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-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 the two streams. In addition, the uptake efficiency ($U_{N-specific}$) 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
(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, O’Brien and Dodds 2010).

Human activities not only alter the concentration of DIN, but they also change the relative proportion of the two major DIN species: nitrate (NO$_3^-$) and ammonium (NH$_4^+$) (Stanley and Maxted 2008, Lassaletta et al. 2009, Martí et al. 2010). Uptake rates and kinetics are expected to differ between NO$_3^-$ and NH$_4^+$, since 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, wherein neither compound is incorporated into biomass, contribute to both 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 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 additionally contribute to the expected differences between NO$_3^-$ and NH$_4^+$ uptake kinetics. Most studies have investigated NO$_3^-$ or NH$_4^+$ uptake separately; thus, we do not know how uptake kinetics differ between these two DIN species under similar environmental conditions. In addition, little is known about differences in uptake kinetics of NO$_3^-$ or NH$_4^+$ for stream biofilms (i.e., the microbial communities that develop on stream substrata associated to increases in DIN availability. Understanding DIN uptake kinetics of stream biofilms is especially important since biofilms are major contributors to nutrient dynamics in stream networks (Pusch et al. 1998, Battin et al. 2003) and may therefore play a role in ameliorating anthropogenic DIN inputs.

In this study, we compared uptake rates and kinetics for NO$_3^-$ and NH$_4^+$ between biofilms developed in two streams differing in background DIN concentrations. We 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 ($U_{\text{max}}$) 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 $U_{\text{max}}$ 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 (2°27’00”E, 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 (2°37’52”E, 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, 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.
**Channel experiments**

We conducted the 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 PVC channels (6 m long and 15 cm wide) on the streambed using a metallic 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 coverage, which were collected from the streambed within <50m upstream from the channel setting. We then exposed them to 24-h fertilization cycles of increasing concentration levels (1x, 4x, 8x, 16x and 32x the background concentration) of either NO$_3^-$ or NH$_4^+$ (n = 3 channels each; Fig. 1a and b). We released two independent solutions of NO$_3^-$ (as NaNO$_3$) and NH$_4^+$ (as NH$_4$Cl) to the corresponding channels at a constant rate, using a 3-output carboy (one per channel), maintaining a constant head in the carboy with a Masterflex (Vernon Hills, Illinois, USA) L/S battery-powered peristaltic pump. To maintain the background stoichiometric ratio between DIN and soluble reactive phosphorus (SRP) throughout the fertilization cycles, we also added phosphate (as NaH$_2$PO$_4$·H$_2$O) proportionally into the solution at each fertilization level.

To estimate N uptake rates of biofilms, 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. We added two independent solutions amended with $^{15}$NO$_3^-$ (as 99% enriched K$^{15}$NO$_3$) or $^{15}$NH$_4^+$ (as 99% enriched $^{15}$NH$_4$Cl) in conjunction with 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$_4$Cl 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$_3^-$
and $^{15}\text{NH}_4^+$ using the ammonia-diffusion technique (Sigman et al. 1997 and Holmes et al. 1998, respectively). For $^{15}\text{NO}_3^-$ determination, 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 $^{15}\text{NH}_4^+$. For $^{15}\text{NH}_4^+$ determination, 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 ashed 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 40ºC for 4 wk. Once the incubation was completed, we removed the filter packets and placed them in a desiccator for 4 d. Filters were then encapsulated in tins and stored until $^{15}$N analysis.

We oven-dried filters with biofilm samples at 60ºC until they reached a constant weight. To estimate the biofilm ash-free dry mass (AFDM; in g m$^{-2}$), we weighed subsamples on a Sartorius (Göttingen, Germany) MC1 analytical balance, and combusted them at 500ºC for 5 h. We determined the chlorophyll-a content of biofilms (in µg/cm$^2$) following McIntire et al. (1996). We submerged frozen filters in a known volume of 90% v/v acetone and kept them in dark conditions at 4ºC 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 using a Shimadzu (Tokyo, Japan) UV spectrometer. To determine the $^{15}$N signature of biofilms, we weighed subsamples of 1-cm diameter to the nearest 0.001 mg on a Mettler-Toledo (Greifensee, Switzerland) MX5 microbalance and encapsulated them in tins. We sent the samples for analysis at the University of California Stable Isotope Facility (Davis, California, USA). The N content (as a percentage of 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 δ$^{15}$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$_{biofilm}$ in µg N/m$^2$) using the following equation:

$$^{15}$N$_{biofilm} = B_{biofilm} \times N/100 \times (M_{Fi} - M_{Fb})$$

(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 ($M_{Fi}$) and at background conditions ($M_{Fb}$).

