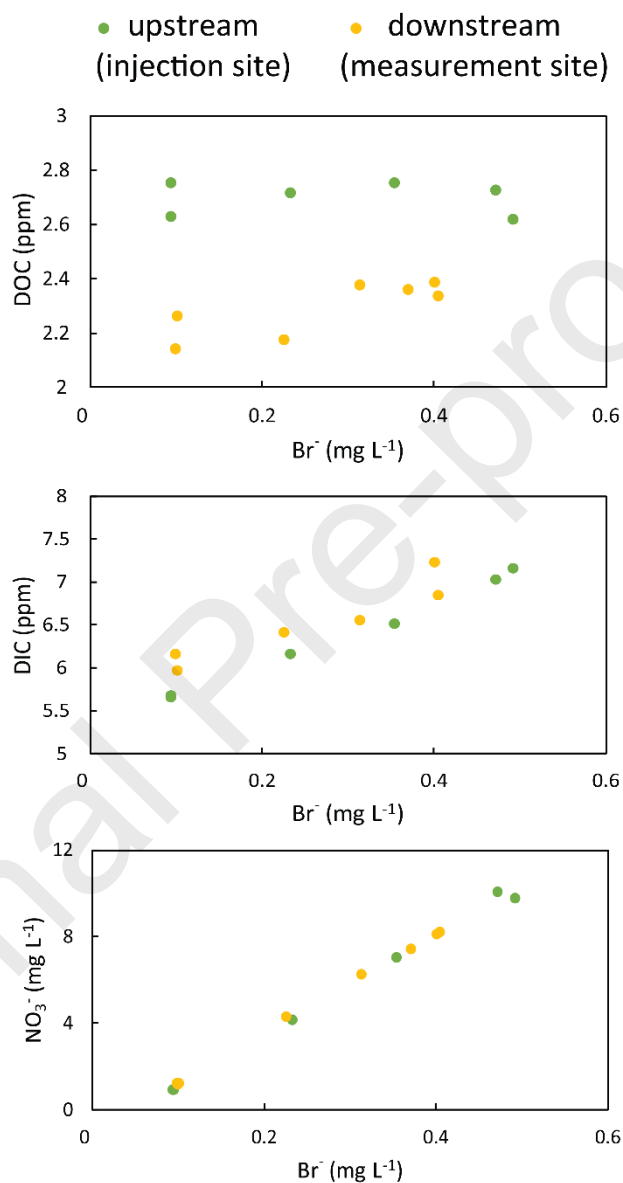
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The concentration changes of NO_3^- and dissolved carbon were consistent with the trends observed for gases. Figure 4 shows NO_3^- , DOC, and DIC as a function of the conservative tracer Br^- at the measurement and injection sites. The concentrations 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

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



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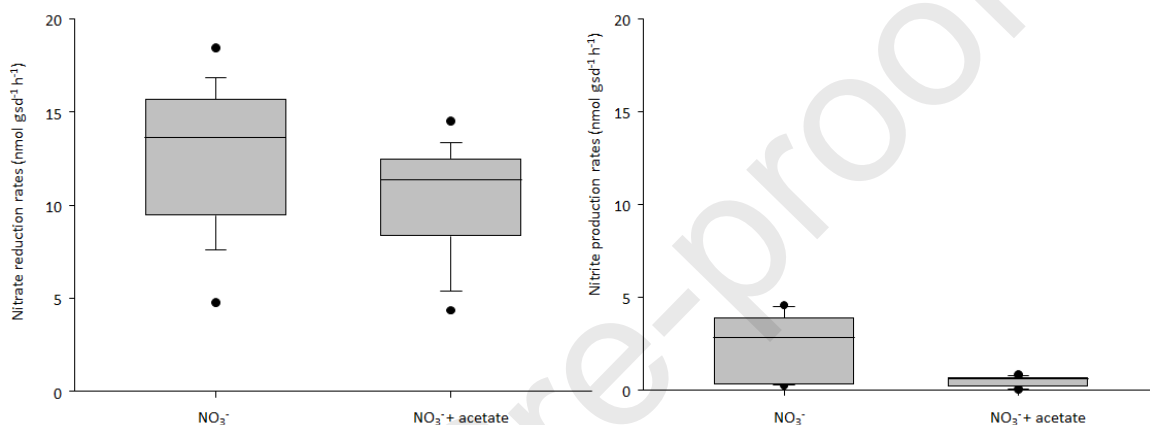
253 Figure 4. NO_3^- , DOC, and DIC concentrations as a function of conservative tracer concentration Br^- during continuous
 254 injection of sodium acetate and potassium nitrate.

