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Influence of nitrate and ammonium availability on uptake kinetics of stream biofilms

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Abstract

Human activity has significantly increased dissolved inorganic nitrogen (DIN) availability and has modified the relative proportion of nitrate (NO_3^-) and ammonium (NH_4^+) species in many stream ecosystems. Understanding the relationship between DIN concentration and DIN uptake is crucial to predict how streams will respond to increased DIN loading. Nonetheless, this relationship remains unclear due to the complex interactions governing DIN uptake. In this study, we aimed to evaluate how biofilms from two streams differing in background DIN concentration would respond to increases in availability and changes in speciation (i.e., NO_3^- or NH_4^+) of DIN. We measured DIN uptake by biofilms in artificial flumes located in each stream, using separate $^{15}N\text{-}NO_3^-$ and $^{15}N\text{-}NH_4^+$ additions in a graded series of increasing DIN concentrations. The ambient uptake rate (U) was higher for NO_3^- than for NH_4^+ in both streams, but only U for NH_4^+ differed between the two streams. In addition, the uptake efficiency ($U_{N\text{-}specific}$) at ambient conditions was higher in the low-N stream for both DIN species. In terms of uptake kinetics, the Michaelis-Menten model best fit the relationship between uptake and concentration in the case of NH_4^+ (for both streams) but not in the case of NO_3^- (neither stream). Moreover, saturation of NH_4^+ uptake occurred at lower rates (lower U_{max}) in the low-N than in the high-N stream, but affinity for NH_4^+ was higher (lower K_s) in the low-N stream. Together, these results indicate that the response capacity of biofilm communities to short-term increases of DIN concentration is primarily determined by the ambient DIN concentrations under which they develop. This study also shows that DIN uptake by benthic biofilms varies not only with DIN availability, but also with DIN speciation, which is often modified by human activities.

Key words: Nitrate, ammonium, biofilm, nitrogen uptake, Michaelis-Menten kinetics, stream, land use, agriculture

Introduction

Human activities have significantly increased the concentration of dissolved inorganic nitrogen (DIN) in streams (Howarth et al. 1996, Carpenter et al. 1998). Understanding how stream DIN uptake (i.e., the process by which stream biota immobilize DIN from the water column) responds to the human alteration of DIN availability has become a research focus for stream ecologists over the past decades (Mulholland & Webster 2010). Some researchers have studied DIN uptake kinetics (i.e., changes in uptake rates in response to changes in concentration) based on the relationship between whole-reach DIN uptake and DIN concentration, using measurements from different streams spanning a broad range of background DIN concentrations (Dodds et al. 2002, Bernot et al. 2006, Newbold et al. 2006, O'Brien et al. 2007). Other studies have focused on DIN uptake kinetics within the same stream by following changes in whole-reach uptake in response to short-term DIN enrichment (Payn et al. 2005, Earl et al. 2006, O'Brien and Dodds 2010, Covino et al. 2010) or by investigating DIN uptake kinetics in mesocosms (Eppley et al. 1969, Kemp and Dodds 2002, O'Brien and Dodds 2008).

According to these studies, there are three mathematical models that describe the relationship between DIN uptake and concentration in streams. The first model corresponds to a first-order response, where uptake rate is directly proportional to concentration of substrate (Dodds et al. 2002). The second model, the efficiency-loss model, follows a power relationship where uptake rate increases with concentration but efficiency declines (O'Brien et al. 2007). The third model follows Michaelis-Menten kinetics, characterized by saturation of uptake when availability exceeds biological demand (Earl et al. 2006). In general, results from inter-stream comparisons suggest that the linear and efficiency-loss models best fit the relationship between DIN uptake and concentration (Dodds et al. 2002, O'Brien et al. 2007). Conversely, results from enrichment experiments within the same stream or in mesocosms

69 (i.e., with the same community) suggest that the Michaelis-Menten model best fits DIN
70 uptake kinetics (Payn et al. 2005, Earl et al. 2006, O'Brien and Dodds 2010).
71 Human activities not only alter the concentration of DIN, but they also change the
72 relative proportion of the two major DIN species: nitrate (NO_3^-) and ammonium (NH_4^+)
73 (Stanley and Maxted 2008, Lassaletta et al. 2009, Martí et al. 2010). Uptake rates and
74 kinetics are expected to differ between NO_3^- and NH_4^+, since energetic costs of assimilation
75 associated with NO_3^- are generally higher than those associated with NH_4^+ (Dortch 1990,
76 Naldi and Wheeler 2002). Furthermore, dissimilatory transformations, wherein neither
77 compound is incorporated into biomass, contribute to both NH_4^+ and NO_3^- 'uptake'.
78 Nitrification (i.e., oxidization of NH_4^+ to NO_3^- by autotrophic or heterotrophic bacteria and
79 archaea) will result in apparent NH_4^+ uptake, whereas NO_3^- 'uptake' may include
80 denitrification (i.e., the respiratory process by which bacteria reduce NO_3^- to N_2). These
81 transformations are carried out by different organisms and governed by different controlling
82 factors (Bothe et al. 2007), and thus may additionally contribute to the expected differences
83 between NO_3^- and NH_4^+ uptake kinetics. Most studies have investigated NO_3^- or NH_4^+
84 uptake separately; thus, we do not know how uptake kinetics differ between these two DIN
85 species under similar environmental conditions. In addition, little is known about differences
86 in uptake kinetics of NO_3^- or NH_4^+ for stream biofilms (i.e., the microbial communities that
87 develop on stream substrata associated to increases in DIN availability. Understanding DIN
88 uptake kinetics of stream biofilms is especially important since biofilms are major
89 contributors to nutrient dynamics in stream networks (Pusch et al. 1998, Battin et al. 2003)
90 and may therefore play a role in ameliorating anthropogenic DIN inputs.
91 In this study, we compared uptake rates and kinetics for NO_3^- and NH_4^+ between
92 biofilms developed in two streams differing in background DIN concentrations. We
93 measured biofilm uptake rates using experiments that separately added ^{15}N-labeled NO_3^- and

NH_4^+ at increasing concentrations of the two DIN species to artificial flumes located in each stream. We predicted that ambient uptake rates would be higher for NO_3^- than for NH_4^+, and in the high-N stream compared to the low-N stream, due to the higher availability of NO_3^- with respect to NH_4^+ as well as the overall higher DIN availability in the high-N stream. In terms of uptake kinetics, we predicted that Michaelis-Menten model would best fit the relationship between DIN uptake and concentration because DIN uptake is mediated by enzymatic processes. In particular, we expected lower maximum uptake (U_{max}) and half-saturation constant (K_s) for NH_4^+ than for NO_3^- because of the lower energetic cost for assimilation of NH_4^+ than of NO_3^-. We further expected U_{max} and K_s to be lower in the low-N stream than in the high-N stream owing to differences in N affinity between stream biofilms resulting from different histories of nutrient exposure.

