Biofilm growth and nitrogen uptake responses to increases in nitrate and ammonium availability

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Abstract

Nitrate (NO_3^-) and ammonium (NH_4^+) are the two major dissolved inorganic nitrogen (DIN) species available in streams. Human activities increase stream DIN concentrations and modify the NO_3^-:NH_4^+ ratio. However, few studies have examined biofilm responses to enrichment of both DIN species. We examined biofilm responses to variation in ambient concentrations and enrichments in either NO_3^- or NH_4^+. We incubated nutrient diffusing substrata (NDS) bioassays with three treatments (DIN-free, +NO_3^- and +NH_4^+) in five streams. Biomass-specific uptake rates (U_{spec}) of NO_3^- and NH_4^+ were then measured using in situ additions of ^{15}N-labeled NO_3^- and NH_4^+. Biomass (estimated from changes in carbon content) and algal accrual rates, as well as U_{spec}-NO_3^- of biofilms in DIN-free treatments varied among the streams in which the NDS had been incubated. Higher ambient DIN concentrations were only correlated with enhanced biofilm growth rates. U_{spec}-NO_3^- was one order of magnitude greater and more variable than U_{spec}-NH_4^+, however similar relative preference index (RPI) suggested that biofilms did not show a clear preference for either DIN species. Biofilm growth and DIN uptake in DIN-amended NDS (i.e., +NO_3^- and +NH_4^+) were consistently lower than in DIN-free NDS (i.e., control). Lower values in controls with respect to amended NDS were consistently more pronounced for algal accrual rates and U_{spec}-NO_3^- and for the +NH_4^+ than for the +NO_3^- treatments. In particular, enrichment with NH_4^+ reduced biofilm U_{spec}-NO_3^- uptake, which has important implications for N cycling in high NH_4^+ streams.

Introduction

Nitrogen (N) is a key element for organisms and its availability can either limit production or favor eutrophication in aquatic ecosystems (Dodds and Welch 2000; Francoeur 2001). Nitrate (NO_3^-) and ammonium (NH_4^+) are the two major dissolved inorganic nitrogen (DIN) species available in running waters. These two DIN species undergo different biogeochemical pathways and their relative availability may affect DIN fate. In streams, DIN cycling is mostly mediated by the benthic microbial assemblages (bacteria, fungi and algae) that develop on submersed substrata (i.e., biofilms; Pusch et al. 1998; Battin et al. 2003).

Microorganisms in biofilms can directly assimilate the two DIN species from the water column. The rates at which they assimilate NO_3^- and NH_4^+ not only depend on the availability of each single DIN species (Dodds et al. 2002; O'Brien et al. 2007; Ribot et al. 2013), but they are also dependent on the relative availability of the two species (Geisseler et al. 2010; Ribot et al. 2013). In addition, NH_4^+ can be directly incorporated into biomass via anabolic pathways while incorporation of NO_3^- into the cells requires an active pumping and a further reduction to NH_4^+; consequently, assimilation of NO_3^- is an energy-consuming process (McCarty 1995). Therefore, microbial assimilation of NO_3^- may be induced by the presence of NO_3^-, and it may be suppressed by the presence of NH_4^+ (Gonzalez et al. 2006). Furthermore, this effect at the biofilm level may have consequences at the ecosystem level as suggested in previous studies (Dugdale et al. 2007; Domingues et al. 2011).

Understanding how biofilms respond to increases in NO_3^- or NH_4^+ is important because human activity increases total DIN availability and changes the relative abundance of the two DIN species (Stanley and Maxted 2008; von Schiller et al. 2008; Lassaletta et al. 2009; Martí et al. 2010). From previous studies we have learned that

streams draining catchments dominated by agricultural practices have higher NO_3^-:NH_4^+ ratios than streams dominated by urban activity. Conversely, urban streams tend to be NH_4^+ enriched at sites where effluent from wastewater treatment plants are subjected to a partial nitrification of the N loads received. Studies addressing the effect of increases in DIN availability on the growth of stream biofilms with explicit consideration of the two DIN species (i.e., NO_3^- and NH_4^+) are scarce (but see von Schiller et al. 2007 and Hoellein et al. 2010). In addition, results from these studies are contradictory, showing either a preference for NH_4^+ as an N source for DIN assimilatory uptake (von Schiller et al. 2007) or no differential effect between the two DIN species on biofilm growth (Hoellein et al. 2010). Furthermore, studies designed to compare biofilm uptake responses to increases in NO_3^- and NH_4^+ concentration have mostly been conducted in the laboratory (Kemp and Dodds 2002; O'Brien and Dodds 2008; Domingues et al. 2011; Bunch and Bernot 2012), with few field experiments (but see Bernot et al. 2006 and Ribot et al. 2013). NH_4^+ has been usually considered the preferred DIN source for DIN uptake (Dortch 1990; Naldi and Wheeler 2002); however, instances when NO_3^- is the main N source for microorganisms are common due to the generally greater NO_3^- availability (Domingues et al. 2011; Bunch and Bernot 2012; Ribot et al. 2013).

The goal of this study was to examine biofilm responses in terms of growth and DIN uptake to variation in ambient concentrations and enrichments of either NO_3^- or NH_4^+. We conducted nutrient diffusing substrata (NDS) bioassays with three treatments (DIN-free, $+NO_3^-$ and $+NH_4^+$) in five streams spanning a range in ambient DIN availability. The NDS allowed us to measure biomass and algal growth under the different treatments in the different streams. In addition, at the end of NDS incubations, we exposed the different biofilms developed on the NDS to ^{15}N additions of either NO_3^-

or NH_4^+ in a single location to measure their capacity for DIN assimilation of the two species as well as their relative preference for the uptake of the two DIN species. Comparison of assimilation rates between biofilms under control and DIN amended conditions allowed us to estimate the effect of DIN species enrichments on N assimilation rates of biofilms. We expected that biofilms in streams with higher ambient DIN concentration would have higher growth rates and higher N demand (i.e., higher DIN uptake rates) than those developed in low DIN concentrations if biofilms were not limited by any other environmental factor. In addition, we expected that responses of biofilms to NH_4^+ enrichments would be higher than those to NO_3^- enrichments because of greater energetic cost of NO_3^- assimilation.

Methods

Study sites

La Tordera catchment (Catalonia, NE Spain) has an area of 868.5 km^2 dominated by siliceous geology, and covers a 1700-m altitudinal gradient from the headwaters to the sea level within a 35 km distance. Climate in this region is typically Mediterranean, with warm, dry summers, and mild, humid winters. Although most of the catchment is forested, agricultural, urban and industrial areas tend to concentrate in the river valley, resulting in a heterogeneous land use template along the lowlands of the river network, which affects stream N concentrations (von Schiller et al. 2008). Within this catchment, we selected five streams draining sub-catchments with different land uses. Three sites have forested land-use 99 % of the watersheds, and the other two sites have human land-use (i.e., agriculture + urban) of 2.7 and 7.1 % (Table 1) mostly adjacent to the stream. These streams were selected to cover a wide range of DIN concentration based on data from 15 streams in la Tordera catchment collected biweekly from September

2004–July 2007 (M. Ribot, unpublished data). Santa Fe del Montseny (MON), Font del Regàs (FR) and Castanyet (CAS) are low DIN concentration streams located at headwater-forested catchments. In contrast, Gualba (GUA) and Santa Coloma (COL) are higher DIN concentration streams located at the river valley and influenced by urban (GUA) and agricultural (COL) activities (Table 1).

Experimental approach

We conducted two separate sets of nutrient diffusing substrata (NDS) bioassays, each one including enrichments of NO_3^- and NH_4^+ (see description below), in each of the five study streams. After incubation in the stream, all NDS were brought together for measurement of N assimilation (^{15}N uptake) at a common location. The first set of NDS bioassays started on June 21st 2006 and lasted for 16 days. After the incubation, we replaced the agar solution of all treatments by fresh DIN-free agar solution to ensure biofilm DIN uptake from the water column. These DIN-free NDS were transferred to COL stream in containers filled with stream water. NDS were left in the stream for 5 days prior to the $^{15}NO_3^-$ addition (see description below) to estimate rates of NO_3^- assimilation by all the biofilms. We repeated the procedure for the second set of NDS bioassays, which started on July 7th and lasted for 21 days, with an acclimation period of 4 days before conducting the $^{15}NH_4^+$ addition (see description below) to estimate rates of NH_4^+ assimilation by all the biofilms. Due to economic and logistic constraints, we could not conduct separate ^{15}N tracer additions in each study stream to quantify in situ biofilm NO_3^- and NH_4^+ uptake rates from the biofilm developed on the NDS. We acknowledge that the acclimation period (4–5 days) of all biofilms in the COL stream may have caused some changes in biofilm composition; and thus, in their uptake responses. However, since the acclimatization time was much shorter than the time

