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

Miquel Ribot[1,5], Daniel von Schiller[2,6], Marc Peipoch[1,7], Francesc Sabater[3,8], Nancy B. Grimm[4,9], AND Eugènia Martí[1,10]

[1] *Biogeodynamics and Biodiversity Group, Centre d'Estudis Avançats de Blanes, CSIC, Accés a la Cala St Francesc 14, 17300, Blanes, Spain*

[2] *Catalan Institute for Water Research, Emili Grahit 101, Edifici H2O, Parc Científic i Tecnològic de la Universitat de Girona, 17003, Girona, Spain*

[3] *Department of Ecology, Faculty of Biology, University of Barcelona, Avinguda Diagonal 645, 08028 Barcelona, Spain*

[4] *School of Life Sciences, Arizona State University, Tempe, Arizona 85287-4501 USA*

Abstract. Human activity has significantly increased dissolved inorganic N (DIN) availability and has modified the relative proportion of NO_3^- and NH_4^+ species in many streams. Understanding the relationship between DIN concentration and DIN uptake is crucial to predicting how streams will respond to increased DIN loading. Nonetheless, this relationship remains unclear because of the complex interactions governing DIN uptake. We aimed to evaluate how biofilms from 2 streams differing in background DIN concentration would respond to increases in availability and changes in speciation (NO_3^- or NH_4^+) of DIN. We measured DIN uptake by biofilms in artificial flumes in each stream, using separate ^{15}N-NO_3^- and ^{15}N-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 streams. Uptake efficiency ($U_{N\text{-}specific}$) at ambient conditions was higher in the low-N than in the high-N stream for both DIN species. A Michaelis–Menten model of uptake kinetics 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 determined primarily by the ambient DIN concentrations under which they develop. Our study also shows that DIN uptake by benthic biofilms varies with DIN availability and with DIN speciation, which often is modified by human activities.

Key words: nitrate, ammonium, biofilm, nitrogen uptake, Michaelis–Menten kinetics, stream, land use, agriculture.

Human activities have significantly increased the concentration of dissolved inorganic N (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 human alteration of DIN availability has become a research focus for stream ecologists (Mulholland and Webster 2010). Some researchers have studied DIN uptake kinetics (i.e., changes in uptake rates [U] in response to changes in concentration) based on the relationship between whole-reach DIN uptake and DIN concentration by 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 researchers have focused on DIN uptake kinetics within the same stream by following changes in whole-reach uptake in response to short-term DIN

[5] E-mail addresses: mribot@ceab.csic.es
[6] dvonschiller@icra.cat
[7] mpeipoch@ceab.csic.es
[8] fsabater@ub.edu
[9] nbgrimm@asu.edu
[10] eugenia@ceab.csic.es

enrichment (Payn et al. 2005, Earl et al. 2006, Covino et al. 2010, O'Brien and Dodds 2010) or by investigating DIN uptake kinetics in mesocosms (Eppley et al. 1969, Kemp and Dodds 2002, O'Brien and Dodds 2008).

Three mathematical models describe the relationship between DIN uptake and concentration in streams. The first model corresponds to a 1st-order response in which uptake flux (μg N m^{-2} s^{-1}) is directly proportional to concentration of substrate (Dodds et al. 2002). The 2nd model, the efficiency–loss model, follows a power relationship in which U increases but efficiency declines with concentration (O'Brien et al. 2007). The 3rd model follows Michaelis–Menten kinetics and is characterized by saturation of uptake when availability exceeds biological demand (Earl et al. 2006). In general, results from interstream 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 in the same stream or in mesocosms (i.e., with the same community) suggest that the Michaelis–Menten model best fits DIN uptake kinetics (Payn et al. 2005, Earl et al. 2006, Covino et al. 2010, O'Brien and Dodds 2010).

Human activities also change the relative proportions of the 2 major DIN species: NO_3^- and NH_4^+ (Stanley and Maxted 2008, Lassaletta et al. 2009, Martí et al. 2010). U and kinetics are expected to differ between NO_3^- and NH_4^+ because energetic costs of assimilation associated with NO_3^- are generally higher than those associated with NH_4^+ (Dortch 1990, Naldi and Wheeler 2002). Furthermore, dissimilatory transformations, in which neither compound is incorporated into biomass, contribute to NH_4^+ and NO_3^- uptake. Nitrification (i.e., oxidization of NH_4^+ to NO_3^- by autotrophic or heterotrophic Bacteria and Archaea) will result in apparent NH_4^+ uptake, whereas apparent NO_3^- uptake may include denitrification (i.e., the respiratory process by which bacteria reduce NO_3^- to N_2). These transformations are carried out by different organisms and governed by different controlling factors (Bothe et al. 2007), and thus, may contribute to the expected differences between NO_3^- and NH_4^+ uptake kinetics. Most researchers have investigated NO_3^- or NH_4^+ uptake separately. Thus, we do not know how uptake kinetics differ between these 2 DIN species under similar environmental conditions. In addition, little is known about differences in uptake kinetics of NO_3^- or NH_4^+ of stream biofilms (i.e., the microbial communities that develop on stream substrata) associated with increases in DIN availability. Understanding DIN uptake kinetics of stream biofilms is especially important because biofilms are major contributors to nutrient dynamics in stream networks (Pusch et al. 1998, Battin et al. 2003) and, therefore, may help ameliorate anthropogenic DIN inputs.