255 3.3 Nitrate reduction rate in the sediment

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

259 10.4 (± 3.2) nmol gram dry sediment⁻¹ h⁻¹ when nitrate and acetate were supplied to the sediments.
 260 The rates were averaged over the duplicate reactors and the different sampling points during the
 261 incubation (6 sampling points, see section 2.5). Some nitrite production was observed (Figure 5)
 262 during nitrate reduction, with somewhat higher nitrite production when nitrate was supplied alone
 263 (2.3 ± 1.9) compared to the nitrite production in sediments that were supplied with nitrate and
 264 acetate (0.5 ± 0.3). The differences were not significantly different neither for nitrate reduction
 265 rates, nor for the nitrite production rates. Ammonium concentrations in the outflow of the reactor
 266 were below the detection limit, therefore no ammonium production (release) rates were deduced.

267



268

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

272 4 Discussion

273 4.1 Assessment of low *in-situ* denitrification

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

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

283 It can be reasonably assumed that the water had time to circulate through the hyporheic
 284 zone and flow back to the stream during the experiment. Indeed, the duration of injection (6h) was
 285 1.5 to 8 times longer than the residence time in the hyporheic zone estimated by Knapp et al. (2017)
 286 for a streambed composed of gravel, sand and silt (45 to 246 minutes). The hyporheic residence

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

290 **4.2 Comparison between potential and *in-situ* denitrification**

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

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

310 This low yet measurable potential activity in the laboratory does not translate into a
311 measurable effective denitrification *in situ*. The actual conditions of oxygenation, temperature, and
312 probability of substrate-bacteria encounter do not allow the denitrification potential to be
313 expressed in the stream. Thus, predicting the biological activity of the stream based on laboratory
314 experiments should be taken with caution. The potential activity measured in the lab gives the
315 maximum value of the effective activity, which can be far from being reached *in situ*. In a
316 comparative study between mesocosms installed in the stream and in the laboratory, Turlan et al.
317 (2007) showed that the rate of nitrate consumption measured in the stream was significantly lower
318 and more variable than the rate measured in the laboratory, even if the laboratory experiments were
319 conducted in a setup very similar to the stream, under non-optimal conditions. By combining a
320 study at the whole stream scale and at the scale of specific profiles of the hyporheic zone, Knapp
321 et al. (2017) further showed that the global rate of degradation of the tracer resazurin measured at
322 the stream scale was significantly lower than the rate measured at a smaller scale in the hyporheic
323 zone. These studies are consistent with our results and emphasize the importance of measuring
324 biological activity rates at the scale at which they are used.

