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Dissolved organic carbon bioreactivity and DOC:DIN stoichiometry control ammonium uptake in an intermittent Mediterranean stream

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Abstract

1. Heterotrophic organisms in streams use dissolved organic carbon (DOC) and dissolved inorganic nitrogen (DIN) from the water column to meet their growth and energy requirements. However, the role of DOC availability in driving DIN uptake in headwater streams is still poorly understood. In this study, we focus on how DOC:DIN stoichiometry and DOC bioreactivity control ammonium (NH_4^+) uptake and heterotrophic aerobic respiration, and how this influence varies among seasons in a forested Mediterranean headwater stream.

2. We estimated in-stream NH_4^+ uptake rates seasonally by conducting whole-reach constant-rate additions of NH_4^+ with and without amendments of either lignin (recalcitrant DOC) or acetate (labile DOC). During each addition, we characterised microbial community composition by molecular analyses, stream metabolism with the single-station method, and heterotrophic aerobic respiration by adding a metabolic tracer (resazurin).

3. The stream was heterotrophic (net ecosystem production <0) regardless of the season, with a microbial community mostly composed of heterotrophic bacteria. In-stream NH_4^+ uptake rates were not related to either background NH_4^+ or DOC concentrations. Instead, these rates increased with increasing the molar ratio of NH_4^+ to nitrate (NO_3^-) ($NH_4^+:NO_3^-$) and DOC to DIN (DOC:DIN). Whole-reach heterotrophic aerobic respiration rates showed the same relationship against stoichiometric ratios as NH_4^+ uptake rates. Furthermore, in-stream NH_4^+ uptake rates were from 5% to >800% higher during the co-additions of acetate than when adding NH_4^+ either alone or with lignin.

4. Our results indicate that in-stream NH_4^+ uptake was largely controlled by heterotrophic bacteria, and that the stoichiometric balance between organic resources and nutrients was key to explaining the variability of in-stream NH_4^+ uptake and heterotrophic aerobic respiration. Moreover, the observed increase in NH_4^+

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uptake during acetate additions suggests that heterotrophic activity was limited by labile DOC availability.

5. Our study highlights that both DOC:DIN stoichiometry and DOC bioreactivity are relevant factors driving the seasonal pattern of in-stream N processing in this forested Mediterranean headwater stream.

KEYWORDS

dissolved organic carbon availability, heterotrophic activity, in-stream nitrogen uptake, stream biofilms, stream metabolism

1 | INTRODUCTION

Headwater streams are considered powerful bioreactors within landscapes because they can retain large amounts of dissolved inorganic nitrogen (DIN) inputs coming from terrestrial ecosystems (Alexander et al., 2007; Peterson et al., 2001). However, although some headwater streams can retain more than half of the DIN inputs (Peterson et al., 2001), in-stream DIN retention is almost negligible in others, especially during periods of low biological activity (Bernal et al., 2012; Marcé et al., 2018; von Schiller et al., 2008). This large variability in in-stream DIN retention has been related to the spatio-temporal irregularity in the environmental drivers controlling stream metabolism and associated biogeochemical processing, such as hydrology (Doyle, 2005; Valett et al., 1997), light availability (Mulholland et al., 2006; Tank et al., 2018) or nutrient concentrations (Dodds et al., 2002; Mulholland et al., 2008; Ribot et al., 2013).

While photoautotrophs play an important role in shaping in-stream DIN uptake in relatively open streams (Tank et al., 2018), heterotrophs and chemoautotrophs, such as nitrifiers, drive ammonium (NH_4^+) uptake, the most reactive form of DIN, in forested headwater streams (Bernhardt et al., 2002; Butturini et al., 2000; Valett et al., 2008). In this case, dissolved organic carbon (DOC) concentration is a major factor controlling NH_4^+ uptake because heterotrophs rely on DOC to assimilate N (Bernhardt & Likens, 2002; Kaplan & Newbold, 1993). Conversely, nitrifiers fix carbon dioxide by oxidising NH_4^+ to nitrate (NO_3^-) and, therefore, are not dependent on DOC concentration. This difference explains why rates of heterotrophic NH_4^+ assimilation and nitrification and the ratio between these two processes are closely dependent on stream background DOC concentrations (Bernhardt & Likens, 2002; Lupon et al., 2020; Rodríguez-Cardona et al., 2016). For instance, an increase in DOC concentration can severely reduce nitrification rates because heterotrophic bacteria outcompete nitrifiers when DOC is available (Butturini et al., 2000; Strauss & Lamberti, 2000; Taylor & Townsend, 2010).

Most currently available research has focused on understanding how DIN or DOC concentration influences in-stream DIN uptake (Bernhardt & Likens, 2002; Johnson et al., 2012; Strauss & Lamberti, 2000, 2002). Yet, recent studies have suggested that the relative concentration of DOC to DIN (i.e., the DOC:DIN ratio) is a more important driver of in-stream DIN uptake than DOC concentration alone (Rodríguez-Cardona et al., 2016; Taylor & Townsend, 2010; Wymore et al., 2016). In line with this idea, some studies suggest that low DOC concentrations can limit N uptake by stream biota, especially when nutrient concentrations are relatively high (e.g., Robbins et al., 2017), whereas others have empirically shown that molar ratios of bioavailable DOC and DIN strongly influence nutrient uptake rates (Graeber et al., 2021; Soares et al., 2017). However, there are still few experimental studies exploring whether seasonal changes in C:N stoichiometry can influence in-stream N uptake rates and contribute to their temporal variability in headwater streams.

The bioreactivity of DOC also can impact in-stream microbial activity because bacteria prefer labile fractions of DOC rather than more humified (i.e., recalcitrant) compounds (Cory & Kaplan, 2012; Kaplan et al., 2008; Kaplan & Newbold, 2003). Regarding stream N cycling, previous studies have shown increases in in-stream DIN uptake in response to experimental additions of labile DOC sources, such as glucose and acetate (Bernhardt & Likens, 2002; Robbins et al., 2017). Moreover, amendments of labile DOC usually lead to increases in biofilm heterotrophic activity measured as either bacterial biomass growth, ecosystem respiration or extracellular enzyme activity (Bernhardt & Likens, 2002; Robbins et al., 2017; Sobczak et al., 2003). Graeber et al. (2021) also found that N uptake by stream heterotrophs was better explained by C:N ratios when considering only the bioreactive fractions of both DOC and dissolved N. While the positive effect of labile DOC on in-stream DIN uptake is well-documented, there is a lack of studies comparing the effect of DOC sources differing in bioreactivity, which limits our capacity to understand the real impact of different DOC sources on whole-reach in-stream NH_4^+ uptake and associated heterotrophic activity (but see Lane et al., 2013).

In this context, Mediterranean headwater streams are well-suited model systems because they show relatively low DOC concentrations (<5 mg C/L; Butturini & Sabater, 2000; Bernal et al., 2005, 2018), which could potentially limit NH_4^+ uptake by stream heterotrophs (Strauss & Lamberti, 2002). Moreover, Mediterranean streams show a marked seasonality in both DOC and DIN concentrations, with peaks during leaf litter fall and marked declines, especially of DIN, during the vegetative period (Bernal et al., 2005; Vázquez et al., 2011, 2013). Stoichiometric ratios also can vary greatly among seasons because the range of variability in concentration is usually higher for DIN than for DOC (Bernal et al., 2005;

Ledesma et al., 2022). Likewise, the composition of dissolved organic matter (DOM), and especially the amount of aromatic and high molecular weight compounds, can vary largely over time in this type of stream (von Schiller et al., 2015). This marked seasonal pattern in stream water chemistry has been attributed to the dynamic hydrological connections with catchment sources and the phenology of riparian trees (Bernal et al., 2013; Butturini et al., 2008; Lupon et al., 2016). The temporal variability in DOC concentration, bioreactivity and DOC:DIN stoichiometry can subsequently influence the composition and activity of streambed microbial communities (Fazi et al., 2013; Merbt et al., 2016) and thereby their capacity to process stream DIN.

The objective of the present study was to explore the influence of DOC:DIN stoichiometry and DOC bioreactivity on in-stream NH_4^+ uptake in a forested and intermittent Mediterranean headwater stream, and how this influence varies across seasons with changing environmental conditions and microbial community composition. We hypothesised that in-stream NH_4^+ uptake increases when increasing both DOC:DIN ratios and DOC bioreactivity because these two factors promote in-stream heterotrophic activity and, thus, N demand. To test this hypothesis, we measured in-stream NH_4^+ uptake using constant-rate additions and examined how it varied with the addition of two different bioreactive DOC sources (either lignin or acetate, recalcitrant and labile, respectively). Moreover, we explored whether in-stream heterotrophic activity responded to the DOC additions by using resazurin, a smart tracer of aerobic respiration (Haggerty, 2013). We expected heterotrophic activity to respond to seasonal changes in stoichiometry, with both aerobic respiration and in-stream NH_4^+ uptake being higher during those seasons with higher DOC:DIN ratios. We also expected higher aerobic respiration and in-stream NH_4^+ uptake with acetate additions, especially when DOC was more limiting, but that lignin additions would cause small effects on these responses regardless of the season.