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):

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

(2)

where $^{15}$N$_{biofilm}$ 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 ($U_{N-specific}$; 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 $U_{N-specific}$ 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 $U_{\text{N-specific}}$.

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 1$\text{st}$-order response model followed the equation:

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

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

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

where $C$ is the DIN concentration, $U_{\text{max}}$ 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:

$$U = aC^b$$  \ \ \ \ (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 $U_{\text{max}}$ 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
assumes asymptotic normality, and needs suitable coef and vcov methods to 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 $W_i$ value.

We conducted all statistical tests with R 2.14.0 (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/). When necessary, data were log-transformed prior to analysis in order to meet assumptions of homogeneity of variance and normality (Zar, 1996).

**Results**

The two study streams differed substantially in environmental conditions during the experiments (Table 1). Mean water temperature and PAR were 1.4 and 7 times 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 times higher in the high-N stream ($t$-test, $p < 0.001$, Table 1). Mean NH$_4^+$ concentration in the low-N stream was half of that in the high-N stream ($t$-test, $p < 0.001$) contrasting to the long-term trend, when the mean NH$_4^+$ concentration of the low-N stream was twice as low as that of the high-N stream (Table 1). Mean SRP concentration was 4 times lower and mean DIN:SRP ratio was 8 times higher in the high-N stream with respect to the low-N stream ($t$-test, $p < 0.001$).

Furthermore, the two study streams showed important differences in biofilm structure (Table 1). The mean AFDM and the mean chlorophyll-a content were significantly higher (5 and 9 times, respectively) in the biofilm of the high-N stream than in the biofilm of the low-N stream ($t$-test, $p < 0.001$).
Results from the two-way ANOVAs showed that both factors (DIN species and stream) as well as their interaction had a statistically significant effect on both $U$ and $U_N$. At ambient concentrations ($p < 0.01$ in all cases). The $U$ (µg N m$^{-2}$ s$^{-1}$) for NO$_3^-$ (mean ± SE $= 3.1 ± 0.6$ in the low-N stream and $4.1 ± 0.8$ in the high-N stream) was higher than $U$ for NH$_4^+$ (0.3 ± 0.02 in the low-N stream and 0.06 ± 0.01 in the high-N stream) in both streams (Fig 2A). Post-hoc comparisons between streams showed that $U$ for NH$_4^+$ significantly differed between streams (Tukey HSD test, $p = 0.001$) whereas $U$ for NO$_3^-$ did not (Tukey HSD test, $p = 0.636$). Similarly, $U_{N\text{-specific}}$ (d$^{-1}$) for NO$_3^-$ (mean ± SE $= 4.1 ± 0.8$ in the low-N stream and 1.0 ± 0.2 in the high-N stream) was higher than $U_{N\text{-specific}}$ for NH$_4^+$ (0.4 ± 0.02 in the low-N stream and 0.01 ± 0.002 in the high-N stream) in both streams (Fig 2B). In contrast to $U$, post-hoc comparisons showed that $U_{N\text{-specific}}$ 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. 3). The relationship between $U$ and concentration for NO$_3^-$ differed between the two streams, but in any case uptake kinetics fitted a Michaelis-Menten model (Fig. 3A-B). In the low-N stream, AIC analysis indicated that the relationship between $U$ and concentration for NO$_3^-$ better fit a 1st-order model with a negative slope (Table 2). Conversely, in the high N-stream the estimated confidence intervals (95%) for the $b$ parameter in the three models crossed 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$_4^+$ concentration in the two study streams (Fig. 3C-D). The AIC analysis selected the Michaelis-Menten model as the best fit for the relationship between $U$ for NH$_4^+$ and NH$_4^+$ concentration in both streams (Table 2). However, uptake kinetic parameters differed between the two streams. The maximum uptake rate ($U_{max}$; in µg N m$^{-2}$ s$^{-1}$) and the half saturation constant ($K_c$; in µg N/L) were...
lower in the low-N stream, and estimated confidence intervals (95%) for the both parameters
did not overlap between streams (Table 2).