150 biofilms were exposed to all the DIN treatments in the different streams, we expected
151 this treatment conditions should dictate biofilm responses. In fact, significant
152 differences in biofilm structural and functional parameters were observed among
153 streams (see "Results").
154
155 **NDS bioassays**
156 We constructed NDS following the method outlined in Tank and Dodds (2003). The
157 NDS consisted of 60 mL plastic containers filled with a 2 % (by mass) agar solution,
158 which was not amended (i.e., DIN-free treatments) or was amended either with nitrate
159 (0.5 M KNO_3; hereafter referred as $+NO_3^-$) or ammonium (0.5 M NH_4Cl; hereafter
160 referred as $+NH_4^+$). We placed pre-combusted and pre-weighed Whatman GF/F glass
161 fiber filters on the top of the plastic containers to cover the agar completely and to serve
162 as the substrata for biofilm colonization. In each stream we placed three plastic baskets
163 in a single pool. Each plastic basket contained two replicates of each treatment (DIN-
164 free, $+NO_3^-$ and $+NH_4^+$) and stream cobbles to hold the baskets on place. Controls were
165 placed upstream to avoid leaching nutrients towards the substratum immediately
166 downstream. We placed the baskets on the streambed of pools of similar water depth
167 and velocity. Stream substratum of all the selected stream reaches was composed of
168 cobbles and pebbles with sand patches. During the study period, a well-developed
169 riparian canopy cover shaded all the selected reaches.
170 During the two NDS incubation periods, we collected stream water samples on 3
171 evenly spaced dates for ambient nutrient concentration analyses. We collected water
172 samples with plastic syringes and filtered them immediately through ashed Whatman
173 (Maidstone, UK) GF/F fiber glass filters into acid-washed plastic containers and stored
174 them on ice for transportation to the laboratory until analysis. On the same dates, we

measured water conductivity and water temperature with a portable WTW conductivity meter (Weilheim, Germany). In addition, we determined discharge on a single cross-sectional transect by measuring mean wetted width, mean depth and mean water velocity (Gordon et al. 1992).

^{15}N constant rate additions

In COL stream, we selected a 250-m reach to run the two ^{15}N additions. In this reach, and prior to the ^{15}N additions, we randomly distributed all NDS from the other four sites along a cross-section located 50 m downstream of the ^{15}N addition point. For each ^{15}N addition (i.e., $^{15}NO_3^-$ and $^{15}NH_4^+$) we prepared a solution amended with either $^{15}NO_3^-$ (as 99 % enriched $K^{15}NO_3$) or $^{15}NH_4^+$ (as 99 % enriched $^{15}NH_4Cl$) in conjunction with NaCl, as a conservative tracer. The amount of $K^{15}NO_3$ and $^{15}NH_4Cl$ and the pump flow rate were set to achieve a target $\delta^{15}N$ enrichment of 10,000 ‰ for each DIN species in the water column. We released the ^{15}N solutions at the top of the reach at a constant rate using a Masterflex (Vernon Hills, Illinois, USA) L/S battery-powered peristaltic pump. The two ^{15}N additions started at midnight (00:00) and lasted for 12 h. The $^{15}NO_3^-$ addition was run on July 12th and the $^{15}NH_4^+$ addition was run on August 1st.

We collected stream water samples at the NDS location for the analysis of the ^{15}N isotopic signature of both DIN species ($^{15}NO_3^-$ and $^{15}NH_4^+$) 24 h prior to the start of the ^{15}N tracer additions and at plateau conditions. To verify plateau conditions during each ^{15}N addition, we automatically recorded conductivity every 10 s at the end of the stream reach using a portable WTW conductivity meter connected to a Campbell Scientific (Logan, Utah, USA) data logger. 24 h after the end of each ^{15}N addition, coinciding with the water collection described above, we also collected the NDS filters, cut them in half and kept them on ice in the field until further laboratory analyses.

Laboratory analyses

One half of each filter was oven-dried at 60 °C until constant weight to estimate biofilm dry mass, C and N content and ^{15}N signature. To estimate the biofilm dry mass we weighed the oven-dried half-filters to the nearest 0.001 mg on a Mettler-Toledo (Greifensee, Switzerland) MX5 microbalance and we subtracted 1/2 of the filter weight. We then encapsulated the half-filters in tins.

The other half of the filter was kept frozen until the measurement of chlorophyll-a (chla) content following McIntire et al. (1996). We submerged the frozen half-filters in a known volume of 90 % v/v acetone and kept them in the dark at 4 °C overnight. We then 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.

We analyzed water samples for the concentrations of NO_3^-, NH_4^+, and soluble reactive phosphorus (SRP) on a Bran + Luebbe (Norderstedt, Germany) TRAACS 2000 autoanalyzer following standard colorimetric methods (APHA 1995). We processed water samples for the analysis of $^{15}NH_4^+$ and $^{15}NO_3^-$ as described in Holmes et al. (1998) and Sigman et al. (1997), respectively. Briefly, for $^{15}NH_4^+$ determination, we amended a known volume of sample with 3 g L^{-1} of MgO and 50 g L^{-1} of NaCl and a Teflon filter packet containing an acidified 1-cm-diameter ashed Whatman GF/D fiber glass filter to trap the volatilized NH_3, and incubated it on a shaker at 40 °C for 4 weeks. For $^{15}NO_3^-$ determination, we amended a known volume of the 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 of MgO and 0.5 mg Devarda's alloy to reduce the NO_3^- to NH_4^+, and treated the remaining sample as for $^{15}NH_4^+$. We also diffused a set of standards of known volume for volume-related fractionation corrections. Once the incubation was completed, we removed the filter

packets and placed them in a desiccator for 4 days. We then encapsulated the filters in tins and stored them until ^{15}N analysis.

Samples for the determination of the ^{15}N signature were analyzed at the University of California Stable Isotope Facility (Davis, California, USA). The C and N content (as a percentage of dry mass) and the abundance of the heavier isotope, expressed as the ^{15}N:^{14}N ratio compared to that of a standard (i.e., N_2 from the atmosphere) using the 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).

Parameter calculations

For each NDS treatment and stream, biomass accrual rates (in µg C cm^{-2} d^{-1}) were calculated by dividing the C content (in µg C cm^{-2}) at the end the of the NDS incubation by the time period of the incubation (in days). Similarly, the algal accrual rates (in µg chla cm^{-2} d^{-1}) were calculated by dividing the chla content (in µg chla cm^{-2}) at the end the of the NDS incubation by the time period of the incubation (in days). We also calculated the C to N molar ratio of the biofilms at the end of the NDS incubation based on the percentage of C and N in dry mass.

To calculate biofilm DIN uptake rates of NO_3^- and NH_4^+ from the $^{15}NO_3$ and $^{15}NH_4$ additions, respectively, we first calculated the amount of ^{15}N tracer contained in biofilm biomass ($^{15}N_{biofilm}$; in µg N m^{-2}) at the end of the addition 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 as dry mass per unit of area (µg m^{-2}), 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 then estimated the DIN uptake rate (U; in µg N m^{-2} s^{-1}) for either NO_3^- or NH_4^+ using the following equation:

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

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 (12 h), $^{15}N_{flux}$ is the stream water ^{15}N flux (as either NO_3^- or NH_4^+) at plateau conditions (µg ^{15}N s^{-1}) and N_{flux} is the total N flux (as either NO_3^- or NH_4^+) based on stream water concentration and discharge (µg N s^{-1}). For each DIN species, we calculated the biomass-specific DIN uptake rate (U_{spec}; s^{-1}) by diving U by the N content in biofilm biomass. We used U_{spec} over U to compare uptake responses among streams and NDS treatments because it avoids confounding effects associated with differences in N biomass accrual rates among all treatments. U_{spec} has been used in the literature as an indicator of N turnover time within a biotic compartment (Dodds et al. 2004).

To assess the biofilm uptake preference for either NO_3^- or NH_4^+, we calculated the relative preference index (RPI) for NO_3^- as proposed by Dortch (1990) using the equation:

$$RPI_{NO3} = (U_{NO3}/\Sigma U_{DIN}) / (NO3/DIN) \quad (3)$$

where U_{NO3} is the biofilm NO_3^- uptake rate (U for NO_3^- from Eq. 2; in µg N m^{-2} s^{-1}) in a given NDS filter, ΣU_{DIN} is the sum of the mean biofilm uptake rate of NO_3^- and NH_4^+ (U for NH_4^+ from Eq. 2; in µg N m^{-2} s^{-1}) within a NDS treatment, NO_3 is the mean nitrate concentration in COL during the two ^{15}N additions and DIN is the sum of the mean concentrations of NH_4^+ and NO_3^- in COL during the two ^{15}N additions. RPI is an

indicator of the relevance of NO_3^- uptake relative to total DIN uptake weighed by the relative importance of NO_3^- concentration to total DIN concentration. For example if NO_3^- uptake is 50 % of DIN uptake, but NO_3^- is only 25 % of DIN, the RPI value is 0.5/0.25 = 2, indicating preference for NO_3^- given the available DIN species. An RPI-NO_3^- value <1 indicates a preference for NH_4^+.

To explore the biofilm response in terms of biomass accrual, algal accrual, C:N ratios and uptake rates of the two DIN species to the enrichments of NO_3^- or NH_4^+, we calculated the response ratio to each DIN species as described in Tank and Dodds (2003). For each variable, we calculated the logarithmic ratio of the values from amended treatments ($+NO_3^-$ or $+NH_4^+$) relative to the control treatment (DIN-free). Response ratios (RRs) can be positive (i.e., treatment values greater than control) or negative (i.e., treatment values lower than control). The RR allows normalizing for the varying effect of NDS treatments on biofilm growth and DIN uptake rates among streams and among replicate locations within each stream, which may mask any treatment effects on areal uptake.