Material and Methods

Study sites

Font del Regàs (2º27'00''E, 41º49'32''N; 929 m asl) is a forested stream situated within the protected area of the Parc Natural del Montseny at the headwaters of the catchment of the river La Tordera. Santa Coloma (2º37'52''E, 41º52'18''N; 425 m asl) is an agricultural stream situated next to gardening plantations in a lower part of the same catchment. Discharge (mean ± SE, in L/s) was 56 ± 12 for Font del Regàs, and 163 ± 35 for Santa Coloma (biweekly samplings from September 2004 to July 2007; Ribot et al. unpublished data), and concentrations (mean ± SE, in µg N/L) of NO_3^- and NH_4^+ were 181 ± 11 and 12 ± 1 for Font del Regàs, and 780 ± 44 and 19 ± 2 for Santa Coloma (biweekly samplings from September 2004 to July 2007; Ribot et al. unpublished data). Hereafter, we refer to Font del Regàs as the low-N stream and to Santa Coloma as the high-N stream.

119 *Channel experiments*

120 We conducted the experiments from 3 to 24 July 2007 in the low-N stream and from
121 23 October to 7 November 2007 in the high-N stream. We placed a set of 6 parallel PVC
122 channels (6 m long and 15 cm wide) on the streambed using a metallic structure that held
123 them together and above the stream water (Fig. 1a). Water from an upstream tank fed all
124 channels continuously with a mean (± SE) flow rate of 1.8 ± 0.018 L/min (from
125 measurements done daily throughout the experiments and in each channel). We filled the
126 channels with stream cobbles of similar size and biofilm coverage, which were collected
127 from the streambed within <50m upstream from the channel setting. We then exposed them
128 to 24-h fertilization cycles of increasing concentration levels (1x, 4x, 8x, 16x and 32x the
129 background concentration) of either NO_3^- or NH_4^+ (n = 3 channels each; Fig. 1a and b). We
130 released two independent solutions of NO_3^- (as $NaNO_3$) and NH_4^+ (as NH_4Cl) to the
131 corresponding channels at a constant rate, using a 3-output carboy (one per channel),
132 maintaining a constant head in the carboy with a Masterflex (Vernon Hills, Illinois, USA)
133 L/S battery-powered peristaltic pump. To maintain the background stoichiometric ratio
134 between DIN and soluble reactive phosphorus (SRP) throughout the fertilization cycles, we
135 also added phosphate (as $NaH_2PO_4 \cdot H_2O$) proportionally into the solution at each fertilization
136 level.

137 To estimate N uptake rates of biofilms, we conducted a tracer addition of either
138 $^{15}NO_3^-$ (n = 3 channels) or $^{15}NH_4^+$ (n = 3 channels) over the last 6 h of each fertilization
139 level. We added two independent solutions amended with $^{15}NO_3^-$ (as 99% enriched $K^{15}NO_3$)
140 or $^{15}NH_4^+$ (as 99% enriched $^{15}NH_4Cl$) in conjunction with NaCl as a conservative tracer at a
141 constant rate using a similar setup as described above. We calculated the amount of $K^{15}NO_3$
142 and $^{15}NH_4Cl$ to produce a target $\delta^{15}N$ enrichment of 3000‰ for both DIN species in the

channels. To verify steady plateau conditions, we automatically recorded conductivity at the end of each channel using a portable WTW conductivity meter (Weilheim, Germany).

Prior to fertilizations, we collected water at the downstream end of each channel for the analysis of ambient nutrient concentration (3 replicates per channel) and $^{15}NH_4^+$ and $^{15}NO_3^-$ signatures (1 replicate per channel). We also collected composite biofilm samples for the analysis of biomass, pigment content, and ^{15}N natural abundance (1 replicate per channel) by scraping 3 randomly selected cobbles and filtering the biomass onto ashed, pre-weighed GF/F filters. Before completion of the fertilization period (when fertilization and ^{15}N addition were running together), we collected another set of water samples for the analysis of nutrient concentration and $^{15}NH_4^+$ and $^{15}NO_3^-$ signatures and of biofilm samples (3 replicates per channel). After that, we stopped the additions, emptied the channels, cleaned them, and filled them again with cobbles from the stream to initiate the experiment with a higher fertilization level (Fig. 1b). We filtered the water samples immediately through ashed Whatman (Maidstone, UK) GF/F glass-fiber filters into acid-washed, plastic containers and stored them on ice for transportation to the laboratory. We estimated the cobble surface by covering it with aluminum foil and weighing it. We stored the filters with biofilm samples on ice in the field, and then froze them (for chlorophyll-a analysis) or oven-dried them (for ash free dry mass and ^{15}N analysis) in the laboratory until further processing. We measured and logged photosynthetically active radiation (PAR) every 10 min using a Skye (Powys, UK) SKP215 quantum sensor connected to a Campbell Scientific data logger. We measured temperature at plateau conditions using a WTW portable conductivity meter.

Laboratory analyses

We analyzed water samples for the concentrations of NO_3^-, NH_4^+, and SRP on a Bran+Luebbe (Norderstedt, Germany) TRAACS 2000 autoanalyzer following standard colorimetric methods (APHA, 1995). We processed water samples for the analysis of $^{15}NO_3^-$

168 and $^{15}NH_4^+$ using the ammonia-diffusion technique (Sigman et al. 1997 and Holmes et al.
169 1998, respectively). For $^{15}NO_3^-$ determination, we amended a known volume of sample with
170 3 g of MgO and 5 g of NaCl and boiled it to remove the NH_4^+. We then added 0.5 mg MgO
171 and 0.5 mg Devarda's alloy to reduce the NO_3^- to NH_4^+, and treated the remaining sample as
172 for $^{15}NH_4^+$. For $^{15}NH_4^+$ determination, we amended a known volume of sample with 3 g/L of
173 MgO and 50 g/L of NaCl and a Teflon filter packet containing a 1-cm-diameter ashed
174 Whatman GF/D fiber glass filter acidified with 25 µL of 2.5 M $KHSO_4$ (to trap the
175 volatilized NH_3), and incubated it on a shaker at 40ºC for 4 wk. Once the incubation was
176 completed, we removed the filter packets and placed them in a desiccator for 4 d. Filters
177 were then encapsulated in tins and stored until ^{15}N analysis.
178 We oven-dried filters with biofilm samples at 60ºC until they reached a constant
179 weight. To estimate the biofilm ash-free dry mass (AFDM; in g m^{-2}), we weighed
180 subsamples on a Sartorious (Göttingen, Germany) MC1 analytical balance, and combusted
181 them at 500ºC for 5 h. We determined the chlorophyll-a content of biofilms (in µg/cm^2)
182 following McIntire et al. (1996). We submerged frozen filters in a known volume of 90%
183 v/v acetone and kept them in dark conditions at 4ºC overnight. We sonicated the filters for 5
184 min and centrifuged them for 10 min at 4000 rpm. We measured the absorbance of the
185 resultant supernatant at 664, 665 and 750 nm before and after acidification using a Shimadzu
186 (Tokyo, Japan) UV spectrometer. To determine the ^{15}N signature of biofilms, we weighed
187 subsamples of 1-cm diameter to the nearest 0.001 mg on a Mettler-Toledo (Greifensee,
188 Switzerland) MX5 microbalance and encapsulated them in tins. We sent the samples for
189 analysis at the University of California Stable Isotope Facility (Davis, California, USA). The
190 N content (as a percentage of dry mass) and the abundance of the heavier isotope, expressed
191 as the ^{14}N:^{15}N ratio compared to that of a standard (N_2 from the atmosphere) using the
192 notation of $\delta^{15}N$ in units of ‰, were measured by continuous-flow isotope-ratio mass

spectrometry (20–20 mass spectrometer; PDZ Europa, Northwich, UK) after sample combustion in an on-line elemental analyzer (PDZ Europa ANCA-GSL).