Discussion

In this study we evaluated the response of biofilm N-uptake rates to changes in DIN
concentration, and determined whether this response varied depending on the DIN species
considered. We used an experimental approach that combined nutrient fertilizations and $^{15}$N-
tracer additions in in situ, artificial flumes. We predicted that uptake rates and kinetics
would differ depending 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. 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, with lower values in the high-N stream. In addition, the
uptake efficiency ($U_{\text{N-specific}}$) 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 $U_{\text{max}}$) 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

The rates of epilithic biofilm uptake ($U$) for both DIN species under ambient
conditions measured in this study were on the same order of magnitude as values reported
from previous studies using whole-stream $^{15}$N-tracer additions (Ashkenas et al. 2004,
Schiller et al. 2009, Sobota et al. 2012). This indicates that the epilithic biofilm uptake rates
measured in our channel experiments were representative of natural field conditions.
We found that ambient $U$ was an order of magnitude higher for NO$_3^-$ than for NH$_4^+$ in the two study streams, even though NH$_4^+$ is theoretically an energetically less costly DIN source and was thus expected to be preferentially assimilated over NO$_3^-$ (Dortch 1990, Naldi and Wheeler 2002). In fact, estimated values of the relative preference index (RPI) were close to 1 in the two streams. This index was proposed by Dortch (1990) as a means to determine the preference for NH$_4^+$ over NO$_3^-$ (if values are <1) or for NO$_3^-$ over NH$_4^+$ (if values are >1). The RPI value of ~1 in our study suggests that biofilms in the two streams have no preference for either DIN species. Thus, the observed higher uptake rates for NO$_3^-$ than for NH$_4^+$ was mostly attributable to the fact that NO$_3^-$ was present at higher concentration than NH$_4^+$. While no difference in ambient $U$ for NO$_3^-$ was observed between streams, ambient $U$ for NH$_4^+$ was an order of magnitude lower in the high-N stream. Higher NO$_3^-$ availability relative to NH$_4^+$ availability in the high-N stream may have favored NO$_3^-$ uptake over NH$_4^+$ uptake in this 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 (Geiseeler et al. 2010). It is known that the expression and further biosynthesis of assimilatory nitrate reductase (i.e., the enzyme responsible for NO$_3^-$ assimilation processes) is induced by the presence of NO$_3^-$ and NO$_2^-$ and suppressed by the presence of NH$_4^+$ (Gonzalez et al. 2006). Thus, in the high-N stream, the concurrence of high NO$_3^-$ concentration and low NH$_4^+$ concentration at ambient conditions may have resulted in lower NH$_4^+$ assimilation rates compared to the low-N stream. Differences in nitrification, which can also contribute to NH$_4^+$ ‘uptake’ within the biofilms, are another potential explanation for the differences in $U$ between the streams. If nitrification rate was constrained by the low substrate (i.e., NH$_4^+$) availability in the high-N 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 $\delta^{15}\text{NO}_3^-$ at plateau conditions in the channels where we did the additions of $^{15}\text{NH}_4^+$, 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 $\delta^{15}\text{NO}_3$ increases, for each fertilization cycle we estimated the contribution of nitrification to total biofilm $\text{NH}_4^+$ ‘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 $\text{NH}_4^+$ uptake in high-$\text{NO}_3^-$ streams of Hubbard Brook (New Hampshire, USA). They hypothesized that when assimilatory processes switch to $\text{NO}_3^-$ uptake (i.e., in high-$\text{NO}_3^-$ 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 $\text{NH}_4^+$ assimilation and lower nitrification by biofilms in the high-N stream explains the differences in $U$ for $\text{NH}_4^+$ between the two streams.