Statistical analyses

We pooled the data from control treatments (DIN-free) from the two NDS incubations to explore differences in biofilm growth at ambient concentrations among streams in which the NDS were incubated. We compared biomass and algal accrual rates and C:N molar ratios using a linear mixed-effects model with stream as fixed factor (n = 5) and incubation date as random factor (n = 2). We included the random effect 'incubation date' in the model to account for the potential temporal variation in biofilm responses between the two sets of NDS bioassays, despite initial analysis indicated that this effect was negligible. However, the inclusion of a non-significant random effect factor does

not influence the inference on fixed effects factors (Zuur et al. 2009). On the other hand, since U_{spec}-NO_3^- and U_{spec}-NH_4^+ for control treatments were calculated separately from the first and the second NDS incubations respectively, we compared U_{spec}-NO_3^-, U_{spec}-NH_4^+ and RPI using one-way ANOVA with stream as fixed factor (n = 5) to explore differences in these variables at ambient concentrations among streams in which the NDS were incubated.

We explored biofilm growth response to enrichments of NO_3^- or NH_4^+ among streams by comparing the RRs of biomass and algal accrual rates and C:N molar ratios using a linear mixed-effects model with stream (n = 5) and NDS treatment (n = 2) as fixed factors and incubation as random factor (n = 2). Again, we included the random effect of 'incubation date' in the model, despite this random effect was shown to be negligible. To explore biofilm DIN uptake response to enrichments of either NO_3^- or NH_4^+ among streams, we compared the RRs of U_{spec}-NO_3^-, U_{spec}-NH_4^+ and RPI using two-way ANOVA with stream (n = 5) and NDS treatment (n = 2) as fixed factors.
We ran Pearson correlations to explore if biofilm growth and DIN uptake were related to the ambient concentrations of NO_3^- and NH_4^+ of the study streams in which the NDS were incubated as well as to explore the relationships between biofilm growth and DIN uptake. Correlations were only explored if the fixed factor 'stream' was significant in the linear mixed-effects or ANOVA models.

We ran all statistical tests with R 2.15.0 (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/.). Linear mixed-effects models were done with the R package 'nlme'. Post-hoc multiple comparisons for nmle models followed significant fixed factor ($p < 0.05$) using the R package 'multcomp'. Post-hoc Tukey HSD tests followed significant ANOVA ($p < 0.05$). When necessary, data were

log-transformed before analysis to meet assumptions of homogeneity of variance and normality (Zar 1996).

Results

Physical and chemical characteristics of the study streams

During the study period, mean discharge was relatively low at all streams and averaged 9.6 L s^{-1} (Table 1). Stream water temperature and conductivity ranged from 14.2 to 21.4 °C and 61 to 310 µS cm^{-1}, respectively, across streams. Concentration of NH_4^+ was low and relatively similar among streams, ranging from 14 to 22 µg N L^{-1}. In contrast, NO_3^- concentration ranged from 140 to 600 µg N L^{-1}, and SRP concentrations ranged from 4 to 46 µg P L^{-1} (Table 1). The lowest NO_3^- and SRP concentrations were observed in two of the forested streams (CAS and FR), whereas the highest concentrations were observed in COL, the stream with the highest percentage of agricultural land use in the drainage area. As a result of the high variability in nutrient concentrations, we observed a wide range in the NO_3^-:NH_4^+ ratio (from 8 to 27) and in the DIN:SRP molar ratio (23 to 95; Table 1).

Biofilm responses to ambient DIN variability

Mean biomass accrual rates of biofilms in DIN-free treatments ranged from 43 to 126 µg C cm^{-2} d^{-1}, and differed significantly among the streams (Fig. 1a; Table 2) with significant differences between GUA and FR (Tukey HSD tests, $p < 0.020$; Fig. 1a). The biomass accrual rates of biofilms in DIN-free treatments were positively correlated with ambient NO_3^- concentration ($r = 0.30$, $p = 0.029$; Fig. 2a) and NH_4^+ concentration ($r = 0.41$, $p = 0.002$; Fig. 2b) among streams. Algal accrual rates of biofilms in DIN-free treatments were similar among streams, except in CAS where rates were 5 times greater

348 (Tukey HSD tests, $p < 0.001$; Fig. 1b; Table 2). Algal accrual rates of biofilms in DIN-
349 free treatments were positively correlated with ambient NH_4^+ concentration among
350 streams ($r = 0.31$, $p = 0.023$; Fig. 2d). Furthermore, algal accrual rates of biofilms in
351 DIN-free treatments were positively correlated with biomass accrual rates in the same
352 treatments ($r = 0.38$, $p = 0.005$; data not shown). The C:N molar ratios of biofilms in
353 DIN-free treatments (mean = 8.9) did not differ significantly among the streams
354 (Fig. 1c; Table 2).

355 U_{spec}-NO_3^- of biofilms in DIN-free treatments was one order of magnitude
356 greater (mean = 0.04 h^{-1} vs. mean = 0.005 h^{-1}) and more variable (CV = 71 % vs
357 CV = 26 %) than U_{spec}-NH_4^+ (Fig. 3a, b). U_{spec}-NO_3^- of biofilms in DIN-free treatments
358 varied significantly depending on the stream in which the NDS were incubated (one-
359 way ANOVA, $F_{4,25} = 7.40$, $p < 0.001$). U_{spec}-NO_3^- was highest in biofilms developed in
360 MON, and FR (Tukey HSD tests, $p < 0.012$). Conversely, U_{spec}-NH_4^+ of biofilms in
361 DIN-free treatments did not differ significantly among the streams (one-way ANOVA,
362 $F_{4,20} = 1.66$, $p = 0.224$). U_{spec}-NO_3^- of biofilms in DIN-free treatments was negatively
363 correlated with the ambient NH_4^+ concentration of the streams ($r = -0.37$ and $p = 0.045$;
364 Fig. 2f). Furthermore, U_{spec}-NO_3^- of biofilms in DIN-free treatments was negatively
365 correlated with algal accrual rates in the same NDS treatments ($r = -0.37$ and $p = 0.046$;
366 data not shown).

367 Mean RPI values of biofilms in DIN-free treatments were close to 1 and similar
368 among biofilms developed in the different streams (one-way ANOVA, $F_{4,25} = 0.54$,
369 $p = 0.712$), indicating no clear preference for any of the two DIN species (Fig. 3c).
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373 **Biofilm responses to NO_3^- and NH_4^+ enrichments**

374 In general, the comparison between DIN-free and DIN-enriched NDS treatments (i.e.,
375 the response ratio, RR) showed that both biofilm growth and DIN uptake had no effect
376 or a negative response to NO_3^- and NH_4^+ enrichments (Figs. 4, 5). The RRs of biomass
377 accrual rates differed significantly among streams (Fig. 4a; Table 3), but they did not
378 differ significantly between $+NO_3^-$ and $+NH_4^+$ treatments (Fig. 4a). Biomass accrual
379 response to DIN enrichments was null in those streams with lower DIN ambient
380 availability and most negative in biofilms developed in COL, the stream with the
381 highest DIN (Tukey HSD tests, $p < 0.036$). In addition, the RRs of biomass accrual rates
382 in $+NO_3^-$ treatments were negatively correlated with ambient NO_3^- ($r = -0.39$,
383 $p = 0.004$) and NH_4^+ concentrations among streams ($r = -0.38$, $p = 0.004$; data not
384 shown). The RRs of biofilm accrual rates in $+NH_4^+$ treatments were also negatively
385 correlated with the ambient NO_3^- concentration among streams ($r = -0.34$ and
386 $p = 0.022$). These correlations suggest that biomass responsiveness decreased with
387 rising DIN concentration among streams.

388 The RRs of algal accrual rates in biofilms differed significantly among the
389 streams and between $+NO_3^-$ and $+NH_4^+$ treatments (Fig. 4b; Table 3). The RRs for the
390 two DIN enrichment treatments were negative in the biofilms developed in the three
391 streams with intermediate ambient DIN concentrations (Tukey HSD tests, $p < 0.030$;
392 Fig. 4b) and null in the two streams located in the extremes of the DIN gradient (Tukey
393 HSD tests, $p < 0.005$; Fig. 4b). On average, the RRs of algal accrual rates were
394 significantly more negative in $+NH_4^+$ than in $+NO_3^-$ treatments (mean = −0.42 and
395 −0.09, respectively; Fig. 4b; Table 3). The RRs of algal accrual rates for both $+NO_3^-$
396 and $+NH_4^+$ treatments were not correlated with either ambient NO_3^- or NH_4^+
397 concentration among streams.

The RRs of the biofilm C:N molar ratio were consistently negative across the streams and for both $+NH_4^+$ and $+NO_3^-$ treatments. Thus, biofilms exposed to DIN enrichments increased their N content relative to their C content. Differences in RRs of C:N were significant among streams, but not between $+NO_3^-$ and $+NH_4^+$ treatments (Fig. 4c; Table 3). The responses to DIN enrichments were more negative in biofilms developed in GUA (Tukey HSD tests, $p < 0.005$).

The RRs of U_{spec}-NO_3^- for biofilms and DIN species enrichments differed significantly depending on the stream in which the biofilms had developed and between $+NO_3^-$ and $+NH_4^+$ treatments (Fig. 5a; Table 4). The interaction between the two factors was also significant (Table 4). The reason for the interaction was the RR of U_{spec}-NO_3^- was null in biofilms grown in $+NO_3^-$ treatments and particularly negative for biofilms grown in $+NH_4^+$ treatments in 4 of the 5 sites (Fig. 5a). However, the pattern was different in GUA. Overall patterns suggest lower biomass-specific uptake of NO_3^- when biofilms are exposed to NH_4^+ enrichment.