2 | MATERIALS AND METHODS

2.1 | Study site

The study was conducted in a third-order reach of the intermittent stream Fuirosos (41° 41′04.5 ″N, 2° 34′46.0 ″E; Figure 1), located within the Montnegre-Corredor Natural Park (Catalonia, NE Spain). Climate in this region is typically Mediterranean, with warm, dry summers and mild winters. Average daily air temperatures range from 3°C in winter to 24°C in summer. Average annual precipitation is c. 660mm, and the number of rainy days does not usually exceed 70 per year (Bernal et al., 2002; Ledesma et al., 2021). Precipitation mostly falls in spring and autumn, with occasional summer storms (Vázquez et al., 2013).

The Fuirosos catchment (18.7km², 50 to 770m a.s.l.) lies on a medium to fine-grained granitoid batholith, resulting in sandy and
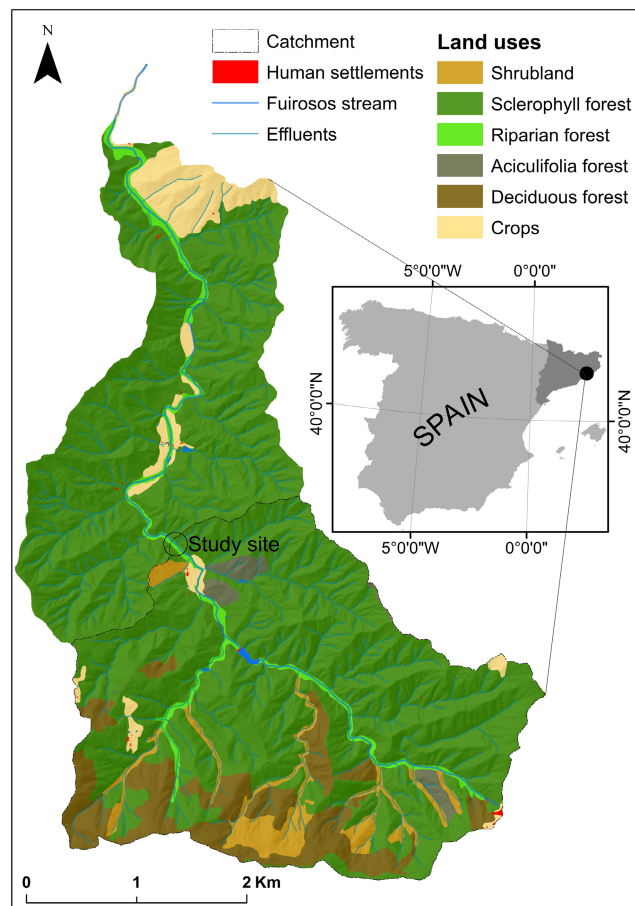
FIGURE 1 Map of the Fuirosos catchment showing the location where experimental additions were conducted (black circle). Land uses in the catchment are also shown with different colours. Data from the Cartographic and Geological Institute of Catalonia (1:5000) (version 2 from 2018, CC BY 4.0 licence).

poorly developed soils. The predominant vegetation is sclerophyllous (72% *Quercus ilex, Quercus suber*), deciduous (15%, *Quercus petraea, Castanea sativa*), and coniferous (2%, *Pinus pinea, Pinus halepensis*) forests (Figure 1). Human activity is low (1.4% of crops) and mainly concentrated in peripheral areas. The stream has a well-developed riparian forest (10–15 m wide) mainly composed of *Alnus glutinosa* and *Platanus acerifolia*, and riparian soils have a thin organic horizon (Bernal et al., 2019).

The stream flows through a 3–5-m-wide channel, with base flow discharge (Q) ranging between 0 and 25L/s (Vázquez et al., 2013). The stream usually dries out up to four months in summer (Butturini et al., 2003). Stream NO_3^- concentration averages $210 \pm 50 \mu g N–NO_3^-/L$ (mean $\pm SD$) and follows a seasonal pattern, with the lowest values in late spring and the highest in winter (Bernal et al., 2005). Mean stream NH_4^+ concentration is generally an order of magnitude lower than that of NO_3^- and exhibits the opposite seasonal pattern, with minima in autumn and winter and maxima in spring (Bernal et al., 2005). Mean stream DOC concentration is relatively low (~4mgC/L), and peaks during autumn, when concentrations can rise up to 8mgC/L (Vázquez et al., 2011).

2.2 | Experimental design

The study was conducted from March 2019 to February 2020. We selected a 100-m-long stream reach draining the upper 9.9 km² of the catchment. The selected reach was flanked by a well-developed riparian forest and no tributaries or other obvious surface inflows were present. The stream reach had a gentle slope (1.9%) and a well-preserved hydromorphology, alternating small runs, riffles, and few shallow pools. During the field campaigns, mean wetted width (w) was 2.4 ± 0.5 m, whereas average water column depth (h) was 7.3 ± 8.3 cm. The streambed substrate was composed of rocks and cobbles (41%–50%) along runs and riffles, and of sands and gravels (32%–47%) in pools.

We carried out four field campaigns: spring (26–28 March), summer (4–6 June), autumn (26–28 November) and winter (18–20 February). All campaigns were done under base flow conditions. However, the winter campaign was preceded by a large extratropic cyclone which occurred 4 weeks before (Ledesma et al., 2021), which could have had a remarkable impact on stream biota composition. For each campaign, in-stream NH_4^+ uptake and aerobic respiration were measured during three consecutive days by conducting short-term constant-rate additions. Additions consisted of the injection of ammonium chloride (NH_4Cl) either with or without a co-injection of a DOC source. This DOC source could be either lignin or acetate. The former is considered to be more recalcitrant, whereas the latter is considered to be more bioreactive and, therefore, more easily used by microorganisms (Baker et al., 1999; Royer & Minshall, 2001). On Day (D)1, we added NH_4Cl alone (hereafter referred as AM treatment); on D2, we added NH_4Cl + lignin as lignin alkaline (hereafter referred as AM+LG treatment); and, on D3, we added NH_4Cl + acetate as sodium acetate (hereafter referred as AM+AC treatment). Additionally, on each day, once nutrient additions reached *plateau* conditions and samples were collected for NH_4^+ and DOC concentrations, we added resazurin (Raz), a metabolic tracer that changes to resorufin (Rru) when reduced by aerobic respiration (Haggerty et al., 2009). We performed an additional Raz addition on D0 (before the three nutrient additions) to obtain a reference value of in-stream aerobic respiration rates under ambient conditions. In all cases, we added sodium chloride (NaCl) as a conservative tracer. These additions were used to calculate rates of in-stream NH_4^+ uptake and aerobic respiration from the longitudinal variation of stream NH_4^+ concentration and Raz:Rru ratios during *plateau* conditions, respectively (Hanrahan et al., 2018; Webster & Valett, 2006). For each step, empirical procedures and calculations are explained below.

2.3 | Physicochemical characterisation of the stream

For each campaign, the stream reach was divided into seven sampling stations distributed in geometric progression. Reach length was slightly adjusted on each campaign based on Q to ensure *plateau* conditions were always reached after 2–3 h of solute addition.

Stream Q was calculated on D0 with a slug addition of NaCl (Gordon et al., 2004). On D0, we also measured w and average h from a 20-cm interval transect at each sampling station.

We installed a dissolved oxygen (DO) concentration sensor (Minidot-PME, San Diego, USA) at the bottom of the reach 3 days before the onset of each field campaign. The sensor recorded stream DO (in mg/L) and stream water temperature (T, in °C) at 10-min intervals during 6–7 days. Incident light (in lux) was recorded with irradiance sensors (HOBO Pendant® UA-002-64, Onset Computer Corporation) installed below the riparian canopy at two representative locations of the reach. These measurements were averaged to represent stream surface irradiance. We converted lux measurements to photosynthetic photons flux density (PPFD, in μmols m⁻² s⁻¹) and computed the sum of PPFD at daily time steps. Daily median values of the three addition days and the three previous days were used to compare DO, T and PPFD among field campaigns. Moreover, these time series were used to calculate daily rates of stream metabolism (see below).