Calculation of uptake rates and data analysis

Differences in ambient nutrient concentrations, biofilm AFDM and biofilm chlorophyll-a content between streams were explored using independent *t*- tests.

To calculate the uptake rates of NO_3^- and NH_4^+ we first calculated the amount of ^{15}N tracer contained in biofilm ($^{15}N_{biofim}$; in µg N/m^2) using the following equation:

$$^{15}N_{biofilm} = B_{biofilm} \times N/100 \times (MF_i - MF_b) \quad (1)$$

where $B_{biofilm}$ is the biofilm biomass as dry mass per unit of area, N is the biofilm N content expressed as percentage of dry mass, MF is the molar fraction of ^{15}N in biofilm at plateau conditions (MF_i) and at background conditions (MF_b).

We estimated the biofilm N uptake rate (U; in µg N m^{-2} s^{-1}) for either NO_3^- or NH_4^+ using the following equation (adapted from von Schiller et al. 2007):

$$U = \frac{^{15}N_{biofilm}}{T_{addition} \times (^{15}N_{flux}/N_{flux})} \quad (2)$$

where $^{15}N_{biofim}$ is the amount of ^{15}N tracer in biofilm biomass from eqn (1), $T_{addition}$ is the duration of the ^{15}N addition (6 h), $^{15}N_{flux}$ is the ^{15}N flux (as either NO_3^- or NH_4^+) at plateau conditions in the channel water and N_{flux} is the total N flux (as either NO_3^- or NH_4^+) at each fertilization level in the channel water based on concentration and channel flow rate (µg N s^{-1}). We then calculated the biomass-specific N uptake rate ($U_{N\text{-}specific}$; d^{-1}) for both biofilm communities and DIN species as a surrogate of N uptake efficiency by dividing the biofilm N uptake rate (µg N m^{-2} s^{-1}) by the N content of dry mass (µg N/m^2).

To compare U and $U_{N\text{-}specific}$ for NO_3^- and NH_4^+ at ambient conditions within and between streams, we used a two-way ANOVA with DIN species (n=2) and stream (n=2) as

factors. Post-hoc Tukey HSD tests following significant ANOVA ($p < 0.05$) were used to further examine the effects of stream and DIN species on both U and $U_{N\text{-}specific}$.

To explore the relationship between U and concentration of each DIN species at the different levels of fertilization, we determined the fit of our experimental data to the 3 mathematical models described in the introduction. The 1st-order response model followed the equation:

$$U = a + bC \quad (3)$$

where U is assumed to increase linearly with DIN concentration (C). The Michaelis-Menten model followed the equation:

$$U = \frac{U_{max}\ C}{K_s + C} \quad (4)$$

where C is the DIN concentration, U_{max} is the maximum uptake rate, and K_s is the concentration at which half the maximum uptake is reached. K_s is an indicator of the biofilm affinity for DIN; high values indicate lower affinity than low values. Finally, the efficiency loss model followed the equation:

$$U = aC^b \quad (5)$$

where U is assumed to increase with DIN concentration (C) as a power law with a slope (b)<1.

The a and b coefficients from each mathematical model (for the Michaelis-Menten model, a corresponds to U_{max} and b corresponds to K_s), were calculated based on Gauss-Newton algorithm, an iterative process which seeks the values of the parameters that minimize the sum of the squared differences between the observed and predicted values of the dependent variable. We then estimated the confidence intervals (95 %) for each coefficient by the generic function confint powered by R software. The default method

239 assumes asymptotic normality, and needs suitable coef and vcov methods to be available.
240 The default method can be called directly for comparison with other methods. We used the
241 Akaike Information Criterion (AIC) to estimate Akaike weights (W_i), which yield the
242 relative likelihood of each model given a particular data set. Within the set of candidate
243 models for the data, we selected the model with the highest W_i value.
244 We conducted all statistical tests with R 2.14.0 (R Foundation for Statistical
245 Computing, Vienna, Austria, http://www.R-project.org/.). When necessary, data were log-
246 transformed prior to analysis in order to meet assumptions of homogeneity of variance and
247 normality (Zar, 1996).
248
249 **Results**
250 The two study streams differed substantially in environmental conditions during the
251 experiments (Table 1). Mean water temperature and PAR were 1.4 and 7 times higher,
252 respectively, in the low-N stream than in the high-N stream. Consistent with the long-term
253 trend (i.e, biweekly sampling), mean NO_3^- concentration was 2 times higher in the high-N
254 stream (*t*-test, $p < 0.001$, Table 1). Mean NH_4^+ concentration in the low-N stream was half
255 of that in the high-N stream (*t*-test, $p < 0.001$) contrasting to the long-term trend, when the
256 mean NH_4^+ concentration of the low-N stream was twice as low as that of the high-N stream
257 (Table 1). Mean SRP concentration was 4 times lower and mean DIN:SRP ratio was 8 times
258 higher in the high-N stream with respect to the low-N stream (*t*-test, $p < 0.001$).
259 Furthermore, the two study streams showed important differences in biofilm structure (Table
260 1). The mean AFDM and the mean chlorophyll-a content were significantly higher (5 and 9
261 times, respectively) in the biofilm of the high-N stream than in the biofilm of the low-N
262 stream (*t*-test, $p < 0.001$).

263 Results from the two-way ANOVAs showed that both factors (DIN species and
264 stream) as well as their interaction had a statistically significant effect on both U and $U_{N\text{-}specific}$
265 at ambient concentrations ($p < 0.01$ in all cases). The U (μg N m^{-2} s^{-1}) for NO_3^- (mean
266 ± SE = 3.1 ± 0.6 in the low-N stream and 4.1 ± 0.8 in the high-N stream) was higher than U
267 for NH_4^+ (0.3 ± 0.02 in the low-N stream and 0.06 ± 0.01 in the high-N stream) in both
268 streams (Fig 2A). Post-hoc comparisons between streams showed that U for NH_4^+
269 significantly differed between streams (Tukey HSD test, $p = 0.001$) whereas U for NO_3^- did
270 not (Tukey HSD test, $p = 0.636$). Similarly, $U_{N\text{-}specific}$ (d^{-1}) for NO_3^- (mean ± SE = 4.1 ± 0.8
271 in the low-N stream and 1.0 ± 0.2 in the high-N stream) was higher than $U_{N\text{-}specific}$ for NH_4^+
272 (0.4 ± 0.02 in the low-N stream and 0.01 ± 0.002 in the high-N stream) in both streams (Fig
273 2B). In contrast to U, post-hoc comparisons showed that $U_{N\text{-}specific}$ for both NO_3^- and NH_4^+
274 differed between streams (Tukey HSD test, $p < 0.001$).
275 Uptake responses to increases in DIN concentration differed substantially between
276 DIN species and streams (Fig. 3). The relationship between U and concentration for NO_3^-
277 differed between the two streams, but in any case uptake kinetics fitted a Michaelis-Menten
278 model (Fig. 3A-B). In the low-N stream, AIC analysis indicated that the relationship
279 between U and concentration for NO_3^- better fit a 1^{st}-order model with a negative slope
280 (Table 2). Conversely, in the high N-stream the estimated confidence intervals (95%) for the
281 b parameter in the three models crossed 0, indicating no significant fit, and AIC analysis
282 resulted in no clear model selection (Table 2).
283 U for NH_4^+ varied with increases in NH_4^+ concentration in the two study streams
284 (Fig. 3C-D). The AIC analysis selected the Michaelis-Menten model as the best fit for the
285 relationship between U for NH_4^+ and NH_4^+concentration in both streams (Table 2).
286 However, uptake kinetic parameters differed between the two streams. The maximum
287 uptake rate (U_{max}; in μg N m^{-2} s^{-1}) and the half saturation constant (K_s; in μg N/L) were