We compared U and kinetics for NO_3^- and NH_4^+ between biofilms developed in 2 streams differing in background DIN concentrations. We measured biofilm U in experiments in which we separately added ^{15}N-labeled NO_3^- and NH_4^+ at increasing concentrations to artificial flumes in each stream. We predicted that ambient uptake flux would be higher for NO_3^- than for NH_4^+ and in the high-N than in the low-N stream because of higher availability of NO_3^- with respect to NH_4^+ and the overall higher DIN availability in the high-N stream. In terms of uptake kinetics, we predicted that the 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 ½-saturation constant (K_s) for NH_4^+ than for NO_3^- because of the lower energetic cost of 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 because of differences in N affinity between stream biofilms resulting from different histories of nutrient exposure.

Methods

Study sites

Font del Regàs (lat 2°27′00″E, long 41°49′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 (lat 2°37′52″E, long 41°52′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) was 56 ± 12 L/s for Font del Regàs and 163 ± 35 L/s for Santa Coloma (biweekly samplings from September 2004–July 2007; MR, DvS, FS, and EM, unpublished data). Concentrations of NO_3^- and NH_4^+ were 181 ± 11 μg N/L and 12 ± 1 μg N/L for Font del Regàs, and 780 ± 44 μg N/L and 19 ± 2 μg N/L for Santa Coloma (biweekly samplings from September 2004–July 2007; MR, DvS, FS, and EM, unpublished data). Hereafter, we refer to Font del Regàs as the low-N stream and to Santa Coloma as the high-N stream.

Channel experiments

We conducted experiments from 3 to 24 July 2007 in the low-N stream and from 23 October to 7 November

2007 in the high-N stream. We placed a set of 6 parallel polyvinyl chloride (PVC) channels (6 m long × 15 cm wide) on the stream bed in a metal structure that held them together and above the stream water (Fig. 1A). Water from an upstream tank fed all channels continuously with a mean (± SE) flow rate of 1.8 ± 0.018 L/min (from measurements done daily throughout the experiments and in each channel). We filled the channels with stream cobbles of similar size and biofilm cover that were collected from the stream bed <50 m upstream from the channel setting. We exposed channels to 5 sequential 24-h fertilization cycles each with an increased concentration (1, 4, 8, 16, and 32× background concentration) of either NO_3^- or NH_4^+ ($n = 3$ channels each; Fig. 1A, B). We released solutions of NO_3^- (as $NaNO_3$) or NH_4^+ (as NH_4Cl) to the corresponding channels at a constant rate from a 3-output carboy (1/channel). We maintained a constant head in each carboy with a Masterflex (Vernon Hills, Illinois) L/S battery-powered peristaltic pump. We also added PO_4^{3-} (as $NaH_2PO_4 \cdot H_2O$) proportionally into the solution at each fertilization level to maintain the background stoichiometric ratio between DIN and soluble reactive P (SRP) throughout the fertilization cycles.

We conducted a tracer addition of either $^{15}NO_3^-$ ($n = 3$ channels) or $^{15}NH_4^+$ ($n = 3$ channels) over the last 6 h of each fertilization level to estimate U of biofilms. We added solutions amended with $^{15}NO_3^-$ (as 99% enriched $K^{15}NO_3$) or $^{15}NH_4^+$ (as 99% enriched $^{15}NH_4Cl$) and NaCl as a conservative tracer at a constant rate using a similar setup as described above. We calculated the amount of $K^{15}NO_3$ and $^{15}NH_4Cl$ needed to produce a target $\delta^{15}N$ enrichment of 3000‰ for both DIN species in the channels. To verify plateau conditions, we logged conductivity every 10 s at the end of each channel with a portable WTW conductivity meter (Weilheim, Germany).

Prior to fertilizations, we collected water at the downstream end of each channel for analysis of ambient nutrient concentrations (3 replicates/channel) and $^{15}NH_4^+$ and $^{15}NO_3^-$ signatures (1 replicate/channel). We also collected composite biofilm samples for the analysis of biomass, pigment content, and natural abundance of ^{15}N (1 replicate/channel) by scraping 3 randomly selected cobbles and filtering the biomass onto combusted, preweighed glass-fiber filters (GF/Fs; Whatman, Maidstone, UK). Before completing each fertilization period (when fertilization and ^{15}N addition were running together), we collected another set of water and biofilm samples (3 replicates/channel) for analysis of nutrient concentration and $^{15}NH_4^+$ and $^{15}NO_3^-$ signatures. Then 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).