On each addition day, we recorded electrical conductivity (EC, in μS/cm) with a conductivity meter (WTW-3310; Xylem Analytics) every 5 min at the bottom of the reach in order to detect *plateau* conditions. This breakthrough curve was used later on to calculate stream Q (in L/s) and velocity (v, in m/s) during the additions.

2.4 | Epilithon and epipsammon sampling and characterisation

On each campaign, we collected two samples of biofilm streambed sediment (i.e., epipsammon, 0–3 cm depth) at each sampling station. The two samples (20–30 g each) were carefully transferred into 50-ml falcon tubes with 5 ml of lysis buffer (40 mM EDTA, 50 mM Tris pH 8.3, 0.75 M sucrose). In addition, we collected two representative cobbles to obtain samples of epilithic biofilm at stations 1, 3, 5 and 7. All samples were kept cold until they arrived at the laboratory. At the laboratory, we extracted the biofilm from each cobble by scraping with a sterilised metal brush and washing them with a ringer's solution. An aliquot of the slurry was filtered through a 0.2-μm polycarbonate membrane and frozen at −20°C until further microbial DNA extractions. The remaining epilithic slurries were filtered through an ignited, pre-weighted GF/F to calculate biofilm biomass, expressed as ash-free dry mass (AFDM) as in Merbt et al. (2015). DNA was extracted using a DNeasy PowerSoil kit (QIAGEN) following the manufacturer's instructions, and the purified DNA extracts stored at −20°C until further processing.

Stream microbial communities were characterised by molecular and bioinformatic analyses. The purified extracted DNA was sequenced using multiplexing PCR amplification and Illumina MiSeq sequencing following the methods of the Research Technology Support Facility at the Michigan State University (https://rtsf.natsci. msu.edu/). The variable V4 region of the 16S rRNA gene (c. 250 nucleotides) was amplified with the primers F515 (5′–GTGCCAGCMGC CGCGGTAA–3′) and R806 (5′–GGACTACHVGGGTWTCTAAT–3′)

(Caporaso et al., 2010), using a paired end base pairs (bp) run. Raw sequence datasets were processed using the UPARSE pipeline (Edgar, 2013). Briefly, after merging of paired reads, filtering by an expected error of 0.25 and read length of 250 bp, c. 79% of the original reads were retained in this step (i.e., 2,180,351 reads). The reads were dereplicated and clustered into zero-radius operational taxonomic units (zOTUs) excluding singletons. More than 93.5% of the merged sequence pool was mapped back into zOTUs (i.e., 2,277,142 reads). A total of 11,545 prokaryotic zOTUs were obtained and taxonomically assigned with SILVA_138 (Quast et al., 2013). Chloroplast, mitochondria, Eukarya and unclassified reads were excluded (representing a 14.3% of the reads). A total of 11,087 prokaryote zOTUs were finally compiled. Only those samples with >8,000 reads were retained for downstream analyses. The whole gene sequence datasets were deposited to the NCBI Sequence Read Archive and are available through BioProject record ID PRJNA881938.

The N cycle functional approach was carried out by functional predictions based on the closest match with representative genomes, which have been recognised to be useful for estimating genomic and metabolic potential (Ortiz-Álvarez et al., 2018). Specifically, we used PICRUSt2 (standalone version) (Douglas et al., 2020) as functional prediction method for microbial communities. A nearest sequenced taxon index (NSTI) value of 1.0 cut-off was chosen for increasing prediction accuracy. We focused on N cycling genes participating in the nitrification process, specifically from ammonia-oxidising archaea (AOA) and bacteria (AOB), as well as on NH_4^+ assimilation, according to the following list of KEGGs (Kyoto Encyclopedia of Genes and Genomes): K10944, K10945, K10946 (methane/ammonia monooxygenase subunits, *pmo-amo-ABC*); K10535 (hydroxylamine dehydrogenase, *hao*); K00265 (glutamate synthase [NADPH/NADH], *gltB*); K00284 (glutamate synthase [ferredoxin-dependent], *gltS*); K01915 (glutamine synthetase, *glnA*); K03320 (ammonium transporter, Amt family–*amt*). To approach the subset of heterotrophic microbial species potentially carrying out NH_4^+ assimilation, we ruled out from the dataset those bacteria presenting a (potentially functional) complete set of genes for the oxygenic photosynthesis or bacteriorhodopsin. Conversely, those potentially classified as aerobic anoxygenic phototrophs were included in the (chemo)-heterotrophic group.

2.5 | Stream metabolism

In order to assess whether the potential contribution of photoautotrophs to in-stream NH_4^+ uptake changed substantially among field campaigns, we estimated gross primary production (GPP, in g $O_2 m^{-2} day^{-1}$) and ecosystem respiration (ER, in g $O_2 m^{-2} day^{-1}$) during each campaign. We used the single-station method because (i) both stream hydromorphology and longitudinal connectivity were similar along the selected reach (Odum, 1956) and (ii) the influence of groundwater inputs was assumed to be minimal during the low flow conditions of the additions (Bernal et al., 2022).

We estimated daily metabolic rates using the Bayesian inverse model "b_Kn0_oipi_tr_plrckm.stan" of the *streamMetabolizer* R

package (Appling et al., 2018). This model produces daily estimates of GPP and ER, and gas exchange rate coefficients (K_{600}) based on sub-daily time series of DO, T, PPFD and h. We used the same model configuration and quality control procedures as done for this same reach in Bernal et al. (2022). Briefly, we assumed that GPP is a linear function of light intensity (van de Bogert et al., 2007) and ER is constant throughout the day. Prior distributions for GPP and ER were based on previous reported values for this stream (0.5 ± 10 and -5 ± 10 g $O_2 m^{-2} day^{-1}$ for GPP and ER, respectively) (Acuña et al., 2004). To minimise equifinality problems, the prior distribution for K_{600} was based on the log–log relationship between Q and K_{600} values estimated from the night-time regression method and hydraulic geometry (Bernal et al., 2022). We ran five Markov chain Monte Carlo (MCMC) chains in parallel on five cores, with 2,000 warmup steps and 1,000 saved steps on each chain. All days showed model convergence (R-hat ~1.0; number effective samples <1,000) and a good fit to DO data (R^2 >0.70, RMSE <0.2).

2.6 | Short-term constant-rate additions and laboratory analysis

For each addition, we first prepared the carboy solution containing NaCl, NH_4Cl and a DOC source; the volume of which was sufficiently large to ensure ~8 h of addition. The solution was added at a constant rate (2.2 ml/s) with a peristaltic pump (GeoPump; GEOTECH) located c. 10–15 m upstream of the first sampling station to ensure a good mixing. The NH_4^+ concentration in the carboy was estimated to increase the background stream NH_4^+ concentration by 2–4-fold, whereas the concentration of either lignin or acetate in the carboy was calculated to increase it by 1.5-fold. Before starting each short-term addition, we collected surface stream water samples (three replicates) from each sampling station to characterise background nutrient concentration. Moreover, we measured EC at each sampling station using a conductivity meter. The same sampling procedure was repeated once the solute addition reached *plateau* conditions. Then, we added Raz to the carboy solution (target stream concentration = 200 μg Raz/L), which was discharged to the stream at the same flow rate used previously. Stream water samples for Raz were collected when the addition reached *plateau* conditions again, following the same procedure as for stream water nutrient samples. To characterise background stream DOC concentration during each campaign, stream water was collected on D0 from stations 1, 4 and 7, and from station 1 on each day before the start of the addition experiment (to ensure no substantial changes occurred during subsequent addition days).

All water samples were filtered in situ through precombusted (450°C, 4 h) glass-fibre filters (0.7-μm pore size GF/F; Whatman), and placed in pre-washed plastic vials rinsed with stream water at least three times and kept dark and cold (<4°C) until arriving at the laboratory. Streamwater samples for both Raz and Rru concentrations were measured immediately upon arrival to laboratory on a

spectroflourometer (Shimadzu/RF-5000, Kyoto, Japan) following the procedure described in Haggerty et al., 2009. Samples for nutrient analyses were frozen until analysed for NH_4^+, NO_3^- and nitrite (NO_2^-) concentrations following standard colorimetric methods (APHA, 1995) on an autoanalyser (FUTURA). DIN was the sum of NH_4^+, NO_3^- and NO_2^- concentrations, the latter being <1% of DIN. Samples for DOC analysis were acidified with HCl and kept in the fridge in pre-washed and pre-combusted (>450°C) glass vials. Non-purgeable organic C was analysed using high temperature catalytic oxidation (Shimadzu TOC/TN analyser) after sparging to remove dissolved inorganic C. All samples were analysed at the Analytical Chemistry Services (LabQA) of the Centre d'Estudis Avançats de Blanes (CEAB).