The RRs of U_{spec}-NH_4^+ for biofilms developed in different streams and DIN species enrichments were similar regardless of the stream considered and the NDS treatment at which they developed (Fig. 5b; Table 4). In general, the RRs of U_{spec}- NH_4^+ were negative, but lower than the RRs of U_{spec}-NO_3^-, indicating a lower effect of DIN enrichments on U_{spec}- NH_4^+ than on U_{spec}-NO_3^-.

The RRs of biofilm RPI differed significantly depending on the stream in which the NDS were incubated and between $+NO_3^-$ and $+NH_4^+$ treatments, with no significant interaction between factors (Fig. 5c; Table 4). The exceptions were COL (both solutes) and CAS (NH_4^+). However, despite these differences, the RRs of RPI were not different from 0 in 7 out of 10 cases (Fig. 5c), indicating no overall preference for any of the two DIN species.

Discussion

Biofilm responses to ambient DIN variability

We expected that differences in ambient NO_3^- and NH_4^+ concentrations among the streams in which the NDS were incubated would affect biofilm development and its N demand from the water column. Specifically, we expected that biofilm growth and DIN uptake would be greater in those biofilms that had developed in streams with higher ambient DIN availability (Dodds et al. 2002; O'Brien et al. 2007; von Schiller et al. 2007; O'Brien and Dodds 2008). We observed that streams with higher ambient NO_3^- and NH_4^+ concentrations showed greater biofilm biomass and algal accrual rates, supporting our expectations and suggesting that biofilms development and its contribution to stream water DIN uptake is enhanced under higher availability of DIN. In addition, DIN was below saturating levels and biofilms were likely not limited by other factors. On the other hand, lack of significant variation in the biofilm C:N ratios at ambient levels suggests that the range of ambient DIN concentration was not broad enough to cause significant stoichiometric differences in the biofilms among the studied streams (Dodds et al. 2004).

Biofilm U_{spec}-NO_3^- was consistently greater than U_{spec}-NH_4^+ regardless of the differences in the concentrations of the two DIN species among the study streams, suggesting that biofilms have a consistently higher reliance on NO_3^- than on NH_4^+ from the water column to meet their N requirements. Our results are in line with previous studies showing that the generally higher NO_3^- availability as a DIN source ultimately drives the use of this DIN species by biofilms to meet their N demand (Fellows et al. 2006; Newbold et al. 2006; Bunch and Bernot 2012). RPI values close to 1, indicating no preference for either DIN species, support this explanation. These results contrast the general idea that microbial assemblages in biofilms preferentially remove NH_4^+ due to

448 the lower energetic cost (Dortch 1990; Naldi and Wheeler 2002). However, the results
449 are in line with empirical data from a previous study which showed an unclear pattern
450 of biofilm preference for NH_4^+ relative to NO_3^- availability (Hoellein et al. 2010).
451 According to previous studies (O'Brien et al. 2007; von Schiller et al. 2007), we
452 expected that variability in U_{spec} of the two DIN species among biofilms would be
453 positively related to differences in ambient DIN concentration of the streams in which
454 the NDS were previously incubated. However, the results did not support our
455 expectations. Greater U_{spec}-NO_3^- was observed in biofilms that developed in 2 of the 3
456 streams with the lowest NO_3^- concentrations, and no differences among streams in
457 biofilm U_{spec}-NH_4^+ were found. In fact, we observed lower biofilm U_{spec}-NO_3^- in
458 streams with higher NH_4^+ concentration, which supports previous studies indicating that
459 NH_4^+ availability may regulate the uptake of DIN in the form of NO_3^- (Gonzalez et al.
460 2006; Dugdale et al. 2007; Domingues et al. 2011). The low range of variation in NH_4^+
461 concentration among streams where biofilms developed (from 14 to 22 µg N/L) may
462 have precluded observing differences in U_{spec}-NH_4^+, despite previous studies have
463 shown that a broader range in the concentration of NH_4^+ can control NH_4^+ uptake rates
464 at whole-reach scale (Dodds et al. 2002; O'Brien and Dodds 2008). Alternatively, lack
465 of U_{spec}-NH_4^+ variation among biofilms developed in the different streams also suggests
466 that biofilms NH_4^+ turnover was similar among streams, regardless of the differences in
467 biomass accrual and algal growth observed, probably due to the lower range of NH_4^+
468 concentration among streams.
469 Variation in biomass accrual rates among streams was positively related to algal
470 accrual rates, indicating that algae had a similar response to that of the bulk biofilm. In
471 this context, the negative correlation between algal accrual rates and U_{spec}-NO_3^-,
472 contrasts with other studies indicating that algae in biofilms rely mostly on NO_3^-

(Bernhardt et al. 2002; Bechtold et al. 2012). It is worth noting that the streams where the NDS were incubated were heavily shaded by riparian vegetation, which may have limited N demand, especially by algae in biofilms (Hill et al. 1995; Sabater et al. 2000; von Schiller et al. 2007). Therefore, it is possible that light-limitation may have masked the effects of other factors such as variation in DIN concentration or relative availability between DIN and SRP among streams, on algal uptake (von Schiller et al. 2007).

Biofilm responses to enrichments in NO_3^- or NH_4^+

We expected a positive response of biofilms to NO_3^- and NH_4^+ enrichments if these DIN species were below saturation under ambient conditions within each stream and if other environmental conditions were favorable. In addition, we expected that the biofilm responses would be more positively pronounced for NH_4^+ than for NO_3^- enrichments because biofilms have a higher preference for the former DIN species. However, we found that biofilm response to either NO_3^- or NH_4^+ enrichments was in general either null or negative for most of the investigated variables, suggesting that biofilms were either above DIN saturation at the ambient conditions at which they developed or that the experimental enrichments affected the structure or the species composition of the biofilms leading to lower biomass accrual rates. Furthermore, algal accrual, U_{spec}-NO_3^- and RPI response ratios were consistently more negative in those biofilms that developed under NH_4^+ enriched conditions compared to NO_3^- enriched conditions, suggesting a differential effect of the two DIN species on biofilm development and biogeochemical activity.

The negative response to DIN enrichments was more pronounced for algal accrual than for bulk biomass accrual. This may be explained by the low light availability (i.e., closed canopy reaches) during the experiments, which had a higher

498 constrain on algal development in biofilms than on whole-bulk biofilm biomass.
499 Interestingly, we also observed that the negative responses of algal growth were more
500 pronounced in $+NH_4^+$ than in $+NO_3^-$ treatments. Instances of lower biofilm and algal
501 growth in DIN-enriched substrates with respect to control treatments are relatively
502 common in the literature (Francoeur 2001; Tank and Dodds 2003; Bernhardt and Likens
503 2004; von Schiller et al. 2007), although these studies have mainly focussed on NO_3^-
504 enrichments. Several mechanisms have been proposed to explain this response: (1)
505 preference of grazing invertebrates for biofilms developed on nutrient-rich substrates,
506 (2) nutrient enrichment up to toxic levels, or (3) changes in the species composition of
507 biofilms (Bernhardt and Likens 2004; Hoellein et al. 2010; Domingues et al. 2011).
508 Despite field observations during both NDS incubations confirmed low presence of
509 grazers on NDS filters, we cannot rule out invertebrates as responsible for differences in
510 biomass accrual between control and DIN-enriched substrates (Steinman 1996).
511 Furthermore, we cannot exclude the fact that $+NH_4^+$ treatments lead to toxic effects
512 (Camargo and Alonso 2006) or that either NO_3^- or NH_4^+ enrichments lead to changes in
513 biofilm assemblage composition because the experiment was not aimed to provide these
514 mechanistic results. Future research could explore the possible toxic effect of NH_4^+
515 enrichments by exploring responses using NDS across streams with a wider gradient of
516 ambient NH_4^+ concentrations.
517 The most relevant biofilm responses to enrichment of the two DIN species were
518 observed for N uptake. In absolute terms, the negative response observed was greater
519 for U_{spec}-NO_3^- than for U_{spec}-NH_4^+ and mostly associated with NH_4^+ enrichments. NO_3^-
520 enrichment caused only minor changes in either U_{spec}-NO_3^- or U_{spec}-NH_4^+ when
521 compared with NH_4^+enrichment. Based on our results, we suggest that biofilm
522 exposures to NH_4^+ enrichment may induce some functional and/or structural changes in