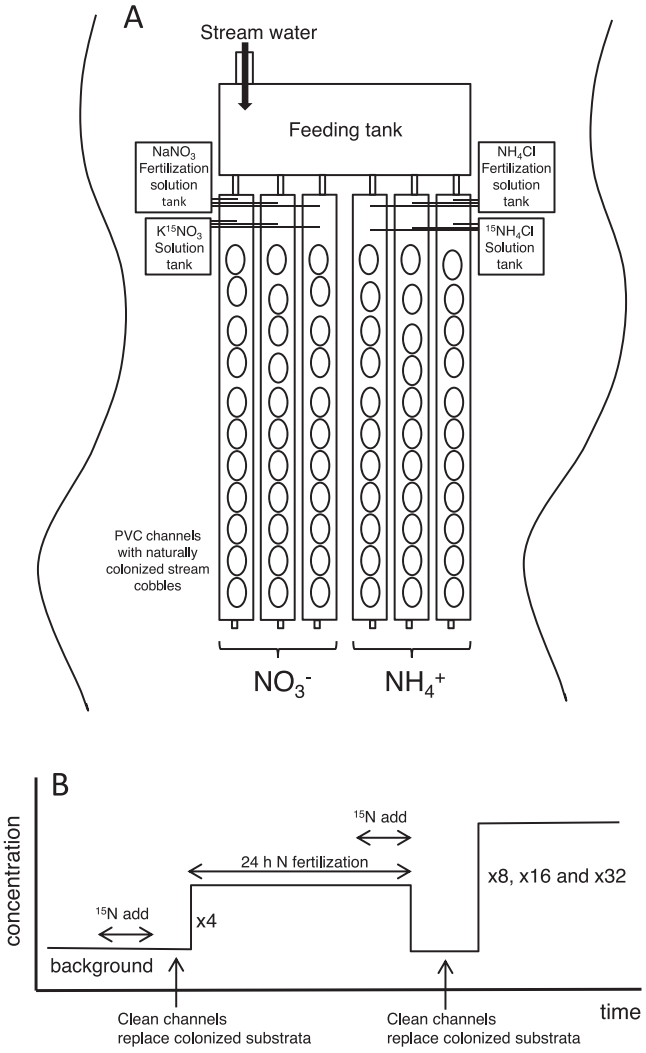

FIG. 1. Scheme of the channel setting used to experimentally approach the objectives of our study. A.—In-situ channel structure. Upstream water supplied the feeding tank, which in turn, fed each polyvinyl chloride (PVC) channel independently. Fertilization and ^{15}N amended solutions for NO_3^- or NH_4^+ reached each single channel independently (3 channels for each dissolved inorganic N [DIN] species). B.—Detail of experimental design to conduct the different fertilization levels (24 h each) and the ^{15}N-tracer additions (add; during the last 6 h of 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 collected upstream of the channel setting.

We filtered the water samples immediately through combusted GF/Fs into acid-washed, plastic containers and stored them on ice for transportation to the

laboratory. We estimated the cobble surface area by covering it with Al foil and weighing the foil. We stored the filters with biofilm samples on ice in the field and froze (for chlorophyll *a* analysis) or oven-dried them (for ash-free dry mass [AFDM] and ^{15}N analysis) in the laboratory until further processing. We logged photosynthetically active radiation (PAR) every 10 min with a SKP215 quantum sensor (Skye; Powys, UK) connected to a Campbell Scientific data logger (Logan, Utah). We measured temperature at plateau conditions with a WTW 340i portable conductivity meter.

Laboratory analyses

We analyzed water samples for concentrations of NO_3^-, NH_4^+, and SRP on a Bran+Luebbe (Norderstedt, Germany) TRAACS 2000 autoanalyzer with standard colorimetric methods (APHA 1995). We processed water samples for analysis of $^{15}NO_3^-$ and $^{15}NH_4^+$ with the NH_3-diffusion technique (Sigman et al. 1997 and Holmes et al. 1998, respectively). To measure $^{15}NO_3^-$, we amended a known volume of sample with 3 g of MgO and 5 g of NaCl and boiled it to remove the NH_4^+. We then added 0.5 mg MgO and 0.5 mg Devarda's alloy to reduce the NO_3^- to NH_4^+, and treated the remaining sample as for $^{15}NH_4^+$. To measure $^{15}NH_4^+$, we amended a known volume of sample with 3 g/L of MgO and 50 g/L of NaCl and a Teflon filter packet containing a 1-cm-diameter combusted Whatman GF/D fiber glass filter acidified with 25 μL of 2.5 M $KHSO_4$ (to trap the volatilized NH_3), and incubated it on a shaker at 40°C for 4 wk. Once the incubation was completed, we removed the filter packets and placed them in a desiccator for 4 d. We encapsulated filters in tins and stored them until ^{15}N analysis.

We oven-dried filters with biofilm samples at 60°C until they reached a constant mass. To estimate the biofilm AFDM (g/m^2), we weighed subsamples on a Sartorious MC1 analytical balance (Göttingen, Germany) and combusted them at 500°C for 5 h. We measured biofilm chlorophyll *a* content (μg/cm^2) following McIntire et al. (1996). We submerged frozen filters in a known volume of 90% volume/volume acetone and kept them in the dark at 4°C overnight. We sonicated the filters for 5 min and centrifuged them for 10 min at 4000 rpm. We measured the absorbance of the resultant supernatant at 664, 665, and 750 nm before and after acidification with a Shimadzu ultraviolet (UV) spectrometer (Tokyo, Japan). To determine the ^{15}N signature of biofilms, we weighed 1-cm-diameter subsamples to the nearest 0.001 mg on a Mettler-Toledo MX5 microbalance (Greifensee, Switzerland) and encapsulated them in tins. We sent the samples for analysis at the University of California Stable Isotope Facility (Davis, California). We measured the N content (as % dry mass) and the abundance of the heavier isotope, expressed as the ^{14}N:^{15}N ratio compared to that of a standard (N_2 from the atmosphere) using the notation of $\delta^{15}N$ in units of ‰, by continuous-flow isotope-ratio mass spectrometry (20–20 mass spectrometer; PDZ Europa, Northwich, UK) after sample combustion in an online elemental analyzer (PDZ Europa ANCA-GSL).

Calculation of U *and data analysis*

We used independent *t*-tests to explore differences in ambient nutrient concentrations, biofilm AFDM, and biofilm chlorophyll *a* content between streams.