2.7 | In-stream NH_4^+ uptake and Raz transformation rates

For each addition, we estimated in-stream NH_4^+ uptake coefficient per unit of reach length (k_{NH4}, 1/m) using the following first-order equation:

$$C_x^* = C_{top}^* \bullet \left(\frac{EC_x^*}{EC_{top}^*} \right) \bullet e^{-k_{NH4} \bullet x} \qquad (1)$$

where C^* is *plateau* NH_4^+ concentration corrected by background concentration (in µg N-NH_4^+/L) and EC^* is background-corrected *plateau EC* (in µS/cm) at each sampling station (x, in m) or at the top of the reach (top) (Newbold et al., 1981; Webster & Valett, 2006). During spring and summer, AM+AC additions showed *plateau* NH_4^+ concentrations lower than background concentrations at the most downstream stations. These samples were excluded from further calculations to avoid negative values.

From k_{NH4}, we calculated three uptake spiralling metrics (Wollheim, 2016): NH_4^+ uptake length (S_{w-NH4}, in m), uptake velocity (v_{f-NH4}, in mm/min) and areal uptake rate (U_{NH4}, in µg N–NH_4^+ m^{-2} min^{-1}). The negative inverse of k_{NH4} equals S_{w-NH4}, which is interpreted as the average distance travelled by a NH_4^+ molecule before being taken up by stream biota or adsorbed onto stream sediments (Stream Solute Workshop, 1990). The v_{f-NH4} indicates NH_4^+ biological demand and it is calculated as:

$$v_{f-NH4} = \frac{Q}{S_{w-NH4} \bullet w} \qquad (2)$$

Finally, U_{NH4} is calculated as:

$$U_{NH4} = C_b \bullet v_{f-NH4} \qquad (3)$$

where C_b is background NH_4^+ concentration (in µg N-NH_4^+/L).

For each Raz addition, we estimated the Raz to Rru transformation rate (k_{Raz}, 1/m) at the reach scale following the coupled parent–daughter steady-state transport analytical solution (Haggerty, 2013):

$$\frac{C_{Rru,x}}{C_{Raz,x}} + P = \left(\frac{C_{Rru,top}}{C_{Raz,top}} + P \right) \bullet e^{k_{Raz} \bullet x} \qquad (4)$$

where C_{Rru} and C_{Raz} are concentration of Rru and Raz (in µmol/L) at each sampling point (x) and at the top (top) of the reach, and P is the ratio of production of daughter (Rru) to decay of parent (Raz) associated with irreversible sorption, photodecay, and other losses. We used P=0.5, which is the average value empirically reported for headwater streams (Haggerty, 2013). Following the spiralling mathematical framework, we calculated the Raz to Rru transformation length (S_{w-Raz}, in m) as the inverse of k_{Raz} and the Raz transformation velocity (v_{f-Raz}, in mm/min) as in Equation 2, which was interpreted as a proxy of aerobic respiration within the reach (Haggerty, 2013).

In order to further explore the influence of DOC bioreactivity on in-stream NH_4^+ uptake, we calculated the percentage difference between v_{f-NH4} estimated for the AM treatment and for either the AM+LG or AM+AC treatments (Δv_{f-NH4}). Values of $\Delta v_{f-NH4} > 0\%$ indicate an increase in NH_4^+ uptake with DOC treatments relative to NH_4^+ uptake in AM treatment, whereas values of $\Delta v_{f-NH4} < 0\%$ indicate a decrease. The same calculation was done with v_{f-Raz}. Finally, we calculated the ratio $v_{f-NH4}:v_{f-Raz}$ to assess how in-stream NH_4^+ uptake changes in relation to aerobic respiration for the different treatments (AM, AM+LG, AM+AC).

2.8 | Data analyses

In order to explore whether hydrological, chemical, and biogeochemical variables differed among seasons, we used non-parametric Kruskal–Wallis tests followed by Dunn's tests because some variables were not normally distributed (Dinno, 2017). Spearman correlations (ρ) were used to explore the relationship among the different physical and chemical variables. In these relationships, we used medians and percentiles of the different variables to reduce potential biases derived from non-normal data distribution, which can lead to misrepresentation of low stoichiometric ratios (Isles, 2020).

Kruskal–Wallis tests followed by Dunn's tests also were applied to test whether in-stream NH_4^+ uptake metrics (S_{w-NH4}, v_{f-NH4} and U_{NH4}) and aerobic respiration (S_{w-Raz} and v_{f-Raz}) varied among treatments or field campaigns. Furthermore, we used the coefficient of variation (CV, in %) to quantify the variability of the different spiralling metrics within each season (pooling all treatments together) or treatment (pooling all seasons together). Finally, we used linear and power regression models to investigate if stream water concentrations and stoichiometry influenced in-stream NH_4^+ uptake. To do so, we related both v_{f-NH4} and v_{f-Raz} to background stream DOC and NH_4^+ concentrations as well as to DOC:DIN and NH_4^+:NO_3^- molar ratios.

For microbial community composition, we calculated indexes related to species diversity and multivariate statistics with the *vegan* R package (Oksanen et al., 2020). We used the rarefied species richness and Shannon index (H) to describe species diversity by season and substrate. The Berger–Parker index was used to describe the proportional importance of the most abundant type in each group. We also used one-way ANOVA to assess differences in diversity

indexes between seasons for each substrate. In this case, our datasets fulfil the assumptions of normality and homoscedasticity.

For distance-based community analyses, Bray–Curtis dissimilarities after Hellinger standardisation (Legendre & Gallagher, 2001) were calculated and represented by non-metric multidimensional scaling (NMDS) (metaMDS function). To test differences between substrates and among seasons, we used permutational multivariate ANOVA (PERMANOVA) using distance matrices (adonis test) carried out based on 1,000 permutations.

All statistical analyses were run in the R software (R Core Team, 2013). In all cases, statistical significance was set at $p < 0.05$, except for Dunn's test, where the significance was set at $p = 0.025$, and the PERMANOVA, for which we evaluated the test results using the statistic value of the Bray–Curtis distance matrix because the p-value was usually highly dependent on the number of permutations.

3 | RESULTS

3.1 | Physicochemical characterisation and stream metabolism

Stream hydrology and water chemistry followed a clear seasonal pattern. Stream Q and v were higher during autumn and winter, whereas stream T showed the opposite seasonal pattern (Table 1). Median stream NH_4^+ concentration was 4.2 [2.8, 8.6] [25th, 75th percentiles] µg N-NH_4^+/L, and peaked in spring (Table 1), whereas median stream NO_3^- concentration was 137.0 [32.2, 2426.9] µg N-NO_3^-/L and peaked in autumn (Table 1). Conversely,

stream DOC concentration showed small seasonal variation among seasons compared to DIN, though concentrations were higher in winter (4.3 mg C/L) than in spring (2.3 mg C/L) (Table 1). Note that stream NH_4^+ concentration showed the opposite seasonal pattern than NO_3^- ($\rho = -0.76$, $p < 0.01$) and DOC ($\rho = -0.64$, $p < 0.05$). There were large differences in median DOC:DIN molar ratios between summer (DOC:DIN = 95.5) and autumn (DOC:DIN = 1.6), whereas median NH_4^+:NO_3^- ratio was <1 during all seasons, reaching its minimum in autumn (Table 1).

Stream DO concentration was >7 mg O_2/L during all field campaigns, and the stream metabolism was dominated by heterotrophic activity (Table 1). ER was higher in autumn (–2.46 g O_2 m^{-2} day^{-1}) than in summer (–1.14 g O_2 m^{-2} day^{-1}), though AFDM in epilithon from cobbles showed no seasonal differences (median = 0.25 [0.18, 0.32] mg/cm^2) (Kruskal–Wallis test, $p > 0.05$). Despite that irradiance varied greatly among seasons, GPP showed small variation and differences among seasons were not statistically significant (Table 1).