288 lower in the low-N stream, and estimated confidence intervals (95%) for the both parameters
289 did not overlap between streams (Table 2).
290
291 **Discussion**
292 In this study we evaluated the response of biofilm N-uptake rates to changes in DIN
293 concentration, and determined whether this response varied depending on the DIN species
294 considered. We used an experimental approach that combined nutrient fertilizations and ^{15}N-
295 tracer additions in *in situ,* artificial flumes. We predicted that uptake rates and kinetics
296 would differ depending on DIN species (NO_3^- vs. NH_4^+) and ambient DIN concentration in
297 the stream (low-N vs. high-N). Our results supported these predictions only partially. The
298 ambient uptake rate (U) was higher for NO_3^- than for NH_4^+ in both streams, but only U for
299 NH_4^+ differed between streams, with lower values in the high-N stream. In addition, the
300 uptake efficiency ($U_{N\text{-}specific}$) at ambient conditions was higher in the low-N stream for both
301 DIN species. In terms of uptake kinetics, the Michaelis-Menten model best fit the
302 relationship between uptake and concentration in the case of NH_4^+ (for both streams), but
303 not in the case of NO_3^- (neither stream). Moreover, saturation of NH_4^+ uptake occurred at
304 lower rates (lower U_{max}) in the low-N stream than in the high-N stream, but affinity for NH_4^+
305 was higher (lower K_s) in the low-N stream.
306 *Biofilm DIN uptake in streams of contrasting DIN availability and speciation*
307 The rates of epilithic biofilm uptake (U) for both DIN species under ambient
308 conditions measured in this study were on the same order of magnitude as values reported
309 from previous studies using whole-stream ^{15}N-tracer additions (Ashkenas et al. 2004,
310 Hamilton et al. 2001, Merriam et al. 2002, Mulholland et al. 2000,Tank et al. 2000, von
311 Schiller et al. 2009, Sobota et al. 2012). This indicates that the epilithic biofilm uptake rates
312 measured in our channel experiments were representative of natural field conditions.

313 We found that ambient *U* was an order of magnitude higher for NO_3^- than for NH_4^+
314 in the two study streams, even though NH_4^+ is theoretically an energetically less costly DIN
315 source and was thus expected to be preferentially assimilated over NO_3^- (Dortch 1990, Naldi
316 and Wheeler 2002). In fact, estimated values of the relative preference index (RPI) were
317 close to 1 in the two streams. This index was proposed by Dortch (1990) as a means to
318 determine the preference for NH_4^+ over NO_3^- (if values are <1) or for NO_3^- over NH_4^+ (if
319 values are >1). The RPI value of ~1 in our study suggests that biofilms in the two streams
320 have no preference for either DIN species. Thus, the observed higher uptake rates for NO_3^-
321 than for NH_4^+ was mostly attributable to the fact that NO_3^- was present at higher
322 concentration than NH_4^+.
323 While no difference in ambient *U* for NO_3^- was observed between streams, ambient
324 *U* for NH_4^+ was an order of magnitude lower in the high-N stream. Higher NO_3^- availability
325 relative to NH_4^+ availability in the high-N stream may have favored NO_3^- uptake over NH_4^+
326 uptake in this stream, as suggested by other authors (Fellows et al. 2006, Newbold et al.
327 2006, Bunch and Bernot 2012). Furthermore, at low NH_4^+ concentration, the presence of
328 NO_3^- can favor NO_3^- assimilation (Geiseeler et al. 2010). It is known that the expression and
329 further biosynthesis of assimilatory nitrate reductase (i.e., the enzyme responsible for NO_3^-
330 assimilation processes) is induced by the presence of NO_3^- and NO_2^- and suppressed by the
331 presence of NH_4^+ (Gonzalez et al. 2006). Thus, in the high-N stream, the concurrence of
332 high NO_3^- concentration and low NH_4^+ concentration at ambient conditions may have
333 resulted in lower NH_4^+ assimilation rates compared to the low-N stream.
334 Differences in nitrification, which can also contribute to NH_4^+ 'uptake' within the
335 biofilms, are another potential explanation for the differences in U between the streams. If
336 nitrification rate was constrained by the low substrate (i.e., NH_4^+) availability in the high-N
337 stream, we would expect the contribution of nitrification to total NH_4^+uptake to be lower in

that stream. In fact, in the two streams we observed an increase in $\delta^{15}NO_3^-$ at plateau conditions in the channels where we did the additions of $^{15}NH_4^+$, which is indicative of nitrification (mean ± SE, 2.6‰ ± 0.5 and 1.9‰ ± 0.9 in the low-N stream and the high-N stream, respectively). Based on these $\delta^{15}NO_3$ increases, for each fertilization cycle we estimated the contribution of nitrification to total biofilm NH_4^+ 'uptake'. In the low-N stream this contribution ranged from 0.2% to 7.6%, whereas it was <0.2% in the high-N stream. These results contrast with findings from Bernhardt et al. (2002), who found a higher contribution of nitrification to total NH_4^+ uptake in high-NO_3^- streams of Hubbard Brook (New Hampshire, USA). They hypothesized that when assimilatory processes switch to NO_3^- uptake (i.e., in high-NO_3^- streams), competition between nitrifiers and heterotrophs is ameliorated, resulting in higher nitrification rates. Our data do not support this mechanism, since nitrification rate was probably lower in the high-N stream. Instead, we suggest that combination of both lower NH_4^+ assimilation and lower nitrification by biofilms in the high-N stream explains the differences in U for NH_4^+ between the two streams.

The $U_{N\text{-}specific}$ values indicate that the biofilm from the high-N stream was less efficient at taking up both NO_3^- and NH_4^+ from the water column than the biofilm from the low-N stream. Lower uptake efficiencies are often found in streams with high DIN concentrations, due to saturation of the assimilative processes (O'Brien et al. 2007). Thus, our results suggest functional differences in the way DIN is cycled within biofilm communities grown under low- and high-N conditions, which in turn may also determine the observed differences in the uptake kinetic response for both DIN species between stream types.