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

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

where $B_{biofilm}$ is the biofilm biomass as dry mass per unit area, N is the biofilm N content expressed as % 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 U (μg N m^{-2} s^{-1}) for NO_3^- or NH_4^+ with the equation (adapted from von Schiller et al. 2007):

$$U = \frac{^{15}N_{biofilm}}{T_{addition}\left(^{15}N_{flux}/N_{flux}\right)} \quad [2]$$

where $^{15}N_{biofilm}$ is the amount of ^{15}N tracer in biofilm biomass from eq. 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 NO_3^- or NH_4^+) at each fertilization level in the channel water based on concentration and channel flow rate (μg N/s). We then calculated the biomass-specific U ($U_{N\text{-}specific}$; d^{-1}) for biofilm communities and DIN species as a surrogate of N uptake efficiency by dividing biofilm U (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 2-way analysis of variance (ANOVA) with DIN species (NO_3^-, NH_4^+) and stream (low-N, high-N) as factors. We used post hoc Tukey Honestly Significant Difference tests after significant ANOVAs ($p < 0.05$)

TABLE 1. Mean (± SE) water temperature, photosynthetically active radiation (PAR), background nutrient concentration for both dissolved inorganic N (DIN) species, soluble reactive P (SRP), and biofilm ash-free dry mass (AFDM) and chlorophyll *a* for both study streams during the experiments. Nutrient data from biweekly samplings from September 2004–July 2007 also provided (in parentheses).

Variable	Low-N stream	High-N stream
Water temperature (°C)	15.4 ± 0.1	11.0 ± 0.2
PAR (mol m^{-2} d^{-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)
AFDM (g/m^2)	0.9 ± 0.1	4.3 ± 0.3
Chlorophyll *a* ($\mu g/cm^2$)	0.3 ± 0.03	2.6 ± 0.2

to further examine the effects of stream and DIN species on 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) and a and b are a constant and the slope, respectively. 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 U, and K_s is the concentration at which ½ U_{max} is reached. K_s is an indicator of the biofilm affinity for DIN. High values indicate lower affinity than low values. 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 exponent $b < 1$.

The parameters a and b from each mathematical model (for the Michaelis–Menten model, U_{max} corresponds to a and K_s corresponds to b), were calculated based on the Gauss–Newton algorithm, an iterative process that 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 estimated the confidence intervals (CIs; 95%) for each coefficient by the generic function *confint* powered by R software (version 2.14.0; R Development Core Team, Vienna, Austria). The default method assumes asymptotic normality, and requires that suitable *coef* and *vcov* methods be available. The default method can be called directly for comparison with other methods. We used the Akaike Information Criterion (AIC) to estimate Akaike weights (w_i), which yield the relative likelihood of each model given a particular data set. Within the set of candidate models for the data, we selected the model with the highest w_i.

We conducted all statistical tests with R. When necessary, data were log(x)-transformed before analysis to meet assumptions of homogeneity of variance and normality (Zar 1996).

Results

Environmental conditions differed substantially between the 2 study streams during the experiments (Table 1). Mean water temperature and PAR were 1.4 and 7× higher, respectively, in the low-N stream than in the high-N stream. Consistent with the long-term trend (i.e., biweekly sampling), mean NO_3^- concentration was 2× higher in the high-N than in the low-N stream (t-test, $p < 0.001$; Table 1). In contrast to the long-term trend, mean NH_4^+ concentration was 2× higher in the low-N stream than in the high-N stream (t-test, $p < 0.001$; Table 1). Mean SRP concentration was 4× lower and mean DIN:SRP ratio was 8× higher in the high-N than in the low-N stream (t-test, $p < 0.001$). Mean biofilm AFDM and chlorophyll *a* content were higher (5 and 9×, respectively) in the high-N than in low-N stream (t-test, $p < 0.001$).

DIN species, stream, and the DIN × stream interaction affected both U and $U_{N\text{-}specific}$ at ambient concentrations (ANOVA, all $p < 0.01$). U_{NO3-} (3.1 ± 0.6 μg N m^{-2} s^{-1} in the low-N stream, 4.1 ± 0.8 μg N m^{-2} s^{-1} in the high-N stream) was higher than U_{NH4+} (0.3 ± 0.02 μg N m^{-2} s^{-1} in the low-N stream, 0.06 ± 0.01 μg N m^{-2} s^{-1} in the high-N stream) in both