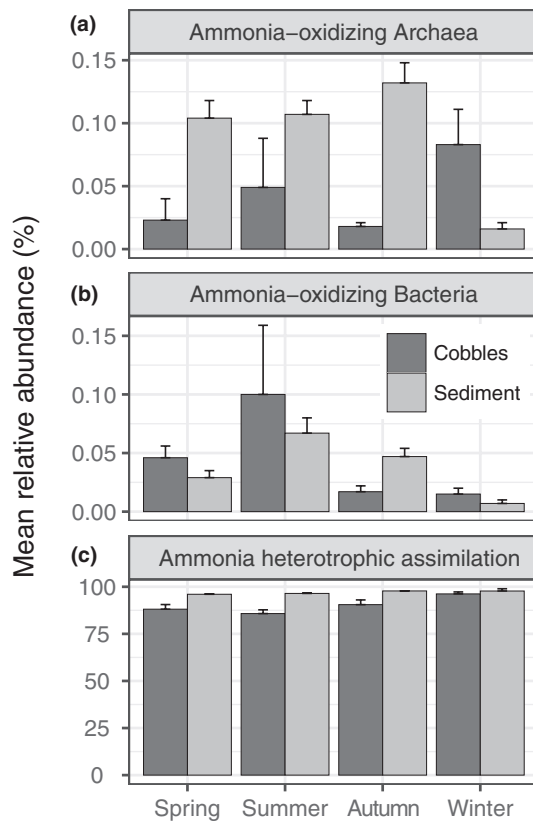### 3.2 | Microbial community composition and functional prediction

Heterotrophic microbial species potentially carrying out NH_4^+ assimilation showed the highest abundances, of between 75% and 90%, in both cobbles and sediments during all seasons (Figure 2c). The AOA and AOB were also detected in both epilithon and epipsammon, but these groups represented a small portion of the total microbial relative abundance with values ranging from 0.01% to 0.13% of the total amplicon DNA mixture (Figure 2a,b).

TABLE 1 Median [25th, 75th percentiles] of physicochemical characteristics and stream metabolism at the study reach.

	Spring	Summer	Autumn	Winter
Physical characteristics				
Q (L/s)	3.7 [3.6, 3.8]^{ab}	1.5 [1.5, 1.6]^a	7.1 [7.1, 8.2]^{ab}	13.2 [13.0, 13.7]^b
v (m/s)	0.031 [0.028, 0.035]^{ab}	0.015 [0.015, 0.017]^a	0.078 [0.077, 0.080]^b	0.067 [0.067, 0.069]^{ab}
T (°C)	14.1 [13.7, 14.2]^{ab}	19.5 [18.8, 19.8]^a	11.7 [11.2, 12.1]^{ab}	9.7 [9.5, 10.1]^b
PPFD (mol m^{-2} day^{-1})	17.1 [16.7, 17.3]^a	5.8 [5.5, 5.9]^{bc}	3.9 [3.5, 4.1]^c	8.3 [6.3, 11.0]^{ab}
Chemical characteristics				
DO (mg O_2/L)	10.0 [9.8, 10.1]^{bc}	7.3 [7.16, 7.4]^a	9.2 [9.0, 9.4]^{ab}	10.2 [10.1, 10.4]^c
NH_4^+ (µg N-NH_4^+/L)	11.0 [8.7, 12.8]^a	7.0 [4.2, 8.4]^b	2.8 [1.4, 4.2]^c	2.8 [2.8, 4.2]^c
NO_3^- (µg N-NO_3^-/L)	127.0 [125.0, 130.0]^a	26.6 [21.0, 29.4]^b	2570.4 [2510.2, 2627.1]^c	327.6 [263.6, 456.4]^d
DOC (mg C/L)	2.3 [2.2, 2.4]^a	2.4 [2.4, 2.4]^{ab}	3.6 [3.5, 3.6]^{bc}	4.3 [4.2, 4.8]^c
DOC:DIN	18.2 [17.4, 20.3]^{ab}	95.5 [86.6, 97.5]^b	1.6 [1.5, 1.6]^a	14.3 [13.8, 15.8]^{ab}
NH_4^+:NO_3^-	0.083 [0.075, 0.095]^{ab}	0.256 [0.225, 0.361]^a	0.0008 [0.0007, 0.0018]^b	0.010 [0.007, 0.012]^{ab}
Stream metabolic rates				
GPP (g O_2 m^{-2} day^{-1})	0.20 [0.20, 0.21]^a	0.04 [0.03, 0.12]^a	0.04 [0.04, 0.05]^a	0.34 [0.31, 0.37]^a
ER (g O_2 m^{-2} day^{-1})	–1.25 [–1.26, –1.23]^{ab}	–1.14 [–1.54, –1.04]^a	–2.46 [–2.47, –2.46]^b	–2.25 [–2.26, –2.12]^{ab}

Note: For each season: discharge (Q), velocity (v), stream water temperature (T), photosynthetic photons flux density (PPFD), dissolved oxygen (DO), electrical conductivity (EC), background stream NH_4^+, NO_3^- and DOC concentrations, DOC:DIN and NH_4^+:NO_3^- molar ratios, gross primary production (GPP), and ecosystem respiration (ER). In all cases, values are medians of the three consecutive addition days, unless indicated differently in the main text. For each variable, different letters (a, b, c and d) indicate statistically significant differences among seasons (multiple comparison Dunn's test, $p < 0.025$).

The emergence of distinct clusters in the NMDS analysis indicated statistically significant differences in the composition of heterotrophic microbes both between substrates and among seasons (Adonis test: $r^2 = 0.61$, $F = 16.2$, $p = 0.001$) (Figure 3). This change in composition was especially noticeable in winter, when epipsammic biofilms in sediments showed a large decrease in species richness (Figure S1). Mean rarefied species richness in spring, summer and autumn ranged from 2315 to 2924 zOTUs, whereas it decreased to 235 in winter (one-way ANOVA, $F_{3,47} = 604.3$, $p < 0.0001$). Likewise, winter epipsammic biofilms showed lower Shannon H index (2.45) but higher Berger–Parker index (0.32) compared to other seasons (mean H ranging from 7.03 to 7.44; mean Berger–Parker index ranging from 0.016 to 0.021). For the two indexes, differences among seasons were statistically significant (one-way ANOVA; for H: $F_{3,47} = 2240.0$, $p < 0.001$; for Berger–Parker index: $F_{3,24} = 104.0$, $p < 0.001$). This result indicates that winter epipsammic biofilms were composed of microbial communities dominated by a lower number of species.

3.3 | In-stream NH_4^+ uptake rates among seasons and treatments

Stream NH_4^+ concentration decreased longitudinally along the reach at *plateau* on all short-term constant-rate additions and our v_{f-NH4} values were within the natural range reported for this stream (Figure S2). For the AM treatment (NH_4^+ addition alone), the three spiralling metrics varied largely among seasons, with CVs ranging from 71.0% for v_{f-NH4} to 99.3% for U_{NH4} (Table 2). For example, S_{w-NH4} was 35 times longer in winter ($S_{w-NH4} = 465.2$ m) than in summer ($S_{w-NH4} = 13.3$ m), whereas v_{f-NH4} was four times higher in summer (2.83 mm/min) than in spring and winter (0.68 and 0.76 mm/min, respectively) (Figure 4a). The additions using a DOC source, either AM+LG or AM+AC, also showed high variability in spiralling metrics among seasons, with CVs usually >80% (Table 2). The only exception was v_{f-NH4} for the AM+AC treatment, which showed the lowest CV (Table 2). Despite this large variability among seasons, we found no statistically significant differences for any spiralling metric when
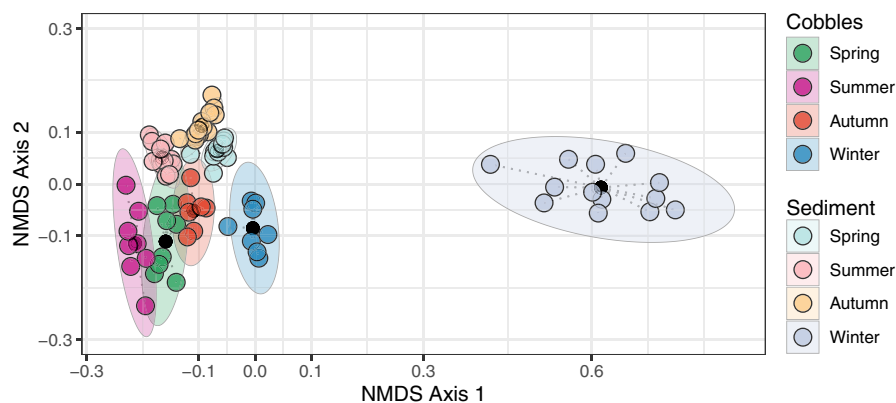

FIGURE 2 Relative abundance of functionally predicted (a) ammonia-oxidisers archaea (AOA), (b) ammonia-oxidisers bacteria (AOB) and (c) ammonia assimilative heterotrophic microorganisms in epilithic biofilms grown in cobbles and epipsammic biofilm grown in sediments for each season. Bars are averages and whiskers are standard deviations.

FIGURE 3 Non-metric multidimensional scaling (NMDS) ordination analysis of community similarities based on Bray–Curtis dissimilarities. Data are representative of the subset of zero-radius operational taxonomic units (zOTU) ascribed to the guild of heterotrophic prokaryotes carrying ammonium assimilation, predicted by means of the N cycle functional approach (PICRUSt2 analyses). Colours are related to the season and substrate where samples were obtained. The ellipsoid areas (confidence interval = 0.95) are indicative of distribution (and dispersion) of each group on the ordination space. Dotted lines join each sample with its corresponding group centroid (black dots).