523 the biofilms resulting in a lower demand for NO_3^-. In addition, NH_4^+ enrichments might
524 have enhanced NH_4^+ sorption and internal N cycling within the biofilms; thereby
525 decreasing the biofilm NO_3^- dependence from the water column (von Schiller et al.
526 2007). An alternative explanation is that the enrichment of NH_4^+ can favor the
527 development of nitrifiers, which is supported by results from previous studies
528 (Bernhardt and Likens 2004; Merbt et al. 2014). Nitrifying microorganisms have lower
529 growth efficiencies compared to other microbial components of the biofilms (Risgaard-
530 Petersen et al. 2004) and they also have a preferential demand for NH_4^+. This potential
531 shift in the microbial composition of biofilms could at least partially explain the more
532 negative effects on U_{spec}-NO_3^- in NH_4^+ enrichments consistently observed for biofilms
533 developed in all streams studied. Future studies following NH_4^+ enrichment in NDS
534 would benefit from measurements of nitrification activity or community composition to
535 elucidate the underlying mechanism driving the observed biofilm response.
536
537 **Conclusions**
538 NDS bioassays have been commonly used to assess nutrient limitation of P and N in a
539 large variety of freshwater environments (Francoeur 2001; Johnson et al. 2009; Keck
540 and Lepori 2012; King et al. 2014). However, NDS have rarely been employed to
541 address other ecologically relevant questions, such as to contrast biofilm responses to
542 different DIN species (but see von Schiller et al. 2007 and Hoellein et al. 2010). In
543 addition, studies using NDS have mostly focused on the biofilm response in terms of
544 biomass accrual, and less attention has been paid on how the nutrient enrichments affect
545 biofilm function, such as the demand of nutrients from the water column. In this regard,
546 we found that the most relevant biofilm responses to enrichment of the two DIN species
547 were observed for N uptake, and more specifically, that NH_4^+ enrichments caused a

clear decrease in U_{spec}-NO_3^-. Knowledge on these responses provides a better understanding of the effects of elevated DIN availability on biofilm development and contribution to in-stream N uptake. We suggest that biofilms developing in streams with high NO_3^- concentration, such as those draining agricultural catchments (Stanley and Maxted 2008; Lassaletta et al. 2009) may have a limited capacity to retain excess NO_3^-. On the other hand, biofilms developing in streams with low NO_3^-:NH_4^+ ratios due to inputs of NH_4^+-rich sources, such as streams receiving wastewater treatment plant effluents (Marti et al. 2004; Martí et al. 2010), may show decreases in the capacity for NO_3^- uptake. Biofilm responses to increases in the concentration of the DIN species, which can be driven by land use changes, may have relevant implications for the export of DIN to downstream ecosystems.

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571 **References**

572 APHA (1995) Standard methods for the examination of water and wastewater.19th edn.
573 American Public Health Association, Washington.

574 Battin TJ, Kaplan LA, Newbold JD, Hansen CME (2003) Contributions of microbial
575 biofilms to ecosystem processes in stream mesocosms. Nature 426:439-442
576 doi:10.1038/nature02152

577 Bechtold HA, Marcarelli AM, Baxter CV, Inouye RS (2012) Effects of N, P, and
578 organic carbon on stream biofilm nutrient limitation and uptake in a semi-arid
579 watershed. Limnol Oceanogr 57:1544-1554 doi:10.4319/lo.2012.57.5.1544

580 Bernhardt ES, Hall RO, Likens GE (2002) Whole-system estimates of nitrification and
581 nitrate uptake in streams of the Hubbard Brook Experimental Forest.
582 Ecosystems 5:419-430 doi:10.1007/s10021-002-0179-4

583 Bernhardt ES, Likens GE (2004) Controls on periphyton biomass in heterotrophic
584 streams. Freshw Biol 49:14-27 doi:10.1046/j.1365-2426.2003.01161.x

585 Bernot MJ, Tank JL, Royer TV, David MB (2006) Nutrient uptake in streams draining
586 agricultural catchments of the midwestern United States. Freshw Biol 51:499-
587 509 doi:10.1111/j.1365-2427.2006.01508.x

588 Bunch ND, Bernot MJ (2012) Nitrate and ammonium uptake by natural stream
589 sediment microbial communities in response to nutrient enrichment. Res
590 Microbiol 163:137-141 doi:10.1016/j.resmic.2011.11.004

591 Camargo JA, Alonso A (2006) Ecological and toxicological effects of inorganic
592 nitrogen pollution in aquatic ecosystems: A global assessment. Environ Int
593 32:831-849 doi:10.1016/j.envint.2006.05.002

594 Dodds WK, Lopez AJ, Bowden WB, Gregory S, Grimm NB, Hamilton SK, Hershey
595 AE, Marti E, Mcdowell WH, Meyer JL, Morrall D, Mulholland PJ, Peterson BJ.,
596 Tank JL, Valett HM, Webster JR. & Wollheim W (2002) N uptake as a function
597 of concentration in streams. J N Am Benthol Soc 21:206-220

598 Dodds WK, Marti E, Tank JL, Pontius J, Hamilton SK, Grimm NB, Bowden WB,
599 Mcdowell WH, Peterson BJ, Valett HM, Webster JR, Gregory S (2004) Carbon
600 and nitrogen stoichiometry and nitrogen cycling rates in streams. Oecologia
601 140:458-467 doi:10.1007/s00442-004-1599-y

602 Dodds WK, Welch EB (2000) Establishing nutrient criteria in streams. J N Am Benthol
603 Soc 19:186-196

604 Domingues RB, Barbosa AB, Sommer U, Galvao HM (2011) Ammonium, nitrate and
605 phytoplankton interactions in a freshwater tidal estuarine zone: potential effects
606 of cultural eutrophication. Aquat Sci 73:331-343 doi:10.1007/s00027-011-0180-
607 0

608 Dortch Q (1990) The interaction between ammonium and nitrate uptake in
609 phytoplankton. mar ecol-prog ser 61:183-201 doi:10.3354/meps061183

610 Dugdale RC, Wilkerson FP, Hogue VE, Marchi A (2007) The role of ammonium and
611 nitrate in spring bloom development in San Francisco Bay Estuar. Coast Shelf
612 Sci 73:17-29 doi:10.1016/j.ecss.2006.12.008

613 Fellows CS, Valett HM, Dahm CN, Mulholland PJ, Thomas SA (2006) Coupling
614 nutrient uptake and energy flow in headwater streams. Ecosystems 9:788-804
615 doi:10.1007/s10021-006-0005-5

616 Francoeur SN (2001) Meta-analysis of lotic nutrient amendment experiments: detecting
617 and quantifying subtle responses. J N Am Benthol Soc 20:358-368
618 doi:10.2307/1468034

619 Geisseler D, Horwath WR, Joergensen RG, Ludwig B (2010) Pathways of nitrogen
620 utilization by soil microorganisms - A review. Soil Biol Biochem 42:2058-2067
621 doi:10.1016/j.soilbio.2010.08.021

622 Gonzalez PJ, Correia C, Moura I, Brondino CD, Moura JJG (2006) Bacterial nitrate
623 reductases: Molecular and biological aspects of nitrate reduction. J Inorg
624 Biochem 100:1015-1023 doi:10.1016/j.jinorgbio.2005.11.024

625 Gordon ND, McMahon TA, Finlayson BL (1992) Stream hydrology: an introduction for
626 ecologists. Stream hydrology: an introduction for ecologists. John Wiley &
627 Sons,

628 Hill WR, Ryon MG, Schilling EM (1995) Light limitation in a stream ecosystem -
629 responses by primary producers and consumers. Ecology 76:1297-1309
630 doi:10.2307/1940936

631 Hoellein TJ, Tank JL, Kelly JJ, Rosi-Marshall EJ (2010) Seasonal variation in nutrient
632 limitation of microbial biofilms colonizing organic and inorganic substrata in
633 streams. Hydrobiologia 649:331-345 doi:10.1007/s10750-010-0276-x

634 Holmes RM, McClelland JW, Sigman DM, Fry B, Peterson BJ (1998) Measuring N-15-
635 NH4+ in marine, estuarine and fresh waters: An adaptation of the ammonia
636 diffusion method for samples with low ammonium concentrations. Marine
637 Chemistry 60:235-243

638 Johnson LT, Tank JL, Dodds WK (2009) The influence of land use on stream biofilm
639 nutrient limitation across eight North American ecoregions Can J Fish Aquat Sci
640 66:1081-1094 doi:10.1139/f09-065

641 Keck F, Lepori F (2012) Can we predict nutrient limitation in streams and rivers?
642 Freshw Biol 57:1410-1421 doi:10.1111/j.1365-2427.2012.02802.x

643 Kemp MJ, Dodds WK (2002) The influence of ammonium, nitrate, and dissolved
644 oxygen concentrations on uptake, nitrification, and denitrification rates
645 associated with prairie stream substrata. Limnol Oceanogr 47:1380-1393

646 King SA, Heffernan JB, Cohen MJ (2014) Nutrient flux, uptake, and autotrophic
647 limitation in streams and rivers. Freshw Sci 33:85-98 doi:10.1086/674383

648 Lassaletta L, Garcia-Gomez H, Gimeno BS, Rovira JV (2009) Agriculture-induced
649 increase in nitrate concentrations in stream waters of a large Mediterranean
650 catchment over 25 years (1981-2005). Sci Total Environ 407:6034-6043
651 doi:10.1016/j.scitotenv.2009.08.002

652 Marti E, Aumatell J, Gode L, Poch M, Sabater F (2004) Nutrient retention efficiency in
653 streams receiving inputs from wastewater treatment plants. Journal of
654 Environmental Quality 33:285-293

655 Martí E, Riera J, Sabater F (2010) Effects of Wastewater Treatment Plants on Stream
656 Nutrient Dynamics Under Water Scarcity Conditions. In: Sabater S, Barceló D
657 (eds) Water Scarcity in the Mediterranean, vol 8. The Handbook of
658 Environmental Chemistry. Springer Berlin / Heidelberg, pp 173-195.
659 doi:10.1007/698_2009_33

660 McCarty GW (1995) the role of glutamine-synthetase in regulation of nitrogen-
661 metabolism within the soil microbial community plant and soil 170:141-147
662 doi:10.1007/bf02183062