Biofilm DIN uptake kinetics

Contrary to expectations from nutrient kinetic theory, increases in NO_3^- availability did not enhance biofilm uptake rates for NO_3^-. In the high-N stream, addition of NO_3^- had no

363 effect on biofilm uptake, suggesting that uptake capacity of biofilm assemblages was most
364 likely saturated at the ambient NO_3^- concentration. Earl et al. (2006) suggested that when N
365 is no longer limiting in streams, a zero-order mathematical model (i.e., constant rate with
366 slope of 0) is more applicable, which is in concordance with results found in the high N-
367 stream. The lack of biofilm uptake response to increases in NO_3^- concentration could be
368 alternatively explained by tight coupling of NO_3^- uptake to availability of other nutrients
369 (Fairchild et al. 1985, Sterner et al. 1992). In this regard, Schanz and Juon (1983) suggested
370 that phosphorus (P) is potentially a limiting element at DIN:P ratios above 20 (others have
371 suggested a transition from N to P limitation at DIN:P ratios around 16-17; Redfield 1958,
372 Grimm and Fisher 1986). Although we added SRP in the fertilization solutions to maintain
373 background DIN:P ratios throughout fertilizations, these ratios were well above the potential
374 P-limitation thresholds, especially in the high-N stream (i.e., mean ± SE, 394 ± 32). In this
375 sense, NO_3^- uptake in the high-N stream may have been constrained by P insufficiency.
376 However, If P was the limiting nutrient, one might expect that increases in P availability
377 should alleviate any P limitation and thus enhance NO_3^- uptake. We believe this alternative
378 explanation is unlikely, since previous nutrient-limitation bioassays in the high-N stream
379 have failed to show P limitation (von Schiller et al. 2007).
380 Increases in NO_3^- availability in the low-N stream provoked a decrease in biofilm
381 uptake rates, indicating a possible inhibitory effect of high NO_3^- concentrations on biofilm
382 uptake in this stream. Inhibitory effects on the uptake of NH_4^+ or NO_2^- at high concentrations
383 are reported in the literature (usually associated with nitrification processes; Kim et al 2006,
384 Vadivelu et al. 2007). However, as far as we know, there is no previous evidence of
385 inhibition of NO_3^- uptake at high NO_3^- concentrations. Nevertheless, inhibitory effects of
386 long-term NO_3^- enrichment on periphyton growth have been reported from nutrient-
387 diffusing substrate experiments (Bernhardt and Likens 2004) and a few studies have shown

388 potentially toxic effects of NO_3^- on freshwater animals and plants (Camargo and Alonso
389 2006; Lambert and Davy 2011). Unfortunately, our experiments do not allow us to
390 determine the mechanisms that could explain the observed pattern, but they provide
391 evidence that a short-term, sharp increase in NO_3^- concentration may have inhibitory effects.
392 Michaelis-Menten kinetics described biofilm uptake responses to increases in NH_4^+
393 concentration in the two streams. Because values of K_s were higher than ambient
394 concentrations of NH_4^+ in both streams, we conclude that biofilm uptake for this DIN source
395 was below saturation at ambient concentrations (Tilman 1982). Therefore, biofilms were
396 able to respond positively to short-term increases in NH_4^+ concentration within a certain
397 range in the two streams. Bunch and Bernot (2012) also compared uptake responses of
398 microbial communities to NH_4^+ and NO_3^- enrichments; and they observed that responses
399 were more immediate and pronounced in the case of NH_4^+ and were delayed and more
400 variable in the case of NO_3^-. They suggested that preference for NH_4^+ as a DIN source by
401 microbial communities dictates stronger and more rapid uptake responses to changes in
402 NH_4^+ than in NO_3^- concentration.
403 Our results agree with those by Bunch and Bernot (2012) in showing rapid response
404 to increases in NH_4^+; however, in this study the values of RPI of ~1 indicated no clear
405 preference for NH_4^+ over NO_3^-, at least under ambient conditions. An alternative explanation
406 for the difference in the kinetic responses between NO_3^- and NH_4^+ involves enzymatic
407 responses to short-term changes in availability. Increased availability of NH_4^+ in NH_4^+-
408 amended channels may have triggered repression of NO_3^- reductase and increased biofilm
409 NH_4^+ uptake to meet N demand (Gonzalez et al. 2006). This could explain the positive
410 biofilm NH_4^+ uptake response to increases in NH_4^+ concentration even though uptake
411 responses for NO_3^- indicated that biofilm demand for this DIN species was saturated at
412 ambient conditions. Previous studies show a Michaelis-Menten response of nitrification

413 rates to increases in NH_4^+ concentration within a similar range of NH_4^+ concentrations used
414 in our study (Koper et al. 2010). Nitrification was likely substrate-limited at the relatively
415 low NH_4^+ concentrations in the two study streams, which would produce a positive response
416 to increased NH_4^+ concentration that conforms to a Michaelis-Menten model. However, our
417 a posteriori calculations of nitrification contribution to the whole-channel uptake suggest
418 that this is only a minor contributor to observed kinetics of NH_4^+ uptake. We suggest that a
419 combination of several of the above-mentioned mechanisms best explains the different
420 kinetic responses of NH_4^+ and NO_3^- in the two study streams.

421 Although NH_4^+ uptake kinetics fit the Michaelis-Menten model in the two streams,
422 the kinetic parameters (i.e., K_s and U_{max}) clearly differed between streams, supporting our
423 predictions. NH_4^+ U_{max} of the biofilm in the high-N stream was 21 times higher than U_{max} of
424 the biofilm in the low-N stream. The high-N stream had higher biofilm biomass as well as
425 more photoautotrophic organisms (as indicated by the chlorophyll-a content) than the low-N
426 stream, which could explain the higher maximum uptake observed in the high-N stream.
427 However, U_{max} weighted by N content of biofilm dry mass, a surrogate measure of uptake
428 efficiency, was only 4 times higher in the high-N stream. Biofilms in the low-N stream were
429 therefore relatively more efficient in the uptake of NH_4^+ than those in the high-N stream,
430 which is in agreement with uptake results measured at ambient DIN conditions.

431 In contrast, the biofilm in the low-N stream showed a higher affinity (i.e., lower K_s)
432 for NH_4^+ than the biofilm in the high N-stream. Higher affinities for substrate are often
433 attributed to microorganisms exposed to lower ambient concentrations (Collos et al. 2005,
434 Martens-Habbena et al. 2009). This explanation may not apply to our study if we only
435 consider ambient NH_4^+ concentration, which was similar and low in the two streams.
436 However, it is more appropriate in discussing nutrient limitation to consider the total DIN
437 concentration, which was two times lower in the low-N stream, since biofilms are capable of

meeting their N demand by uptake of either DIN species. Alternatively, differences in NH_4^+ affinity between streams may be caused by boundary-layer constraints arising from differences in biofilm structure (Dodds et al. 2002). In support of this idea, the higher AFDM content per unit area in the high N-stream implies thicker biofilms and higher diffusion limitation for DIN to reach all cells in the biofilm (Stewart 2003, Teissier et al. 2007). Diffusion limitation has been demonstrated for inorganic carbon uptake and nitrification activity in model biofilms; both processes were restricted to the surface layer of different thickness (Gieseke et al. 2005). As a result, the thickness of the biofilm in the high-N stream may contribute to increase the range of NH_4^+ concentration within which there is a positive response of NH_4^+ uptake rate. It is worth noting that the constraints due to diffusion in thicker biofilms operate for both N assimilation and nitrification; and thus, this can contribute to amplify the NH_4^+ concentration range before saturation because the two processes may be subjected to different kinetics.