TABLE 2 Median [25th, 75th percentiles] of in-stream NH_4^+ uptake length (S_{w-NH4}), uptake velocity (v_{f-NH4}), and areal uptake rate (U_{NH4}) measured for each treatment.

Spiralling metric	Treatment		
	AM	**AM+LG**	**AM+AC**
S_{w-NH4} (m)	155.4 [106.1, 246.6][a] (97%)	217.7 [134.3, 96.4][a] (92%)	50.0 [12.4, 96.4][a] (96%)
v_{f-NH4} (mm/min)	1.05 [0.74, 1.71][ab] (71%)	0.62 [0.54, 1.15][a] (92%)	3.53 [2.80, 4.46][b] (56%)
U_{NH4} (µg N-NH_4^+ m^{-2} min^{-1})	5.6 [2.3, 11.6][a] (99%)	3.5 [1.5, 7.1][a] (98%)	23.5 [16.6, 37.1][a] (87%)

Note: AM: NH_4^+ addition alone, AM+LG: NH_4^+ + lignin addition, and AM+AC: NH_4^+ + acetate addition. For each spiralling metric, different letters (a and b) indicate statistically significant differences between treatments (multiple comparison Dunn's test, $p < 0.025$). The coefficient of variation for each group (CV) is indicated in brackets and represents the seasonal variability of each spiralling metric for each treatment.
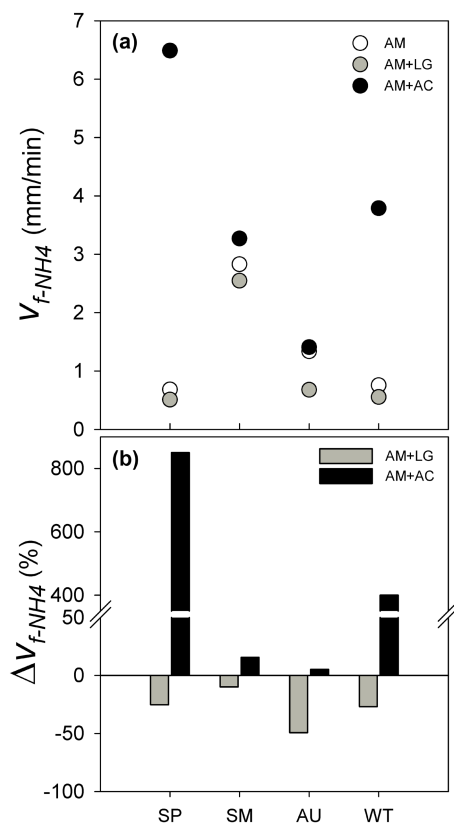

FIGURE 4 (a) In-stream NH_4^+ uptake velocity (v_{f-NH4}), and (b) percentage difference in v_{f-NH4} (Δv_{f-NH4}) between the DOC addition treatments and the AM treatment grouped by season. Positive values of Δv_{f-NH4} indicate an increase in v_{f-NH4} during either the AM+LG or the AM+AC treatments compared to the AM treatment, whereas negative values indicate the opposite. AM: NH_4^+ addition alone, AM+LG: NH_4^+ + lignin addition; AM+AC: NH_4^+ + acetate addition. SP, spring; SM, summer; AU, autumn; WT, winter.

data from the three treatments was grouped by season (in all cases, Kruskal–Wallis test, $p > 0.05$).

In-stream NH_4^+ uptake varied among treatments, although differences were not consistent among spiralling metrics. For S_{w-NH4} and U_{NH4}, there were no statistically significant differences among treatments (Table 2). By contrast, v_{f-NH4} statistically differed among treatments, being higher for the AM+AC than for the AM+LG

treatment (Table 2). Relative differences in v_{f-NH4} between treatments with and without DOC amendments (i.e., Δv_{f-NH4}) varied depending on the DOC source and among seasons. For the AM+LG treatment, Δv_{f-NH4} was negative, especially in autumn (Δv_{f-NH4} = −49.3%) (Figure 4b). By contrast, Δv_{f-NH4} was positive for the AM+AC treatment, being higher in spring and winter ($\Delta v_{f-NH4} > 400\%$) than in summer and autumn ($\Delta v_{f-NH4} < 16\%$) (Figure 4b).

3.4 | Raz transformation rates among seasons and treatments

The Raz:Rru ratio decreased along the reach at *plateau* on all short-term constant-rate additions, except for the AM+LG treatment in autumn, when the lack of in-stream Raz:Rru longitudinal change preclude the estimation of S_{w-Raz} and v_{f-Raz}. Transformation of Raz to Rru under ambient conditions (D0) varied among seasons, but in all cases v_{f-Raz} was lower during D0 than for the respective AM treatment (D1): spring (0.10 vs. 0.15 mm/min), summer (0.14 vs. 0.17 mm/min), autumn (0.03 vs. 0.06 mm/min), and winter (0.04 vs. 0.10 mm/min).

Considering the entire set of Raz transformation rates (ambient and treatments), differences between seasons were observed (Figure 5a). For S_{w-Raz}, median values were shorter in summer (270.8 [243.7, 298.4] m) than in autumn (3059.1 [2776.4, 3341.8] m) and winter (3818.3 [3639.2, 3947.4] m) (Dunn's test; $\chi^2 = 12.13$, $p = 0.010$). For v_{f-Raz}, median values were higher in summer (0.15 [0.13, 0.16] mm/min) than in autumn (0.067 [0.065, 0.068] mm/min) (Dunn's test; $\chi^2 = 9.29$, $p = 0.015$). When data were split by treatment, there were no differences in Raz:Rru transformation (Krsukal–Wallis test: $\chi^2 = 0.88$, $p = 0.83$; Kruskal–Wallis test: $\chi^2 = 2.27$, $p = 0.52$, for S_{w-Raz} and v_{f-Raz}, respectively), probably because of the high variability among seasons within each treatment (CVs ranged from 30.3% to 106.8%). The percentage difference in v_{f-Raz} (Δv_{f-Raz}) of the two DOC treatments (AM+LG and AM+AC) with respect to the AM treatment was negative in almost all cases (−15.2 [−30.7, −11.4] %) (Figure 5b).

Differences in v_{f-NH4}:v_{f-Raz} ratios were not statistically significant among seasons (Kruskall–Wallis test: $\chi^2 = 0.92$, $p = 0.82$) or treatments (Kruskal–Wallis test: $\chi^2 = 6.14$, $p = 0.05$). However, this ratio was several times higher for the AM+AC treatment than for the other two treatments in both spring and winter (Figure 6).
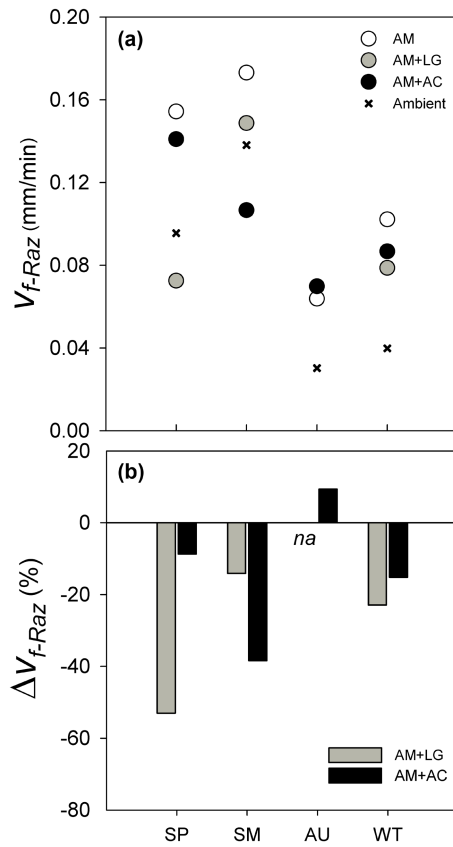
FIGURE 5 (a) In-stream Raz transformation velocity (v_{f-Raz}), and (b) percentage difference in v_{f-Raz} (Δv_{f-Raz}) between the DOC addition treatments and the AM treatment grouped by season. Positive values of Δv_{f-Raz} indicate an increase in v_{f-Raz} during either AM+LG or AM+AC treatments, whereas negative values indicate the opposite. Ambient: day before the three additions; AM: NH_4^+ addition alone; AM+LG: NH_4^+ + lignin addition; AM+AC: NH_4^+ + acetate addition. SP, spring; SM, summer; AU, autumn; WT, winter; na, not available.
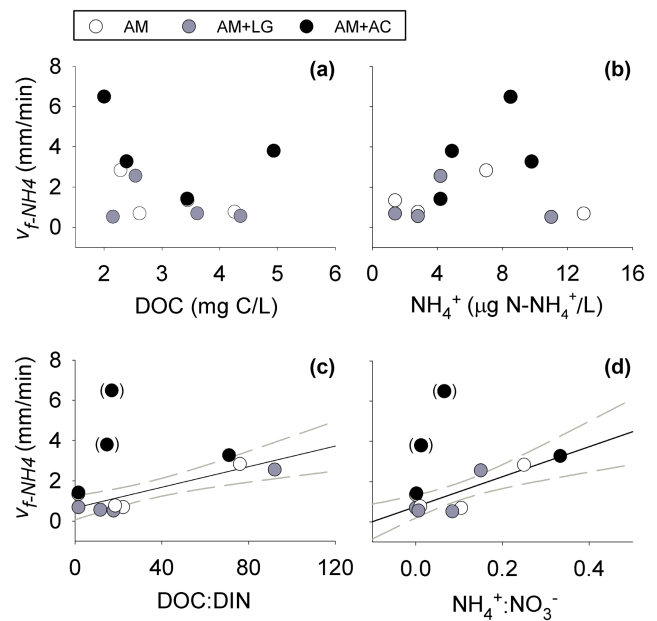
3.5 | Relationship between solute concentrations, in-stream NH_4^+ uptake and Raz transformation rates