663 McIntire CD, Gregory SV, Steinman AD, Lamberti GA (1996) Modeling benthic algal
664 communities: an example from stream ecology. Algal ecology: freshwater
665 benthic ecosystems. Academic Press. doi:10.1016/b978-012668450-6/50050-3

666 Merbt S, Auguet J-C, Blesa A, Martí E, Casamayor E (2015) Wastewater Treatment
667 Plant Effluents Change Abundance and Composition of Ammonia-Oxidizing
668 Microorganisms in Mediterranean Urban Stream Biofilms Microb Ecol 69:66-74
669 doi:10.1007/s00248-014-0464-8

670 Naldi M, Wheeler PA (2002) N-15 measurements of ammonium and nitrate uptake by
671 Ulva fenestrata (chlorophyta) and Gracilaria pacifica (rhodophyta): Comparison
672 of net nutrient disappearance, release of ammonium and nitrate, and N-15
673 accumulation in algal tissue. J Phycol 38:135-144 doi:10.1046/j.1529-
674 8817.2002.01070.x

675 Newbold JD, Bott TL, Kaplan LA, Dow CL, Jackson JK, Aufdenkampe AK, Martin
676 LA, Van Horn DJ, De Long AA (2006) Uptake of nutrients and organic C in
677 streams in New York City drinking-water-supply watersheds. J N Am Benthol
678 Soc 25:998-1017

679 O'Brien JM, Dodds WK (2008) Ammonium uptake and mineralization in prairie
680 streams: chamber incubation and short-term nutrient addition experiments.
681 Freshw Biol 53:102-112 doi:10.1111/j.1365-2427.2007.01870.x

682 O'Brien JM, Dodds WK, Wilson KC, Murdock JN, Eichmiller J (2007) The saturation
683 of N cycling in Central Plains streams: N-15 experiments across a broad
684 gradient of nitrate concentrations. Biogeochemistry 84:31-49
685 doi:10.1007/s10533-007-9073-7

686 Pusch M et al. (1998) The role of micro-organisms in the ecological connectivity of
687 running waters. Freshw Biol 40:453-495 doi:10.1046/j.1365-2427.1998.00372.x

688 Ribot M, von Schiller D, Peipoch M, Sabater F, Grimm NB, Marti E (2013) Influence
689 of nitrate and ammonium availability on uptake kinetics of stream biofilms.
690 Freshw Sci 32:1155-1167 doi:10.1899/12-209.1

691 Risgaard-Petersen N, Nicolaisen MH, Revsbech NP, Lomstein BA (2004) Competition
692 between ammonia-oxidizing bacteria and benthic microalgae. Appl Environ
693 Microbiol 70:5528-5537 doi:10.1128/aem.70.9.5528-5537.2004

694 Sabater F, Butturini A, Marti E, Munoz I, Romani A, Wray J, Sabater S (2000) Effects
695 of riparian vegetation removal on nutrient retention in a Mediterranean stream. J
696 N Am Benthol Soc 19:609-620 doi:10.2307/1468120

697 Sigman DM, Altabet MA, Michener R, McCorkle DC, Fry B, Holmes RM (1997)
698 Natural abundance-level measurement of the nitrogen isotopic composition of
699 oceanic nitrate: an adaptation of the ammonia diffusion method. Marine
700 Chemistry 57:227-242

701 Stanley EH, Maxted JT (2008) Changes in the dissolved nitrogen pool across land cover
702 gradients in Wisconsin streams. Ecol Appl 18:1579-1590 doi:10.1890/07-1379.1

703 Steinman AD (1996) Effects of grazers on freshwater benthic algae. Algal ecology:
704 freshwater benthic ecosystems. doi:10.1016/b978-012668450-6/50041-2

705 Tank JL, Dodds WK (2003) Nutrient limitation of epilithic and epixylic biofilms in ten
706 North American streams. Freshw Biol 48:1031-1049 doi:10.1046/j.1365-
707 2427.2003.01067.x

708 von Schiller D, Marti E, Riera JL, Ribot M, Marks JC, Sabater F (2008) Influence of
709 land use on stream ecosystem function in a Mediterranean catchment. Freshw
710 Biol 53:2600-2612 doi:10.1111/j.1365-2427.2008.02059.x

711 von Schiller D, Marti E, Riera JL, Sabater F (2007) Effects of nutrients and light on
712 periphyton biomass and nitrogen uptake in Mediterranean streams with
713 contrasting land uses. Freshw Biol 52:891-906 doi:10.1111/j.1365-
714 2427.2007.01742.x

715 Zar JH (1996) Biostatistical analysis. 3rd edition. Prentice–Hall, Upper Saddle River,
716 New Jersey.

717 Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM (2009) Mixed effects models
718 and extensions in ecology with R (Statistics for biology and health). Springer
719 Science+Business Media, LLC2009.

720

721 **Tables**

722 **Table 1.** Physical and chemical characteristics of the streams in which the nutrient

723 diffusing substrata (NDS) were incubated.

	Font del Regàs	Castanyet	Santa Fe del Montseny	Gualba	Santa Coloma
Stream code	FR	CAS	MON	GUA	COL
Forested area (%)	99.7	99.6	99.4	96.0	92.6
Urban area (%)	0.0	0.0	0.0	0.6	3.7
Agricultural area (%)	0.2	0.4	0.0	2.1	3.4
Longitude 2º E	27'00''	37'25''	27'42''	30'17''	39'32''
Latitude 41º N	49'32''	53'28''	46'37''	44'02''	51'48''
Mean altitude (m)	429	572	1419	940	554
Discharge (L s^{-1})	21.7 ± 4.4	2.5 ± 1.4	9.3 ± 0.5	11.2 ± 3.1	11.5 ± 4.5
Water temperature (ºC)	16.6 ± 0.4	19.8 ± 0.9	14.2 ± 0.8	19.8 ± 0.9	21.4 ± 1.0
Conductivity (µS cm^{-1})	198.0 ± 3.2	214.0 ± 10	60.6 ± 0.4	123.9 ± 7.7	309.7 ± 8.8
NH_4^+ (µg N L^{-1})	14 ± 3	19 ± 2	16 ± 3	17 ± 3	22 ± 1
NO_3^- + NO_2^- (µg N L^{-1})	144 ± 33	140 ± 85	189 ± 23	270 ± 9	600 ± 263
SRP (µg P L^{-1})	4 ± 1	8 ± 5	20 ± 2	20 ± 1	46 ± 39
NO_3^-:NH_4^+	11.8 ± 3.9	8.0 ± 5.5	12.9 ± 3.4	16.5 ± 2.6	27.7 ± 11.8
DIN:SRP (molar)	95.3 ± 27.7	50.3 ± 6.4	22.9 ± 2.9	32.3 ± 1.8	84.4 ± 33.3

724 Data reported are the mean ± SE of samples collected on three different dates during

725 each of the two NDS incubation periods (n = 6).

726 Note that streams are listed in order of increasing DIN availability (sum of NH_4^+ and

727 NO_3^- concentrations).

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731 **Table 2.** Results from the linear mixed-effects model with stream as fixed factor and
732 incubation as random factor on the biomass accrual rate, algal accrual rate and C:N
733 molar ratio of biofilms in DIN-free treatments.

Variable	*df*	*F*	*p*
Biomass accrual rate			
Stream	4	5.80	**<0.001**
Incubation			0.922
Algal accrual rate			
Stream	4	14.64	**<0.001**
Incubation			0.173
C:N molar ratio			
Stream	4	0.20	0.940
Incubation			0.664

734 Significance of the random factor incubation was obtained with the Likelihood Ratio
735 Test.

736 Values highlighted in bold indicate significant effects ($p < 0.05$).

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749 **Table 3.** Results from the linear mixed-effects model with stream and NDS treatment as
750 fixed factors and incubation as random factor on biofilm growth response ratio (RR) to
751 DIN enrichments in the form of NO_3^- and NH_4^+ among streams in terms of biomass
752 accrual rate, algal accrual rate and C:N molar ratio.

Variable	*df*	*F*	*p*
Biomass accrual rate			
Stream	4	3.99	**0.005**
Treatment	1	0.06	0.813
Stream x treatment	4	0.75	0.558
Incubation			0.150
Algal accrual rate			
Stream	4	10.17	**<0.001**
Treatment	1	13.85	**<0.001**
Stream x treatment	4	2.00	0.101
Incubation			0.221
C:N molar ratio			
Stream	4	5.09	**<0.001**
Treatment	1	0.50	0.483
Stream x treatment	4	0.88	0.480
Incubation			0.734

753 Significance of the random factor incubation was obtained with the Likelihood Ratio
754 Test.

755 Values highlighted in bold indicate significant effects ($p < 0.05$).

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759 **Table 4.** Results from two-way ANOVA with stream and NDS treatment as fixed
760 factors on biofilm uptake response ratio (RR) to DIN enrichments in the form of NO_3^-
761 and NH_4^+ among streams in terms of biomass-specific uptake rate of NO_3^- (U_{spec}-NO_3^-),
762 NH_4^+ (U_{spec}-NH_4^+) and relative preference index (RPI).