Finally, we cannot rule out differences between the two streams in environmental conditions, such as light availability and temperature, as causes of observed differences in biofilm uptake kinetics for NH_4^+. Although we aimed to conduct experiments in the two streams within similar ranges of environmental conditions, a large flood occurred in the high-N stream, forcing us to postpone the experiment until the biofilm communities recovered fully. As a result, temperature and light availability were higher in the low-N stream than in the high-N stream during the experiments, which could have enhanced biofilm activity and kinetic responses in the low-N stream. However, the relevance of temperature for nutrient uptake kinetics remains unclear; some studies have shown no evidence of sensitivity of Michaelis-Menten parameters to temperature (Smith et al 2011). Although light availability was higher in the low-N stream, the chlorophyll a content in the high-N stream was ~9 times higher than in the low-N stream. Thus, this factor could not

463 have caused the kinetic differences observed, at least for the photoautotrophic component of
464 the biofilms. These arguments suggest that observed differences in biofilm uptake kinetics
465 between streams are more influenced by stream differences in DIN concentrations and
466 relative proportions of the two DIN species than by differences in other environmental
467 factors.

468 *Conclusions*

469 Biofilm uptake responses to short-term changes in DIN concentration in the two
470 investigated Mediterranean streams during the study period varied depending on ambient
471 conditions, including DIN concentrations, where biofilm developed, as well as on the DIN
472 species considered. Under short pulses of increased DIN concentration, these particular
473 stream biofilms were more reactive to changes in NH_4^+ concentration than to changes in
474 NO_3^- concentration, yet ambient uptake rates for NO_3^- far exceeded those for NH_4^+, largely
475 because the former N species was present at much higher concentration. The greater kinetic
476 response to NH_4^+ may be attributable to repression of enzymes associated with NO_3^- uptake,
477 or a different process (nitrification) contributing to total uptake. The lack of response to
478 NO_3^- suggests this species is at saturating concentrations. Our results contrast with findings
479 from laboratory-scale experiments, in which NO_3^- kinetics conformed to the Michaelis-
480 Menten model (Eppley et al. 1969, Kemp and Dodds 2002, Maguer et al. 2011). In our
481 study, stream biofilm communities were able to respond to increases in NH_4^+ concentration,
482 which is an energetically cheaper N source than NO_3^- and is also the substrate for
483 nitrification. However, clear differences in NH_4^+ response by biofilms were observed
484 between the two streams, likely owing to differences in biofilm characteristics, interactions
485 which other N species, such as NO_3^-, or adaptive changes in affinity.

486 As pointed out by other studies, human activities associated with different land uses
487 not only may enrich the adjacent streams with DIN but may also alter the proportion of DIN

488 species in those ecosystems. In this regard, streams draining catchments dominated by
489 agricultural practices tend to be enriched in NO_3^- whereas streams draining urbanized
490 catchments are often NH_4^+-enriched (Stanley and Maxted 2008; Lasaletta et al. 2009; Martí
491 et al. 2010). Given widespread changes in land use, our findings have implications for
492 understanding and managing N losses to downstream ecosystems, since the distinct N
493 species that reach stream ecosystems could be potentially retained by the in-stream biofilm
494 communities (i.e., NH_4^+) or exported downstream, with the subsequent enrichment of
495 receiving waters (i.e., NO_3^-).
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Acknowledgements

We thank A. Poch, S. Merbt and L. Proia for excellent field assistance and J. L. Riera for assistance in statistical analyses. We are also grateful to the Font del Regàs landowners and Massaneda Garden for allowing access to the study sites during the experiments. This study was funded by the Spanish Ministry of Education and Science through NICON project (ref: CGL2005-7362). M. Ribot was supported by a contract with the Spanish Ministry of Science and Innovation through the ISONEF project (CGL2008-05504-C02-02/BOS). M. Peipoch was funded by a Formación de Personal Investigador PhD fellowship from the Spanish Ministry of Science and Innovation. D. von Schiller's work was additionally funded by a Juan de la Cierva postdoctoral contract (JCI-2010-06397) from the Spanish Ministry of Science and Innovation. N. B. Grimm was supported by funds from the Spanish Council for Scientific Research (CSIC).

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698 **Table legends**

699 **Table 1.** Water temperature, photosynthetically active radiation (PAR), background nutrient
700 concentration for both dissolved inorganic nitrogen (DIN) species and soluble reactive
701 phosphorus (SRP) and biofilm characteristics for both study streams during the experiments.
702 Nutrient data from biweekly samplings from September 2004 to July 2007 are also provided
703 (in brackets). All data are reported as the mean ± SE.

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	Low-N stream	High-N stream
Water temperature (ºC)	15.4 ± 0.1	11.0 ± 0.2
PAR (mol m^{-2} day^{-1})	9.5 ± 3.4	1.4 ± 0.3
NO_3^- (µg N/L)	222 ± 2 (181 ± 11)	400 ± 27 (780 ± 44)
NH_4^+ (µg N/L)	15 ± 1 (12 ± 1)	8 ± 1 (19 ± 2)
SRP (µg P/L)	11 ± 0.3 (4 ± 0.5)	3 ± 0.3 (15 ± 2.6)
DIN:SRP (molar)	48 ± 1 (192 ± 32)	394 ± 32 (429 ± 106)
Ash free dry mass (g/m^2)	0.9 ± 0.1	4.3 ± 0.3
Chlorophyll- a ($µg/cm^2$)	0.3 ± 0.03	2.6 ± 0.2

705

706 **Table 2.** Statistical parameters of linear, Michaelis-Menten and efficiency loss models used
707 to evaluate the model that best fits the relationship between uptake rate (*U*) and DIN
708 concentration (*C*) for both streams and DIN species (NO_3^- and NH_4^+). $^+$). The Akaike
709 information criterion (AIC) was used to estimate Akaike weights,Wi, which give the relative
710 likelihood of each model. The highest relative likelihoods are marked in bold. For the
711 Michaelis-Menten model, *a* corresponds to the maximum uptake rate (U_{max}; µg N m^{-2} s^{-1})
712 and *b* corresponds to the half saturation constant (K_s;µg N/L). The 95% confidence intervals
713 of the values are also reported in brackets.
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715