There was no statistically significant relationship between v_{f-NH4} and either DOC or NH_4^+ stream background concentrations (in both cases, $p > 0.05$; Figure 7a,b). However, a strong positive relationship emerged between v_{f-NH4} and DOC:DIN and NH_4^+:NO_3^- ratios when the spring and winter values from AM+AC treatment (black circles in brackets in Figure 7), which showed disproportionate increases in v_{f-NH4}, were excluded from the analysis (v_{f-NH4} vs. DOC:DIN: $r^2 = 0.72$, $F_{1,8} = 20.5$, $p = 0.002$; v_{f-NH4} vs. NH_4^+:NO_3^-, $r^2 = 0.71$, $F_{1,8} = 19.7$, $p = 0.002$) (Figure 7c,d).

There was no statistically significant relationship between v_{f-Raz} and either DOC and NH_4^+ stream background concentrations (Figure 8a,b), whereas a moderate positive relationship emerged between v_{f-Raz} and both DOC:DIN ($r^2 = 0.54$, $F_{1,9} = 12.7$, $p = 0.006$) and NH_4^+:NO_3^- molar ratios ($r^2 = 0.54$, $F_{1,9} = 12.8$, $p = 0.006$) (Figure 8c,d). There was no relationship between v_{f-NH4} and v_{f-Raz} ($r^2 = 0.05$, $F_{1,9} = 1.56$, $p = 0.243$).
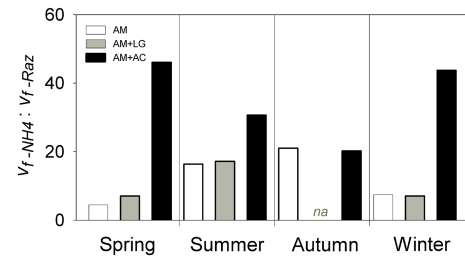
FIGURE 6 Bar plot of the ratio between v_{f-NH4} and v_{f-Raz} for each season and treatment. AM: NH_4^+ addition alone, AM+LG: NH_4^+ + lignin addition; AM+AC: NH_4^+ + acetate addition; na, not available. High values indicate higher NH_4^+ uptake velocity per unit of Raz transformation velocity, which was used as a proxy for in-stream aerobic respiration.
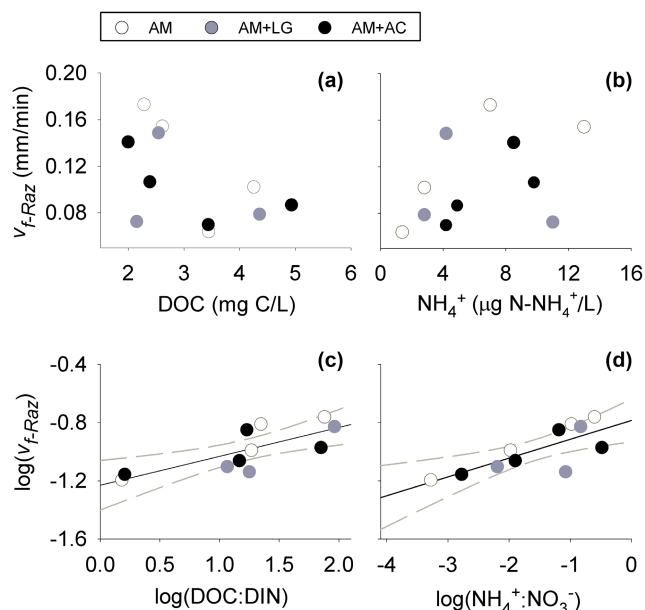

FIGURE 7 Relationship between v_{f-NH4} and stream median concentrations of (a) DOC, (b) NH_4^+, and median stoichiometric ratios of (c) DOC:DIN and (d) NH_4^+:NO_3^-. Colours indicate different treatments: AM: NH_4^+ addition alone, AM+LG: NH_4^+ + lignin addition; AM+AC: NH_4^+ + acetate addition. The solid line indicates the linear regression between the two variables, only shown when statistically significant ($p < 0.05$). Black circles in brackets (corresponding to the values of spring and winter from the AM+AC treatment) are excluded from the linear regressions. Dashed lines indicate the 95% confidence interval.

4 | DISCUSSION

4.1 | Stoichiometry as a driver of seasonal variation in in-stream NH_4^+ uptake

Despite our relatively small dataset, we captured a large variability in in-stream NH_4^+ uptake rates (0.7–2.8 mm/min⁻). These estimates of v_{f-NH4} fall within the low range observed for headwater streams worldwide (0.20–59.20 mm/min; Ribot et al., 2017), but concur with

FIGURE 8 Relationships between v_{f-Raz} and stream median concentrations of (a) DOC, (b) NH_4^+, and the stoichiometric ratio of (c) DOC:DIN, and (d) NH_4^+:NO_3^-. Colours indicate different treatments: AM: NH_4^+ addition alone, AM+LG: NH_4^+ + lignin addition; AM+AC: NH_4^+ + acetate addition. The solid line indicates the linear regression between the two variables, only shown when statistically significant ($p < 0.05$). Dashed lines indicate the 95% confidence interval.

previous studies performed in the same stream (Peipoch et al., 2016; von Schiller et al., 2008). Furthermore, the range of stream concentrations was within that reported in previous studies: from 2.2 to 6.8 mg C/L and from 14 to 2200 μg N/L, for DOC and DIN, respectively (Bernal et al., 2005; Guarch-Ribot & Butturini, 2016; von Schiller et al., 2008). Thus, we feel confident that our experiment captured the natural variability of in-stream NH_4^+ uptake rates that potentially could be attributed to seasonal changes in background solute concentrations.

The observed high seasonal variability in v_{f-NH4} supports the idea that in-stream biota is highly responsive to changes in environmental conditions. We ruled out stream water temperature as a potential driver of these seasonal differences, given that values were relatively high throughout the year ($T > 9°C$). The fact that in-stream NH_4^+ uptake peaked in summer, when stream DIN concentrations were the lowest, could suggest a higher biological NH_4^+ demand under low DIN concentrations. However, during the rest of the year, in-stream NH_4^+ uptake did not respond to changes in DIN concentration. The lack of a significant relationship between v_{f-NH4} and either NH_4^+ or NO_3^- background concentration disagrees with previous studies reporting a negative relationship between DIN concentration and in-stream DIN uptake velocity (Dodds et al., 2002; Mulholland et al., 2008). Likewise, we found no relationship between DOC concentration and v_{f-NH4}, which contrasts with the idea that in-stream DIN uptake increases with increasing DOC availability (Bernhardt & Likens, 2002; Johnson et al., 2012; Robbins

et al., 2017; Rodríguez-Cardona et al., 2016). Instead, in-stream NH_4^+ uptake was strongly related to DOC:DIN ratios, which showed higher values in summer than in autumn. This variable alone explains 72% of the observed variability in v_{f-NH4}, supporting our hypothesis that biological NH_4^+ demand is strongly conditioned by the relative concentration of DOC with respect to DIN.