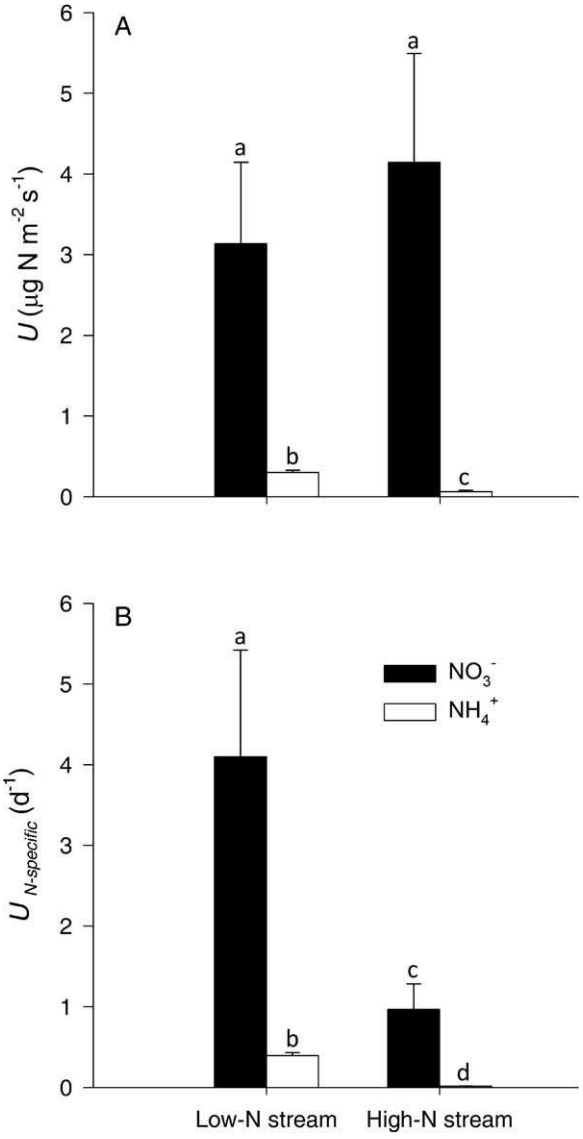

FIG. 2. Mean (±1 SE; $n = 3$) uptake rate (U) (A) and biomass-specific N uptake rate ($U_{N\text{-}specific}$) (B) at ambient concentrations for the 2 dissolved inorganic N species (NO_3^- and NH_4^+) and study streams. Bars with the same letters are not significantly different ($p > 0.05$) based on post hoc Tukey Honestly Significant Difference test.

streams (Fig. 2A). U_{NH4+} differed between streams (Tukey HSD test, $p = 0.001$), whereas U_{NO3-} did not (Tukey HSD test, $p = 0.636$). $U_{N\text{-}specific}$ for NO_3^- (4.1 ± 0.8 d^{-1} in the low-N stream, 1.0 ± 0.2 d^{-1} in the high-N stream) was higher than $U_{N\text{-}specific}$ for NH_4^+ (0.4 ± 0.02 in the low-N stream, 0.01 ± 0.002 in the high-N stream) in both streams (Fig. 2B). In contrast to U, $U_{N\text{-}specific}$ for both NO_3^- and NH_4^+ differed between streams (Tukey HSD test, $p < 0.001$).

Uptake responses to increases in DIN concentration differed substantially between DIN species and streams (Fig. 3A–D). The relationship between U and NO_3^- concentration differed between streams, but uptake kinetics did not fit Michaelis–Menten model in either stream (Fig. 3A, B). In the low-N stream, AIC analysis indicated that the relationship between U and NO_3^-concentration better fit a 1st-order model with a negative slope (Table 2). Conversely, in the high N-stream, 95% CIs for b in all 3 models contained 0, indicating no significant fit, and AIC analysis resulted in no clear model selection (Table 2).

U for NH_4^+ varied with increases in NH_4^+ concentrations (Fig. 3C, D). The AIC analysis indicated the Michaelis–Menten model as the best fit for the relationship between U and NH_4^+concentration in both streams (Table 2). However, uptake kinetic parameters differed between streams. U_{max} and K_s were lower in the low-N than in the high-N stream, and 95% CIs did not overlap (Table 2).

Discussion

We evaluated the response of biofilm U to changes in DIN concentration, and tested whether this response varied among DIN species. We used an experimental approach that combined nutrient fertilizations and ^{15}N-tracer additions in situ in artificial flumes. We predicted that U and uptake kinetics would depend on DIN species (NO_3^- vs NH_4^+) and ambient DIN concentration in the stream (low-N vs high-N). Our results supported these predictions only partially. U was higher for NO_3^- than for NH_4^+ in both streams, but only U_{NH4+} differed between streams, with lower values in the high-N stream. In addition, $U_{N\text{-}specific}$ 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 U 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 U_{max} in the low-N stream than in the high-N stream, but affinity for NH_4^+ was higher (lower K_s) in the low-N stream.

Biofilm DIN uptake in streams of contrasting DIN availability and speciation

U of epilithic biofilm for both DIN species under ambient conditions in our study were similar to values reported from previous studies using whole-stream ^{15}N-tracer additions (Mulholland et al. 2000, Tank et al. 2000, Hamilton et al. 2001, Merriam et al. 2002, Ashkenas et al. 2004, von Schiller et al. 2009, Sobota et al. 2012). This result indicates that values of