325 **4.3 Hypothesis for the low *in situ* denitrification**

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

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

336 The low impact of the hyporheic processes has several plausible explanations. A first one
337 would be the low available amount of organic carbon being responsible for the low intrinsic
338 activity of the hyporheic zone. Indeed, sediment organic carbon content has been shown to be a
339 major control of potential denitrification rate (Wu et al., 2021). In our case, during the incubation
340 of stream sediment in the laboratory, the addition of acetate did not increase nitrate reduction rates.
341 This suggests the presence of a small denitrifying bacterial population, probably due to a low
342 carbon content of the sediment as nitrate concentrations in the stream are high and not a limiting
343 factor. The low amount of ammonium released from the sediment also indicates a low level of
344 carbon degradation, and therefore probably a low initial amount of carbon in the sediment. Another
345 explanation for low hyporheic activity would be that biological activity is concentrated in the
346 benthic reactive layer, the surface portion of the hyporheic zone. Yet the thickness of the benthic
347 reactive layer has been estimated being only 2 cm by Knapp et al. (2017). In small stream beds,
348 the first few centimeters of sediment can be unstable (Schippa and Pavan, 2009). A sudden increase
349 in flow driven by a large rainfall event can displace stream bottom sediments, especially if they
350 are loosely joined, such as gravels. Sediment instability has a strong impact on biological activity
351 (Atkinson et al., 2008). It favors the renewal of pore water and thus limits the development of
352 anoxic zones conducive to denitrification, although anoxic micro-niches seem to be able to develop
353 in some oxygenated sediments (Briggs et al., 2015). Finally, the metabolic activity of the
354 streambed is influenced by the strength and direction of the water exchanges between the stream
355 and the hyporheic zone (Wang et al., 2022). The recirculation of stream water into the sediments
356 is particularly limited in gaining streams (Boano et al., 2008; Caruso et al., 2016; Trauth et al.,
357 2014), that are streams fed by resurgent groundwater, which exerts an ascending pressure on the
358 hyporheic zone. The resulting residence time in the hyporheic zone may be too short for substantial
359 denitrification to occur. It is likely that denitrification occurs specifically at critical hydrological
360 moments (eg flood events) (Singh et al., 2022). The impact of hyporheic processes on stream
361 chemistry not only depends on the denitrification potential of the hyporheic zone, but also on the
362 time the water spends in the hyporheic zone (Bech et al., 2023; Trauth et al., 2014).

363 **4.4 Interest of *in-situ* gas measurement for microbial activity** 364 **assessment**

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

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

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

383 5 Conclusion

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

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

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

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Journal Pre-proofs

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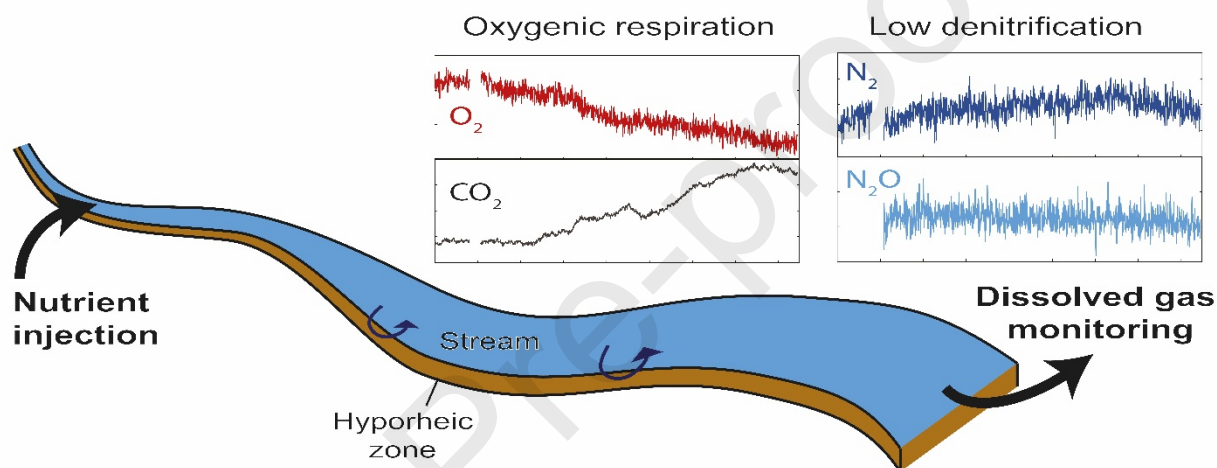
Abstract

662

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

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

678



679

680 Highlights

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

684

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

687

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

695

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

700

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

703

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

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CRedit author statement

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716 Editing, Supervision

717

Declaration of interests

719

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

722

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

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