Our results align with previous studies showing that DOC:DIN stoichiometry better predicts in-stream DIN uptake than DOC or DIN concentration alone (Rodríguez-Cardona et al., 2016; Wymore et al., 2016). In the same vein, our findings are concordant with a global meta-analysis showing that microbial N assimilation, especially under aerobic conditions, is highly responsive to ambient resource stoichiometry (i.e., organic carbon: N ratios) (Helton et al., 2015; Taylor & Townsend, 2010). While these studies focus on comparing DOC:DIN stoichiometry across ecosystems, our study shows that seasonal differences in water stoichiometry can be sufficiently large to drive changes in in-stream NH_4^+ uptake within a given stream. In addition, we found a positive relationship between v_{f-NH4} and the NH_4^+:NO_3^- ratio, suggesting that NH_4^+ uptake is also conditioned by the relative availability of the different forms of DIN. Overall, our results indicate that the concentration of a single solute, either DOC or DIN, is not enough to explain the natural variability of v_{f-NH4}, pointing towards co-limitation of organic substrates and nutrients. Further studies simultaneously considering C and N uptake and including other macronutrients such as phosphorus could help to better understand how DOC bioreactivity and stoichiometric availability ultimately shape in-stream DOC and nutrient uptake in this and other headwater streams (but see Graeber et al., 2021).

4.2 | The role of stream heterotrophs as drivers of seasonal variation in in-stream NH_4^+ uptake

Our results indicate that microbial heterotrophs were mainly responsible for NH_4^+ uptake in Fuirosos. Two results give rise to this conclusion. Firstly, we found no correlation between v_{f-NH4} and irradiance, which, together with low GPP throughout the year (median = 0.20 mg O_2 m^{-2} day^{-1}), suggests that photoautotrophic activity plays a small role in NH_4^+ uptake in this forested stream. Secondly, we found that heterotrophic prokaryotes dominate on both epilithic and epipsammic microbial biofilms during all seasons and that nitrifying organisms showed a small relative mean abundance (<1%), suggesting that heterotrophic NH_4^+ assimilation was an important biogeochemical pathway in the Fuirosos stream. Interestingly, both AOA and AOB were ubiquitous in cobbles and sediments, an indication that nitrification was likely to be occurring. Overall, these results are in agreement with previous studies conducted in forested headwater streams where dense riparian tree canopies limit photoautotrophic activity while promoting the activity of heterotrophs and their role in in-stream NH_4^+ uptake (Fellows et al., 2006; Tank et al., 2018).

Heterotrophic bacteria were likely responsible for in-stream NH_4^+ uptake during all seasons, yet heterotrophic aerobic respiration

(measured from Raz additions) showed high temporal variability. Indeed, the range of values observed across seasons in Fuirosos (v_{f-Raz} from 0.03 to 0.14 mm/min) was similar to that reported for streams across Europe and the United States of America (USA) (0.02–0.28 mm/min) (Argerich et al., 2011; Haggerty et al., 2014; Ledford et al., 2021). Moreover, we found that v_{f-Raz} was positively related to both DOC:DIN and NH_4^+:NO_3^- molar ratios, suggesting that observed patterns of aquatic heterotrophic aerobic respiration and associated in-stream NH_4^+ uptake could result from seasonal changes in stream water stoichiometry.

Seasonal differences in bacterial community composition, which were substantial as highlighted by the NMDS analysis, also could be responsible for the observed changes in v_{f-Raz}. However, our data do not allow to assess how changes in bacterial composition could influence in-stream NH_4^+ uptake and associated heterotrophic aerobic respiration. For instance, v_{f-NH4} showed small differences between spring and winter, even though species richness substantially decreased between the two seasons. These changes in microbial community composition likely derived from the in situ effects of the extratropical cyclone Gloria, a large climatic perturbation that hit our region 1 month before the winter campaign, bringing extreme rainfall amounts and consequent extraordinarily high stream discharge (Ledesma et al., 2021). Yet, despite these major changes in the bacterial community, the capacity of stream biota to process NH_4^+ remained, indicating that this ecosystem function was resilient to large perturbations, likely because of the high functional redundancy of microbial communities (Louca et al., 2017, 2018).

4.3 | DOC bioreactivity influences in-stream NH_4^+ uptake

In line with our hypothesis, we found that the relationships between stoichiometric ratios and in-stream NH_4^+ demand vanished when acetate was released into the stream. Furthermore, acetate additions led to disproportionate increases in v_{f-NH4}, especially in spring and winter, suggesting that stream biota was limited by labile DOC. Our finding provides additional evidence that heterotrophic bacteria are more capable of processing DIN when labile DOC is available (Lane et al., 2013; Lupon et al., 2020; Robbins et al., 2017). By contrast, in-stream NH_4^+ uptake in autumn was not affected by acetate addition, even though DOC:DIN ratios were low (<2). We suggest that, in this case, stream biota met their N requirements with NO_3^- rather than NH_4^+ because NO_3^- concentration was very high in autumn (>2500 μg N–NO_3^-/L), which led to extremely low NH_4^+:NO_3^- ratios (<0.001). The same pattern has been reported previously in other forested headwater streams, such as Hubbard Brook (NH, USA) (Bernhardt et al., 2002).

In agreement with our expectations, no increases in in-stream NH_4^+ uptake were observed during lignin additions for any season. This pattern was reflected by the consistent differences observed in v_{f-NH4} between the AM+AC and AM+LG treatments, which were statistically significant despite the large variation in environmental conditions among seasons. Likewise, Lane et al. (2013) showed that glucose induced higher in-stream DIN uptake than vanilla, a more complex organic molecule. Noteworthy, in-stream NH_4^+ uptake substantially decreased during some lignin additions, suggesting some inhibitory effect of lignin on the activity of stream biota. This result could be explained by an occluding effect of these high molecular-weight compounds, which may stick to the biofilm surface, delaying the entry of smaller and more labile moieties (Freeman et al., 1990; Freeman & Lock, 1992).

Surprisingly, our results indicated that DOC bioreactivity did not influence heterotrophic aerobic respiration. Although values of v_{f-Raz} were usually higher for the AM treatment compared to ambient conditions, they tended to decrease rather than increase after adding either acetate or lignin. This pattern suggests that NH_4^+ additions stimulated in-stream heterotrophic aerobic respiration, but changes in DOC bioreactivity did not. Yet, when in-stream NH_4^+ demand was expressed per unit of aerobic respiration (Δv_{f-NH4}:Δv_{f-Raz}), the ratio was higher for the AM+AC treatment than for the other two treatments in spring, summer, and winter. These results indicate that in-stream NH_4^+ uptake was more efficient when a labile source of DOC was present, suggesting an increase in C-use efficiency, a process strongly affected by the availability and composition of organic matter substrates (Manzoni et al., 2012; Mehnaz et al., 2019).

5 | CONCLUSIONS AND IMPLICATIONS

The present study provides insights into the role of DOC availability in controlling whole-reach NH_4^+ uptake in a Mediterranean headwater stream. We found that in-stream NH_4^+ uptake was driven largely by heterotrophic microbes, with in-stream NH_4^+ demand and heterotrophic aerobic respiration varying markedly across seasons. A substantial fraction of this seasonal variability was explained by water stoichiometry, with NH_4^+ demand increasing with increasing DOC:DIN and NH_4^+:NO_3^- ratios. Our findings provide evidence that DOC:DIN ratios can explain differences in whole-reach N uptake not only across streams (e.g., Rodríguez-Cardona et al., 2016; Stutter et al., 2018; Taylor & Townsend, 2010), but also across seasons within the same stream. Furthermore, our study adds to the growing evidence that labile DOC availability limits heterotrophic activity in forested headwater streams (Bernhardt & Likens, 2002; Strauss & Lamberti, 2000) by showing that acetate can largely increase N uptake by biota at the whole-reach scale. Yet, our results also suggest that the response of heterotrophic bacteria to increases in labile DOC largely depends on the availability of nutrients and, ultimately, on resource stoichiometry, as shown previously in mesocosms and bioassay experiments (Graeber et al., 2021; Soares et al., 2017).

Overall, our results highlight that the functioning of headwater streams, and in particular their capacity to process N, can be highly sensitive to natural or human-induced changes in DOC availability, resulting in either different C:N stoichiometry or DOC bioreactivity. In this sense, restoration strategies focused on balancing resource stoichiometry could contribute to partially solve environmental

problems derived from nutrient excesses (Stutter et al., 2018). Likewise, it is of particular interest to understand how future climate change will impact DOC and DIN concentrations and the stoichiometry of lateral groundwater inputs, and its consequences on in-stream N processing in headwater streams.

CONFLICT OF INTEREST STATEMENT

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study will be openly available in a public repository (HydroShare) that issues datasets with DOIs if the paper is accepted. Nucleotide sequence data is uploaded in electronic format to the NCBI Sequence Read Archive, available through BioProject record ID PRJNA881938.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.