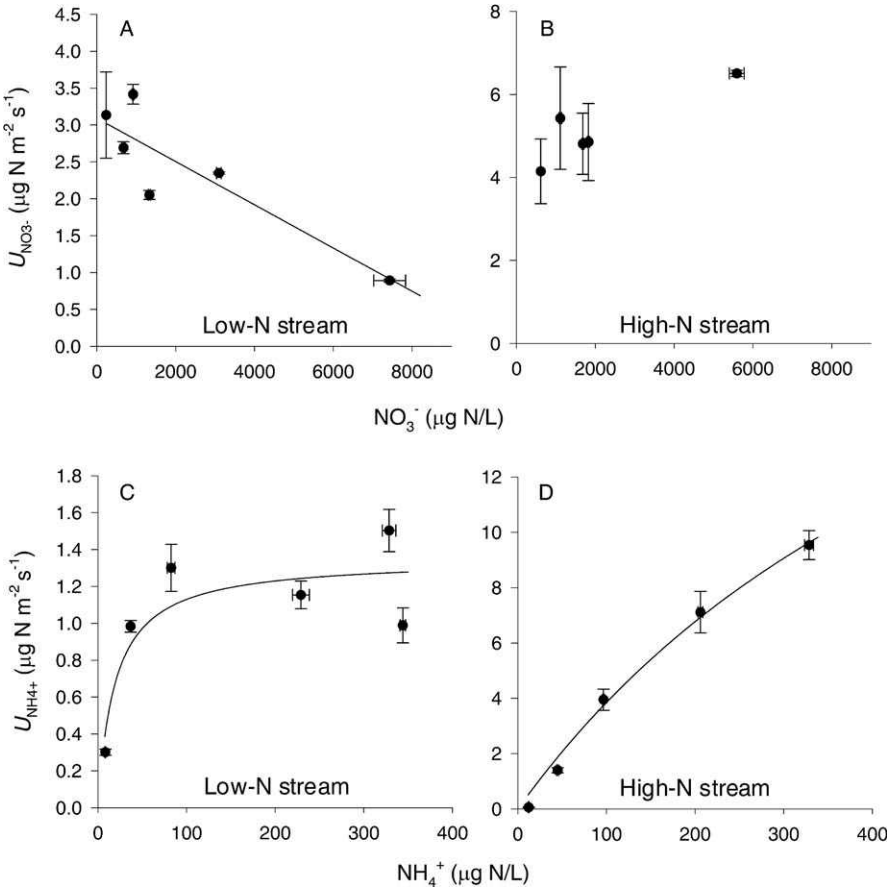

FIG. 3. Mean (±1 SE; $n = 3$) uptake rates (U) for NO_3^- (U_{NO3-}) (A, B) and NH_4^+ (U_{NH4+}) (C, D) in the low-N (A, C) and high-N (B, D) streams. The first point in each panel corresponds to U measured at ambient concentration. Lines represent the selected regression model from Akaike Information Criterion analysis (see Table 2 for regression statistics).

U in our channel experiments were representative of natural field conditions.

Ambient U_{NO3-} was 10× higher than U_{NH4+} in both streams, even though NH_4^+ is theoretically an energetically less costly DIN source and, thus, was expected to be preferentially assimilated over NO_3^- (Dortch 1990, Naldi and Wheeler 2002). Estimated values of the relative preference index (RPI) were ~1 in the 2 streams. This index was proposed by Dortch (1990) as a means to determine the preference for NH_4^+ over NO_3^- (values < 1) or for NO_3^- over NH_4^+ (values > 1). The RPI value of ~1 in our study suggests that biofilms in the 2 streams have no preference for either DIN species. Thus, the observed higher U_{NO3-} than U_{NH4+} was mostly attributable to the higher concentrations of NO_3^- than of NH_4^+.

Ambient U_{NO3-} did not differ between streams, but U_{NH4+} was 10× lower in the high-N than in the low-N stream. Higher NO_3^- availability relative to NH_4^+ availability in the high-N stream may have favored uptake of NO_3^- over NH_4^+ in the high-N stream, as suggested by other authors (Fellows et al. 2006, Newbold et al. 2006, Bunch and Bernot 2012). Furthermore, at low NH_4^+ concentration, the presence of NO_3^- can favor NO_3^- assimilation (Geisseler et al. 2010). Expression and biosynthesis of assimilatory nitrate reductase (the enzyme responsible for NO_3^- assimilation processes) is induced by NO_3^- and NO_2^- and suppressed by NH_4^+ (Gonzalez et al. 2006). Thus, the concurrence of high NO_3^- and low NH_4^+ concentration at ambient conditions in the high-N stream may have led to lower NH_4^+ assimilation rates than in the low-N stream.

Differences in nitrification, which can contribute to NH_4^+ uptake in biofilms, are another potential explanation for the differences in U between streams. If nitrification rate were constrained by the low substrate (NH_4^+) availability in the high-N stream, then we would expect the contribution of nitrification to total NH_4^+ uptake to be lower in that stream. In both streams, $\delta^{15}NO_3^-$ increased during plateau conditions in the channels where we did $^{15}NH_4^+$

TABLE 2. Mean (95% CI) for statistical parameters of linear ($U = a + bC$), Michaelis–Menten ($U = aC/b + C$), and efficiency–loss ($U = aC^b$) models used to evaluate the model that best fit the relationship between uptake rate (U) and dissolved inorganic N (DIN) concentration (C) for both streams and DIN species (NO_3^- and NH_4^+). The Akaike Information Criterion (AIC) was used to estimate Akaike weights (w_i), which give the relative likelihood of each model. The highest relative likelihoods are marked in bold. For the Michaelis–Menten model, a corresponds to the maximum uptake rate (U_{max}; μg N m^{-2} s^{-1}) and b corresponds to the ½-saturation constant (K_s; μg N/L).