	Variable	*df*	*F*	*p*
U_{spec}-NO_3^-				
	Stream	4	9.57	**<0.001**
	Treatment	1	58.13	**<0.001**
	Stream x treatment	4	6.12	**<0.001**
U_{spec}-NH_4^+				
	Stream	4	1.99	0.118
	Treatment	1	1.06	0.311
	Stream x treatment	4	1.92	0.129
RPI				
	Stream	4	5.38	**0.001**
	Treatment	1	4.81	**0.034**
	Stream x treatment	4	2.30	0.075

763 Values highlighted in bold indicate significant effects ($p < 0.05$).

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Figure legends

Figure 1. Biomass accrual rate (**a**), algal accrual rate (**b**) and C:N molar ratio (**c**) of biofilms developed on nutrient diffusing substrata (NDS) for the different streams and nutrient treatments in which the NDS were incubated. Data reported are the mean ± SE.

Figure 2. Relationships between biofilm variables and ambient concentrations of NO_3^- and NH_4^+ in the streams in which the NDS were incubated. Biomass accrual rates and NO_3^- (**a**) or NH_4^+ (**b**), algal accrual rates and NO_3^- (**c**) or NH_4^+ (**d**), and biomass-specific uptake for NO_3^- (U_{spec}-NO_3^-) and NO_3^- (**e**) or NH_4^+ (**f**). Results are for Pearson correlations. Values highlighted in *bold* indicate significant correlations ($p < 0.05$).

Figure 3. Biomass-specific uptake for NO_3^- (U_{spec}-NO_3^-; **a**), for NH_4^+ (U_{spec}-NH_4^+; **b**) and relative preference index (RPI; **c**) of biofilms developed on nutrient diffusing substrata (NDS) in the different streams and nutrient treatments. Note that the y-axis from panel **b** is one order of magnitude lower than that from panel **a**. In panel **c**, the *horizontal dashed line* at *1* denotes the shift from NH_4^+ to NO_3^- preference. Values <1 indicate preference for NH_4^+, whereas values >1 indicate preference for NO_3^-. Data reported are the mean ± SE.

Figure 4. Biofilm growth response ratio (RR) to enrichments of NO_3^- and NH_4^+ in terms of biomass accrual rate (**a**), algal accrual rate (**b**) and C:N molar ratio (**c**) for the different streams in which the nutrient diffusing substrata (NDS) were incubated. Data reported are the mean ± SE.

Figure 5. Biofilm DIN uptake response ratio (RR) to enrichments of NO_3^- and NH_4^+ in terms of biomass-specific uptake for NO_3^- (U_{spec}-NO_3^-; **a**) and for NH_4^+ (U_{spec}-NH_4^+; **b**), and relative preference index (RPI; **c**) for the different streams in which the nutrient diffusing substrata (NDS) were incubated. Data reported are the mean ± SE.

Figure 1

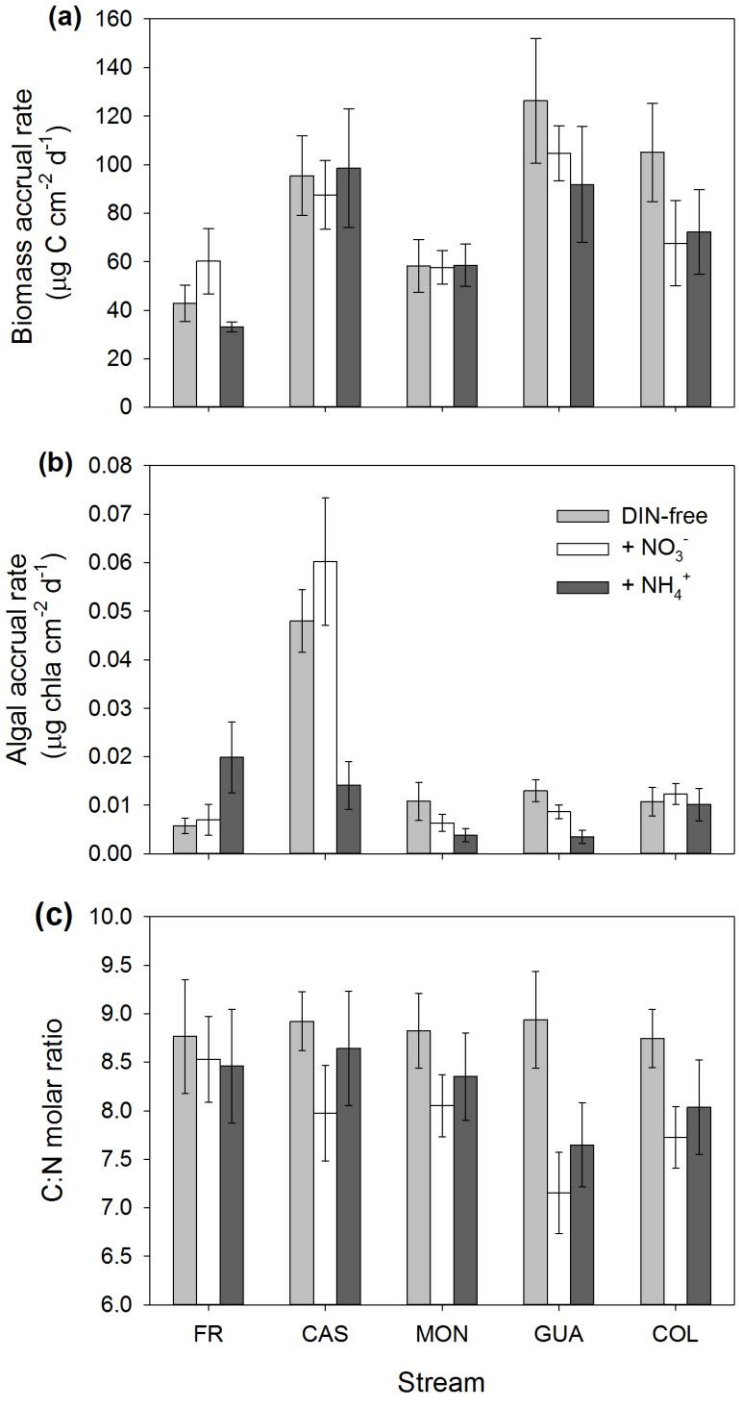

(a)
Biomass accrual rate
(µg C cm-2 d-1)
160
140
120
100
80
60
40
20
0
(b)
Algal accrual rate
(µg chla cm-2 d-1)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
DIN-free
+ NO3-
+ NH4+
(c)
C:N molar ratio
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
FR
CAS
MON
GUA
COL
Stream

Figure 2

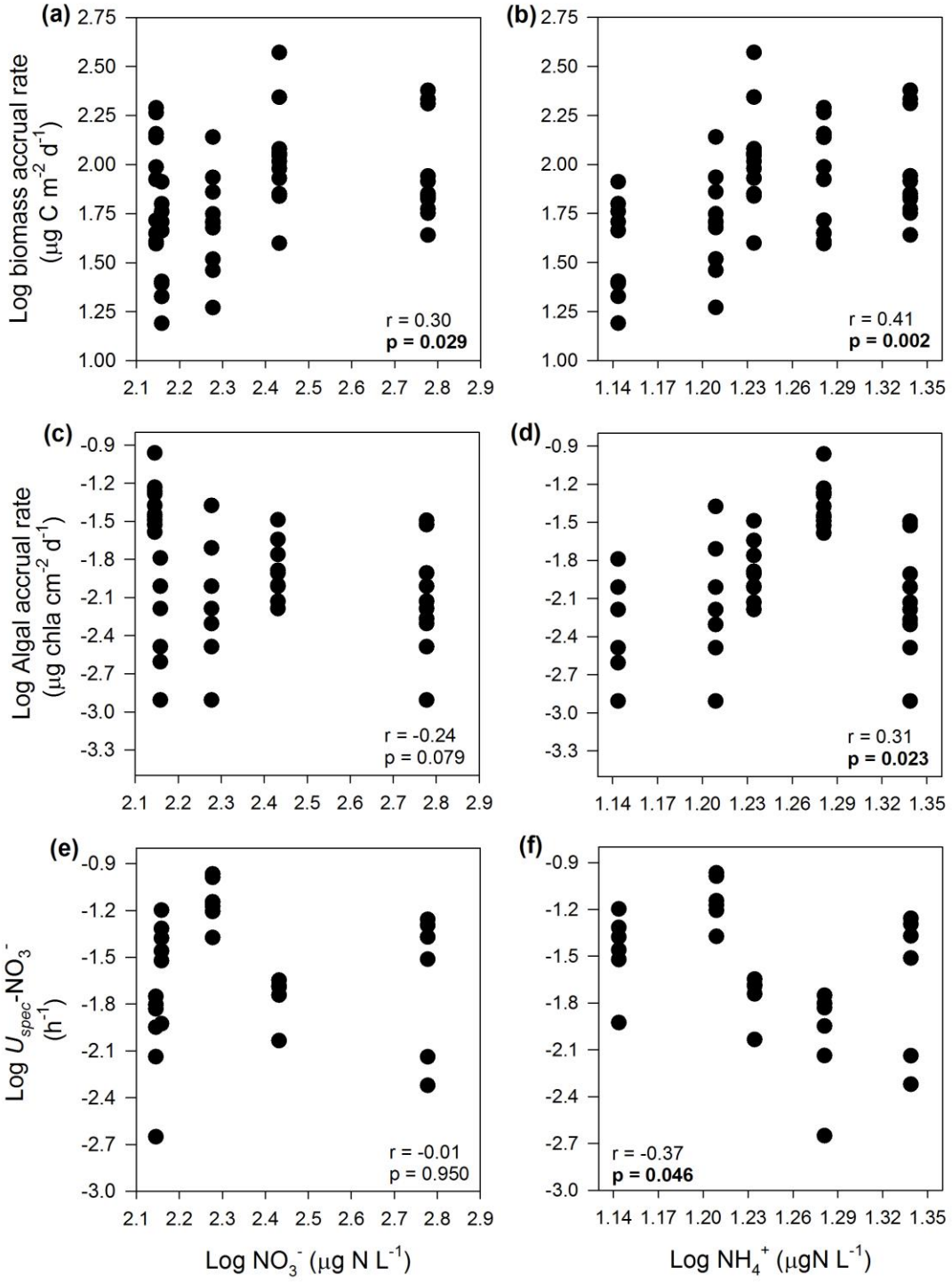

(a)
(b)
(c)
(d)
(e)
(f)
Log biomass accrual rate (µg C m-2 d-1)
Log Algal accrual rate (µg chla cm-2 d-1)
Log U spec -NO3- (h-1)
r = 0.30
p = 0.029
r = 0.41
p = 0.002
r = -0.24
p = 0.079
r = 0.31
p = 0.023
r = -0.01
p = 0.950
r = -0.37
p = 0.046
Log NO3- (µg N L-1)
Log NH4+ (µgN L-1)