The $U_{\text{N-specific}}$ values indicate that the biofilm from the high-N stream was less efficient at taking up both $\text{NO}_3^-$ and $\text{NH}_4^+$ 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 $\text{NO}_3^-$ availability did not enhance biofilm uptake rates for $\text{NO}_3^-$. In the high-N stream, addition of $\text{NO}_3^-$ had no
effect on biofilm uptake, suggesting that uptake capacity of biofilm assemblages was most likely saturated at the ambient NO$_3^-$ concentration. Earl et al. (2006) suggested that when N is no longer limiting in streams, a zero-order mathematical model (i.e., constant rate with slope of 0) is more applicable, which is in concordance with results found in the high N-stream. The lack of biofilm uptake response to increases in NO$_3^-$ concentration could be alternatively explained by tight coupling of NO$_3^-$ uptake to availability of other nutrients (Fairchild et al. 1985, Sterner et al. 1992). In this regard, Schanz and Juon (1983) suggested that phosphorus (P) is potentially a limiting element at DIN:P ratios above 20 (others have suggested a transition from N to P limitation at DIN:P ratios around 16-17; Redfield 1958, Grimm and Fisher 1986). Although we added SRP in the fertilization solutions to maintain background DIN:P ratios throughout fertilizations, these ratios were well above the potential P-limitation thresholds, especially in the high-N stream (i.e., mean ± SE, 394 ± 32). In this sense, NO$_3^-$ uptake in the high-N stream may have been constrained by P insufficiency. However, if P was the limiting nutrient, one might expect that increases in P availability should alleviate any P limitation and thus enhance NO$_3^-$ uptake. We believe this alternative explanation is unlikely, since previous nutrient-limitation bioassays in the high-N stream have failed to show P limitation (von Schiller et al. 2007).

Increases in NO$_3^-$ availability in the low-N stream provoked a decrease in biofilm uptake rates, 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 are reported in the literature (usually associated with nitrification processes; Kim et al 2006, Vadivelu et al. 2007). However, as far as we know, there is no previous evidence of inhibition of NO$_3^-$ uptake at high NO$_3^-$ concentrations. Nevertheless, inhibitory effects of long-term NO$_3^-$ enrichment on periphyton growth have been reported from nutrient-diffusing substrate experiments (Bernhardt and Likens 2004) and a few studies have shown
potentially toxic effects of NO$_3^-$ on freshwater animals and plants (Camargo and Alonso 2006; Lambert and Davy 2011). Unfortunately, our experiments do not allow us to determine the mechanisms that could explain the observed pattern, but they provide evidence that a short-term, sharp increase in NO$_3^-$ concentration may have inhibitory effects. Michaelis-Menten kinetics described biofilm uptake responses to increases in NH$_4^+$ concentration in the two streams. Because values of $K_s$ were higher than ambient concentrations of NH$_4^+$ in both streams, 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 two streams. Bunch and Bernot (2012) also compared uptake responses of microbial communities to NH$_4^+$ and NO$_3^-$ enrichments; and they observed that responses were more immediate and pronounced in the case of NH$_4^+$ and were delayed and more variable in the case of NO$_3^-$. 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, in this study the values of RPI of ~1 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 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 studies show a Michaelis-Menten response of nitrification...
rates to increases in NH$_4^+$ concentration within a similar range of NH$_4^+$ concentrations used in our study (Koper et al. 2010). Nitrification was likely substrate-limited at the relatively low NH$_4^+$ concentrations in the two 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 this is only a minor contributor to observed kinetics of NH$_4^+$ uptake. We suggest that a combination of several of the above-mentioned mechanisms best explains the different kinetic responses of NH$_4^+$ and NO$_3^-$ in the two study streams.

Although NH$_4^+$ uptake kinetics fit the Michaelis-Menten model in the two streams, the kinetic parameters (i.e., $K_s$ and $U_{max}$) clearly differed between streams, supporting our predictions. NH$_4^+$ $U_{max}$ of the biofilm in the high-N stream was 21 times higher than $U_{max}$ of the biofilm in the low-N stream. The high-N stream had higher biofilm biomass as well as more photoautotrophic organisms (as indicated by the chlorophyll-a content) than the low-N stream, which could explain the higher maximum uptake 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 times higher in the high-N stream. Biofilms in the low-N stream were therefore relatively more efficient in the uptake of NH$_4^+$ than those in the high-N stream, which is in agreement with uptake results measured at ambient DIN conditions.