	Low-N stream				High-N stream			
NO_3^-	a	b	AIC	W_i	a	b	AIC	W_i
Linear, $U = a + bC$	**3.1 (2.7 – 3.5)**	**-2.9e^{-4} (-4.0e^{-4} - -1.8e^{-4})**	**33.4**	**0.97**	4.3 (3.1 – 5.5)	4.0e^{-4} (-2.3e^{-5} – 8.2e^{-4})	55.1	0.36
Michaelis-Menten, $U = a\,C / b + C$	2.1 (1.6 – 2.6)	-85.8 (-131.9 - -7.6)	48.0	0	6.5 (4.8 – 9.2)	384 (-36.5 – 1282)	55.6	0.28
Efficiency Loss, $U = a\,C^b$	11.9 (5.3 – 27.1)	-0.2 (-0.4 - -0.1)	48.1	0.03	1.3 (0.3 – 5.6)	0.2 (-1.0e^{-2} – 0.4)	55.1	0.37
NH_4^+	a	b	AIC	W_i	a	b	AIC	W_i
Linear, $U = a + bC$	0.8 (0.5 – 1.0)	1.6e^{-3} (2.9e^{-4} – 2.9e^{-3})	17.3	0	0.3 (-0.5 - 1.1)	3.0e^{-2} (2.5e^{-2} - 3.4e^{-2})	45.1	0.03
Michaelis-Menten, $U = a\,C / b + C$	**1.3 (1.2 – 1.5)**	**17.1 (7.8 – 34.9)**	**2.6**	**0.98**	**28.0 (17.4 – 113)**	**628 (307 – 3449)**	**38.9**	**0.77**
Efficiency Loss, $U = a\,C^b$	0.4 (0.2 – 0.7)	0.2 (9.3e^{-2} – 0.3)	10.9	0.02	8.2e^{-2} (3.0e^{-2} - 0.2)	0.8 (0.7 - 1.0)	41.7	0.19

716

Figure legends

Figure 1. Scheme of the channel setting used to experimentally approach the objectives of this study. (A) In-situ channels structure. Upstream water supplied the feeding tank, which in turn fed each channel independently. Fertilization and ^{15}N amended solutions for NO_3^- or NH_4^+ reached each single channel independently (3 channels for each DIN species). (B) Detail of experimental design to conduct the different fertilization levels (over 24h each) and the ^{15}N tracer additions (over the last 6 h for each fertilization treatment) to measure biofilm N uptake for each DIN species (3 channels for each DIN species treatment). For each N fertilization cycle, we used a new set of colonized substrata from the stream that was collected upstream of the channel setting

Figure 2. Uptake rate (U; A) and biomass-specific N uptake rate ($U_{N\text{-}specific}$; B) at ambient concentrations for the two DIN species (NO_3^- and NH_4^+) and study streams. Each value is the mean ± SE of 3 replicates (one per channel). Different letters indicate significant differences ($p < 0.05$) based on post-hoc Tukey HSD test, after a significant two-way ANOVA test.

Figure 3. Uptake kinetics of NO_3^- and NH_4^+ in the low-N stream (A and C) and the high-N stream (B and D). The first point in each panel corresponds to the uptake rate (U) measured at ambient DIN concentration. Subsequent points correspond to measurements of U throughout experimental fertilizations. Each point is the mean ± SE of 3 replicates (one per channel). Lines represent the selected regressional model from AIC analysis (see Table 2 for regression statistics).

Figure 1

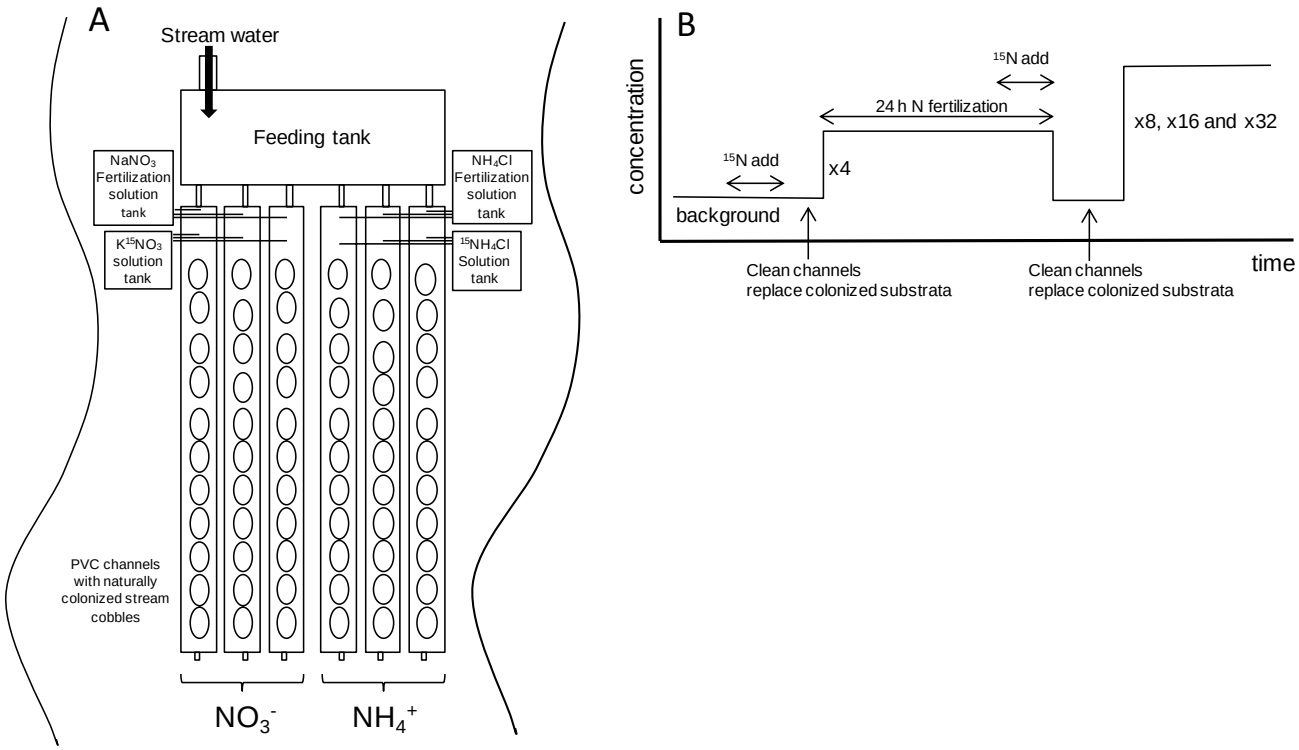

A
Stream water
Feeding tank
NaNO3 Fertilization solution tank
K15NO3 solution tank
NH4Cl Fertilization solution tank
15NH4Cl Solution tank
PVC channels with naturally colonized stream cobbles
NO3-
NH4+
B
concentration
15N add
24 h N fertilization
x8, x16 and x32
15N add
x4
background
Clean channels replace colonized substrata
Clean channels replace colonized substrata
time