Model	Low-N stream				High-N stream			
	a	b	AIC	w_i	a	b	AIC	w_i
NO_3^-								
Linear	**3.1 (2.7–3.5)**	**−0.00029 (−0.0004 to −0.00018)**	**33.4**	**0.97**	4.3 (3.1–5.5)	0.00040 (–0.000023–0.00082)	55.1	0.36
Michaelis–Menten	2.1 (1.6–2.6)	−85.8 (−131.9 to −7.6)	48.0	0	6.5 (4.8–9.2)	384 (−36.5–1282)	55.6	0.28
Efficiency–loss	11.9 (5.3–27.1)	−0.2 (−0.4 to −0.1)	48.1	0.03	1.3 (0.3– .6)	0.2 (−0.010–0.4)	55.1	0.37
NH_4^+								
Linear	0.8 (0.5–1.0)	0.0016 (0.00029–0.0029)	17.3	0	0.3 (−0.5–1.1)	0.030 (0.025–0.034)	45.1	0.03
Michaelis–Menten	**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	0.4 (0.2–0.7)	0.2 (0.093–0.3)	10.9	0.02	0.082 (0.030–0.2)	0.8 (0.7–1.0)	41.7	0.19

additions, a result indicative of nitrification (2.6 ± 0.5‰ and 1.9 ± 0.9‰ in the low-N and the high-N streams, respectively). Based on these $\delta^{15}NO_3$ increases, we estimated the contribution of nitrification to total biofilm NH_4^+ uptake for each fertilization cycle. This contribution ranged from 0.2 to 7.6% in the low-N stream, 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 because nitrification rate was probably lower in the high-N than in the low-N stream. Instead, we suggest that combination of lower NH_4^+ assimilation and lower nitrification by biofilms in the high-N stream explains the differences in U_{NH4+} between streams.

$U_{N\text{-}specific}$ values indicate that the biofilm from the high-N stream took up both NO_3^- and NH_4^+ from the water column less efficiently than the biofilm from the low-N stream. Lower uptake efficiencies often occur in streams with high DIN concentrations because of saturation of 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 lead to differences in the uptake kinetics 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 U_{NO3-}. In the high-N stream, addition of NO_3^- had no effect on biofilm U, suggesting that uptake capacity of biofilm assemblages probably was saturated at the ambient NO_3^- concentration. Earl et al. (2006) suggested that when N is not limiting in streams, a 0-order mathematical model (i.e., constant rate with slope = 0) is more applicable than a higher-order model, a suggestion in concordance with our results in the high N-stream. Alternatively, the lack of biofilm uptake response to increases in NO_3^- concentration might be explained by tight coupling of NO_3^- uptake to availability of other nutrients (Fairchild et al. 1985, Sterner et al. 1992). Schanz and Juon (1983) suggested that P is a potentially limiting element at DIN:P > 20 (others have suggested a transition from N to P limitation at DIN:P ≈ 16–17; Redfield 1958, Grimm and Fisher 1986). We added

SRP in the fertilization solutions to maintain background DIN:P, but ratios were well above the potential P-limitation thresholds, especially in the high-N stream (394 ± 32 μg P/L). In this sense, NO_3^- uptake in the high-N stream may have been constrained by P insufficiency. However, if P were the limiting nutrient, then increases in P availability should alleviate P limitation and, thus, enhance NO_3^- uptake. We think this alternative explanation is unlikely because previous nutrient-limitation bioassays in the high-N stream failed to show P limitation (von Schiller et al. 2007).

Increases in NO_3^- availability in the low-N stream produced a decrease in biofilm *U*, indicating a possible inhibitory effect of high NO_3^- concentrations on biofilm uptake in this stream. Inhibitory effects on the uptake of NH_4^+ or NO_2^- at high concentrations have been reported in the literature (usually associated with nitrification processes; Kim et al. 2006, Vadivelu et al. 2007). However, as far as we know, no previous evidence exists for inhibition of NO_3^- uptake at high NO_3^- concentrations. However, inhibitory effects of long-term NO_3^- enrichment have been reported for periphyton growth in nutrient-diffusing substrate experiments (Bernhardt and Likens 2004), and a few investigators have shown potentially toxic effects of NO_3^- on freshwater animals and plants (Camargo and Alonso 2006, Lambert and Davy 2011). Our experiments do not allow us to identify the mechanisms underlying observed patterns but do provide evidence that a short-term, sharp increase in NO_3^- concentration may be inhibitory.

Michaelis–Menten kinetics described biofilm uptake responses to increases in NH_4^+ concentration in both streams. Values of K_s were higher than ambient concentrations of NH_4^+ in both streams, so we conclude that biofilm uptake for this DIN source was below saturation at ambient concentrations (Tilman 1982). Therefore, biofilms were able to respond positively to short-term increases in NH_4^+ concentration within a certain range in the 2 streams. Bunch and Bernot (2012) also compared uptake responses of microbial communities to NH_4^+ and NO_3^- enrichments. They observed that responses to NH_4^+ were immediate and pronounced, whereas responses to NO_3^- were delayed and more variable. They suggested that preference for NH_4^+ as a DIN source by microbial communities dictates stronger and more rapid uptake responses to changes in NH_4^+ than in NO_3^- concentration.

Our results agree with those by Bunch and Bernot (2012) in showing rapid response to increases in NH_4^+. However, the values of RPI of ~1 in our study indicated no clear preference for NH_4^+ over NO_3^-, at least under ambient conditions. An alternative explanation for the difference in the kinetic responses between NO_3^- and NH_4^+ involves enzymatic responses to short-term changes in availability. Increased availability of NH_4^+ in NH_4^+-amended channels may have triggered repression of NO_3^- reductase and increased biofilm NH_4^+ uptake to meet N demand (Gonzalez et al. 2006). This mechanism could explain the positive biofilm NH_4^+ uptake response to increases in NH_4^+ concentration even though uptake responses for NO_3^- indicated that biofilm demand for this DIN species was saturated at ambient conditions. Previous investigators have found a Michaelis–Menten response of nitrification rates to increases in NH_4^+ concentration within a range of NH_4^+ concentrations similar to that used in our study (Koper et al. 2010). Nitrification probably was substrate-limited at the relatively low NH_4^+ concentrations in the 2 study streams, which would produce a positive response to increased NH_4^+ concentration that conforms to a Michaelis–Menten model. However, our a posteriori calculations of nitrification contribution to the whole-channel uptake suggest that nitrification is only a minor contributor to observed kinetics of NH_4^+ uptake. We suggest that a combination of several mechanisms best explains the different kinetic responses of NH_4^+ and NO_3^- in the study streams.