In contrast, the biofilm in the low-N stream showed a higher affinity (i.e., lower $K_s$) for NH$_4^+$ than the biofilm in the high N-stream. Higher affinities for substrate are often attributed to microorganisms exposed to lower ambient concentrations (Collos et al. 2005, Martens-Habbena et al. 2009). This explanation may not apply to our study if we only consider ambient NH$_4^+$ concentration, which was similar and low in the two streams. However, it is more appropriate in discussing nutrient limitation to consider the total DIN 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
have caused the kinetic differences observed, at least for the photoautotrophic component of
the biofilms. These arguments suggest that observed differences in biofilm uptake kinetics
between streams are more influenced by stream differences in DIN concentrations and
relative proportions of the two DIN species than by differences in other environmental
factors.

Conclusions

Biofilm uptake responses to short-term changes in DIN concentration in the two
investigated Mediterranean streams during the study period varied depending on ambient
conditions, including DIN concentrations, where biofilm developed, as well as on the DIN
species considered. Under short pulses of increased DIN concentration, these particular
stream biofilms were more reactive to changes in NH$_4^+$ concentration than to changes in
NO$_3^-$ concentration, yet ambient uptake rates for NO$_3^-$ far exceeded those for NH$_4^+$, largely
because the former N species was present at much higher concentration. The greater kinetic
response to NH$_4^+$ may be attributable to repression of enzymes associated with NO$_3^-$ uptake,
or a different process (nitrification) contributing to total uptake. The lack of response to
NO$_3^-$ suggests this species is at 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 also the substrate for
nitrification. However, clear differences in NH$_4^+$ response by biofilms were observed
between the two streams, likely owing to differences in biofilm characteristics, interactions
which other N species, such as NO$_3^-$, or adaptive changes in affinity.

As pointed out by other studies, human activities associated with different land uses
not only may enrich the adjacent streams with DIN but may also alter the proportion of DIN
species in those ecosystems. In this regard, streams draining catchments dominated by agricultural practices tend to be enriched in NO$_3^-$ whereas streams draining urbanized catchments are often NH$_4^+$-enriched (Stanley and Maxted 2008; Lasaletta et al. 2009; Martí et al. 2010). Given widespread changes in land use, our findings have implications for understanding and managing N losses to downstream ecosystems, since the distinct N species that reach stream ecosystems could be potentially retained by the in-stream biofilm communities (i.e., NH$_4^+$) or exported downstream, with the subsequent enrichment of receiving waters (i.e., NO$_3^-$).
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Table legends

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

<table>
<thead>
<tr>
<th></th>
<th>Low-N stream</th>
<th>High-N stream</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water temperature (°C)</td>
<td>15.4 ± 0.1</td>
<td>11.0 ± 0.2</td>
</tr>
<tr>
<td>PAR (mol m⁻² day⁻¹)</td>
<td>9.5 ± 3.4</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>NO₃⁻ (µg N/L)</td>
<td>222 ± 2</td>
<td>400 ± 27</td>
</tr>
<tr>
<td></td>
<td>(181 ± 11)</td>
<td>(780 ± 44)</td>
</tr>
<tr>
<td>NH₄⁺ (µg N/L)</td>
<td>15 ± 1</td>
<td>8 ± 1</td>
</tr>
<tr>
<td></td>
<td>(12 ± 1)</td>
<td>(19 ± 2)</td>
</tr>
<tr>
<td>SRP (µg P/L)</td>
<td>11 ± 0.3</td>
<td>3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>(4 ± 0.5)</td>
<td>(15 ± 2.6)</td>
</tr>
<tr>
<td>DIN:SRP (molar)</td>
<td>48 ± 1</td>
<td>394 ± 32</td>
</tr>
<tr>
<td></td>
<td>(192 ± 32)</td>
<td>(429 ± 106)</td>
</tr>
<tr>
<td>Ash free dry mass (g/m²)</td>
<td>0.9 ± 0.1</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>Chlorophyll- a (µg/cm²)</td>
<td>0.3 ± 0.03</td>
<td>2.6 ± 0.2</td>
</tr>
</tbody>
</table>
Table 2. Statistical parameters of linear, Michaelis-Menten and efficiency loss models used to evaluate the model that best fits the relationship between uptake rate ($U$) and DIN concentration ($C$) for both streams and DIN species ($\text{NO}_3^-$ and $\text{NH}_4^+$). 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 ($U_{\text{max}}; \mu g \text{ N m}^{-2} \text{s}^{-1}$) and $b$ corresponds to the half saturation constant ($K_s; \mu g \text{ N/L}$). The 95% confidence intervals of the values are also reported in brackets.