Figure 2

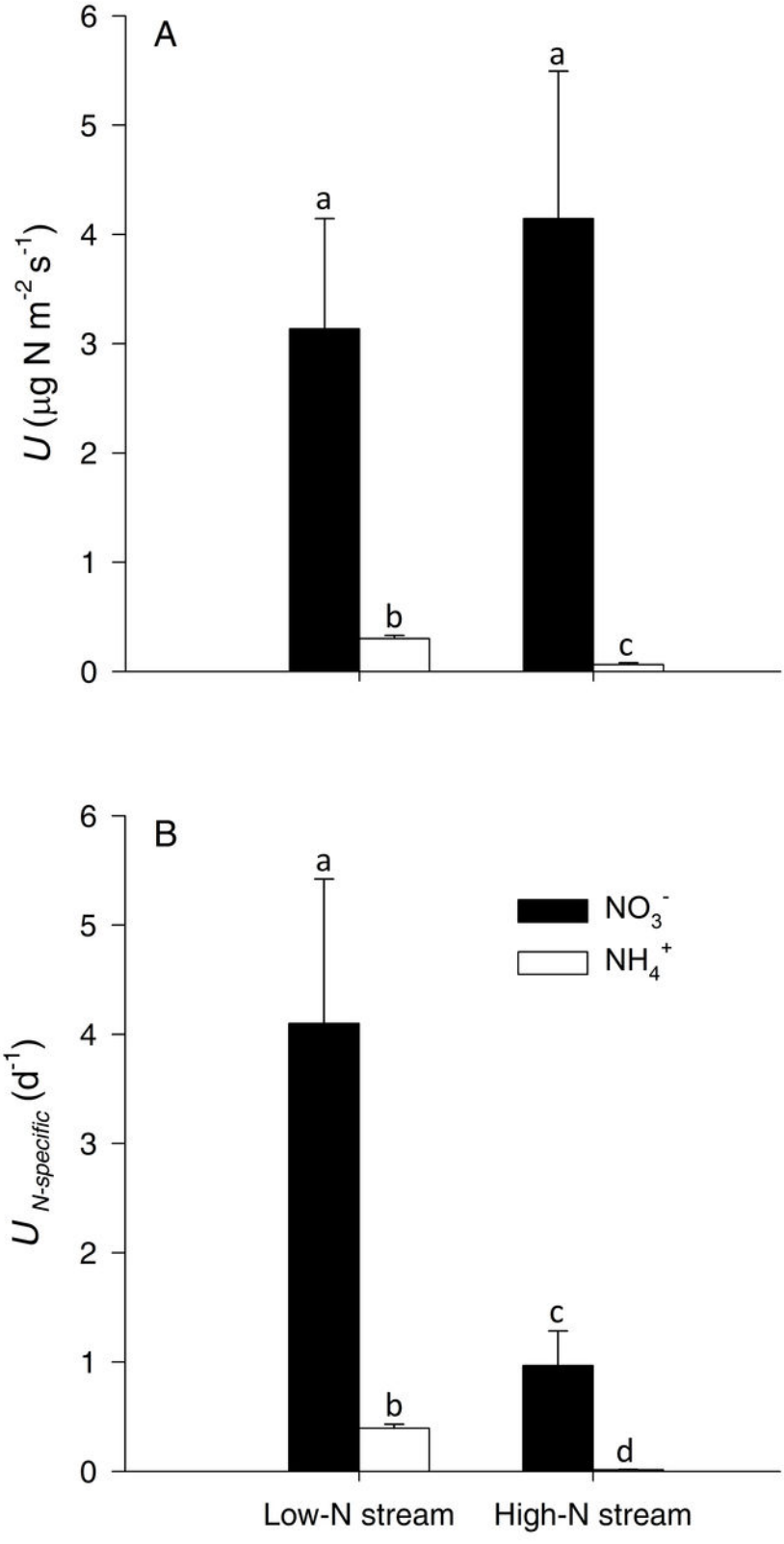
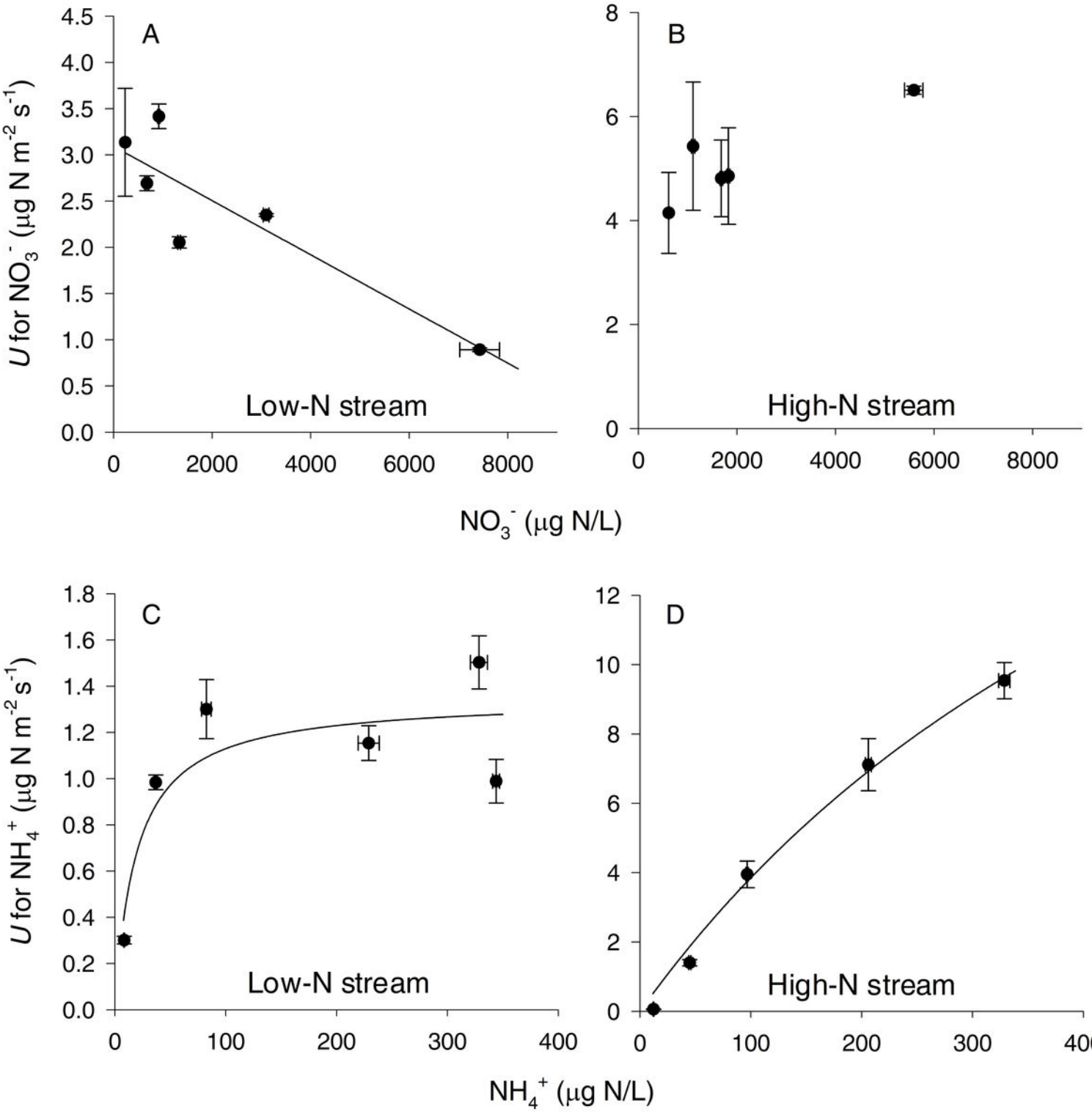

A
B
U (μg N m^{-2} s^{-1})
$U_{N\text{-}specific}$ (d^{-1})
NO_3^-
NH_4^+
a
b
c
d
0
1
2
3
4
5
6
Low-N stream
High-N stream

Figure 3


A
B
C
D
Low-N stream
High-N stream
Low-N stream
High-N stream
U for NO3- (µg N m-2 s-1)
U for NH4+ (µg N m-2 s-1)
NO3- (µg N/L)
NH4+ (µg N/L)