NH_4^+ uptake kinetics fit the Michaelis–Menten model in the 2 streams, but the kinetic parameters (K_s and U_{max}) clearly differed between streams, supporting our predictions. NH_4^+ U_{max} of the biofilm in the high-N stream was 21× higher than U_{max} of the biofilm in the low-N stream. The high-N stream had higher biofilm biomass and more photoautotrophic organisms (as indicated by chlorophyll *a* content) than the low-N stream, a result that could explain the higher U_{max} observed in the high-N stream. However, U_{max} weighted by N content of biofilm dry mass, a surrogate measure of uptake efficiency, was only 4× higher in the high-N stream. Therefore, biofilms were relatively more efficient in NH_4^+ uptake in the low-N than in the high-N stream, a result that is in agreement with uptake results measured at ambient DIN conditions.

In contrast, biofilms showed a higher affinity (lower K_s) for NH_4^+ in the low-N stream than in the high N-stream. Higher affinities for substrate often are attributed to exposure of microorganisms to lower ambient concentrations (Collos et al. 2005, Martens-Habbena et al. 2009). This explanation may not apply to our study if we consider only ambient NH_4^+ concentration, which was similar and low in the 2

streams. However, when discussing nutrient limitation, it is more appropriate to consider total DIN concentration, which was 2× lower in the low-N than in the high-N stream, because biofilms can meet their N demand by uptake of either DIN species. Alternatively, differences in NH_4^+ affinity between streams could 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 limitation of diffusion of DIN to all cells in the biofilm (Stewart 2003, Teissier et al. 2007). Limitation by diffusion has been demonstrated for uptake of inorganic C and nitrification activity in model biofilms, with both processes restricted to the surface layer of the biofilm (Gieseke et al. 2005). As a result, the thickness of the biofilm in the high-N stream may contribute to an increase in the range of NH_4^+ concentrations within which U_{NH4+} responds positively. Constraints resulting from diffusion limitation in thicker biofilms operate for both N assimilation and nitrification and, thus, can amplify the range of NH_4^+ concentrations that can be reached before saturation occurs because the 2 processes may have different kinetics.

We cannot rule out differences in environmental conditions, such as light availability and temperature, between the 2 streams as potential causes of differences in biofilm uptake kinetics for NH_4^+. We tried to conduct experiments in streams with similar environmental conditions, but a large flood in the high-N stream forced us to postpone the experiment until the biofilm communities recovered fully. As a result, temperature and light availability were higher in the low-N than in the high-N stream during the experiments and could have enhanced biofilm activity and kinetic responses in the low-N stream. However, the effect of temperature on nutrient uptake kinetics is unclear, and Smith (2011) found no evidence of sensitivity of Michaelis–Menten parameters to temperature. Light availability was higher in the low-N stream, but biofilm chlorophyll *a* content was 9× higher in the high-N than in the low-N stream. Thus, this factor could not have caused the observed kinetic differences, at least for the photoautotrophic component of the biofilms. Thus, observed differences in biofilm uptake kinetics between streams seem to be more influenced by differences in DIN concentrations and relative proportions of DIN species than by differences in other environmental factors.

Conclusions

Biofilm uptake responses to short-term changes in DIN concentration in the 2 Mediterranean streams investigated during the study period depended on ambient conditions, including DIN concentrations, where biofilm developed, and the DIN species considered. Under short pulses of increased DIN concentration, the stream biofilms in our study were more reactive to changes in NH_4^+ than to changes in NO_3^- concentration, but ambient U_{NO3-} far exceeded ambient U_{NH4+}, largely because NO_3^- was present at much higher concentrations. The greater kinetic response to NH_4^+ may be attributable to repression of enzymes associated with NO_3^- uptake or the contribution of a different process (nitrification) to total uptake. Lack of response to NO_3^- suggests this species was present in saturating concentrations. Our results contrast with findings from laboratory-scale experiments, in which NO_3^- kinetics conformed to the Michaelis–Menten model (Eppley et al. 1969, Kemp and Dodds 2002, Maguer et al. 2011). In our study, stream biofilm communities were able to respond to increases in NH_4^+ concentration, which is an energetically cheaper N source than NO_3^- and is the substrate for nitrification. However, we found clear differences between streams in biofilm responses to NH_4^+ that probably arose from differences in biofilm characteristics, interactions with other N species, such as NO_3^-, or adaptive changes in affinity.

Human activities associated with different land uses may enrich adjacent streams with DIN and alter the proportion of DIN species in the streams. Thus, streams draining catchments dominated by agricultural practices tend to be NO_3^- enriched, whereas streams draining urbanized catchments are often NH_4^+ enriched (Stanley and Maxted 2008, Lassaletta et al. 2009, Martí et al. 2010). Given widespread changes in land use, our results have implications for understanding and managing N losses to downstream ecosystems. The N species that reach stream ecosystems potentially could be retained by in-stream biofilm communities (NH_4^+) or exported downstream with the subsequent enrichment of receiving waters (NO_3^-).

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