| DIN Species | Low-N stream | | | High-N stream | | |
|-------------|--------------|-----| -----|--------------|-----|
| | $a$ | $b$ | AIC | $W_i$ | $a$ | $b$ | AIC | $W_i$ |
| $\text{NO}_3^-$ | | | | | | | | |
| Linear, $U = a + bC$ | 3.1 | $-2.9e^4$ | 33.4 | 0.97 | 4.3 | $4.0e^4$ | 55.1 | 0.36 |
| | (2.7 – 3.5) | (-4.0e^4 - -1.8e^4) | | | (3.1 – 5.5) | (-2.3e^4 - 8.2e^4) | | |
| Michaelis-Menten, $U = a \frac{C}{b} + C$ | 2.1 | -85.8 | 48.0 | 0.00 | 6.5 | 384 | 55.6 | 0.28 |
| | (1.6 – 2.6) | (-131.9 - -7.6) | | | (4.8 – 9.2) | (-36.5 - 1282) | | |
| Efficiency Loss, $U = a C^b$ | 11.9 | -0.2 | 48.1 | 0.03 | 1.3 | 0.2 | 55.1 | 0.37 |
| | (5.3 – 27.1) | (-0.4 - -0.1) | | | (0.3 – 5.6) | (-1.0e^2 - 0.4) | | |
| $\text{NH}_4^+$ | | | | | | | | |
| Linear, $U = a + bC$ | 0.8 | $1.6e^3$ | 17.3 | 0.00 | 0.3 | $3.0e^2$ | 45.1 | 0.03 |
| | (0.5 – 1.0) | (2.9e^3 - 2.9e^3) | | | (-0.5 - 1.1) | (2.5e^3 - 3.4e^3) | | |
| Michaelis-Menten, $U = a \frac{C}{b} + C$ | 1.3 | 17.1 | 2.6 | 0.98 | 28.0 | 628 | 38.9 | 0.77 |
| | (1.2 – 1.5) | (7.8 – 34.9) | | | (17.4 – 113) | (307 – 3449) | | |
| Efficiency Loss, $U = a C^b$ | 0.4 | 0.2 | 10.9 | 0.02 | 8.2e^2 | 0.8 | 41.7 | 0.19 |
| | (0.2 – 0.7) | (9.3e^2 - 0.3) | | | (3.0e^2 - 0.2) | (0.7 - 1.0) | | |

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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

\[ \text{NO}_3^- \quad \text{NH}_4^+ \]

PVC channels with naturally colonized stream cobbles

N\text{aNO}_3\text{ solution tank}

K\text{NO}_3\text{ solution tank}

\text{NH}_4\text{Cl solution tank}

B

Clean channels replace colonized substrata

\[ ^{15}\text{N add} \]

24 h N fertilization

x4

2x, 4x, 8x, 16x, and 32x

Clean channels replace colonized substrata

\[ ^{15}\text{N add} \]
Figure 3

(A) Relationship between $U$ for NO$_3^-$ (μg N m$^{-2}$ s$^{-1}$) and NO$_3^-$ (μg N/L) in Low-N stream. 

(B) Relationship between $U$ for NO$_3^-$ (μg N m$^{-2}$ s$^{-1}$) and NO$_3^-$ (μg N/L) in High-N stream. 

(C) Relationship between $U$ for NH$_4^+$ (μg N m$^{-2}$ s$^{-1}$) and NH$_4^+$ (μg N/L) in Low-N stream. 

(D) Relationship between $U$ for NH$_4^+$ (μg N m$^{-2}$ s$^{-1}$) and NH$_4^+$ (μg N/L) in High-N stream.