Figure 3

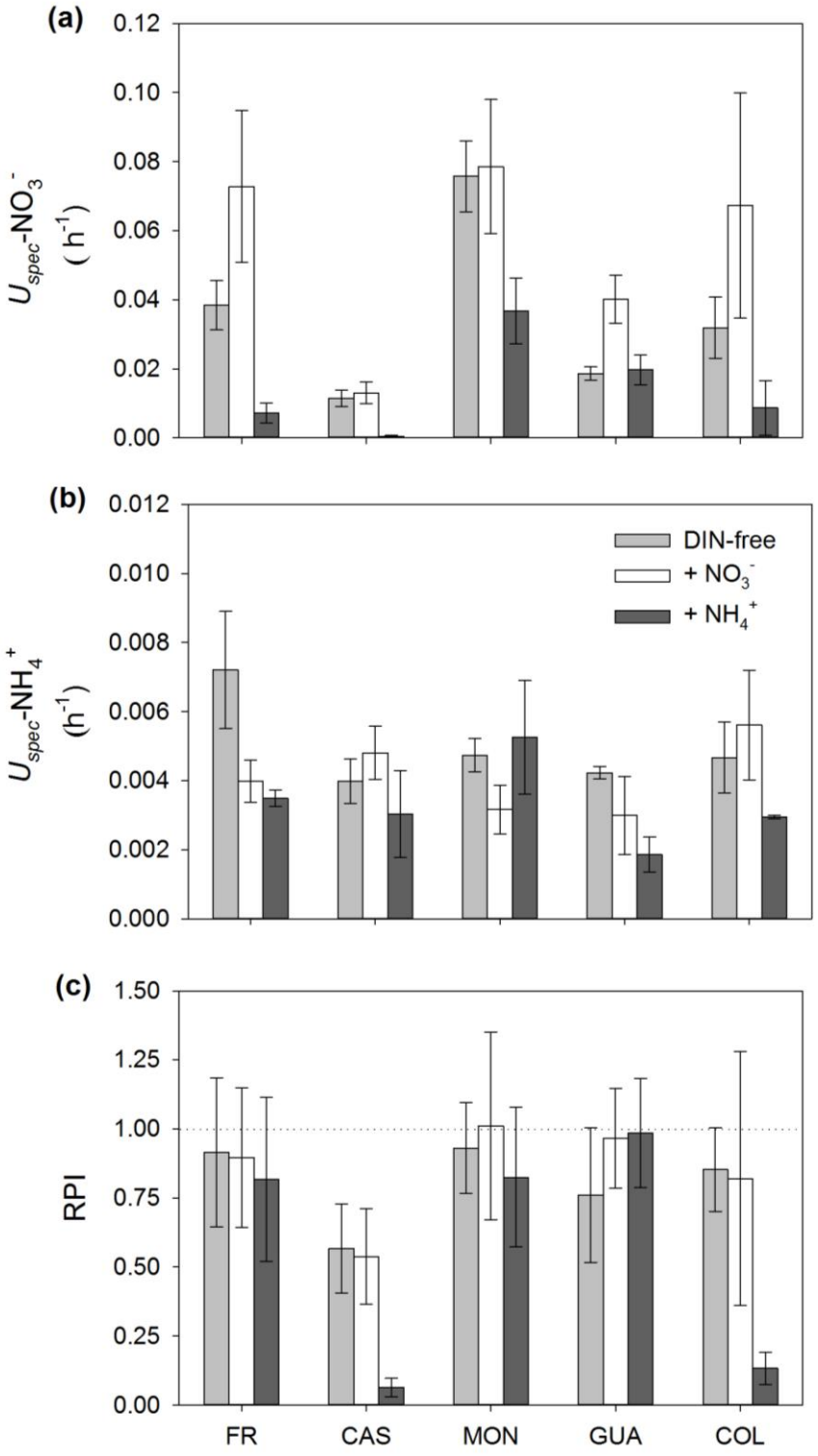

(a)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
U_{spec}-NO_3^- (h^{-1})
(b)
0.012
0.010
0.008
0.006
0.004
0.002
0.000
U_{spec}-NH_4^+ (h^{-1})
DIN-free
+ NO_3^-
+ NH_4^+
(c)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
RPI
FR
CAS
MON
GUA
COL

Figure 4

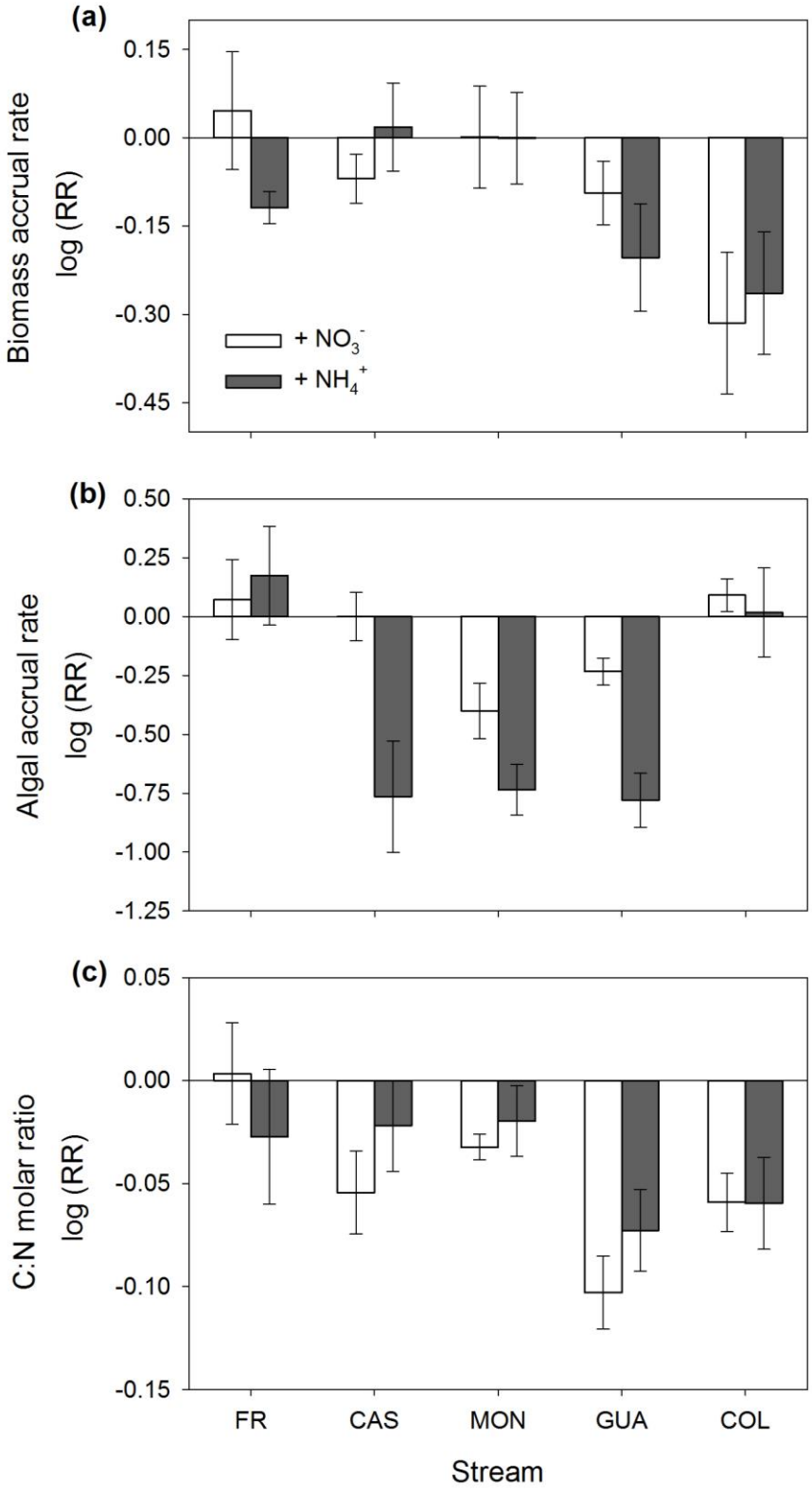
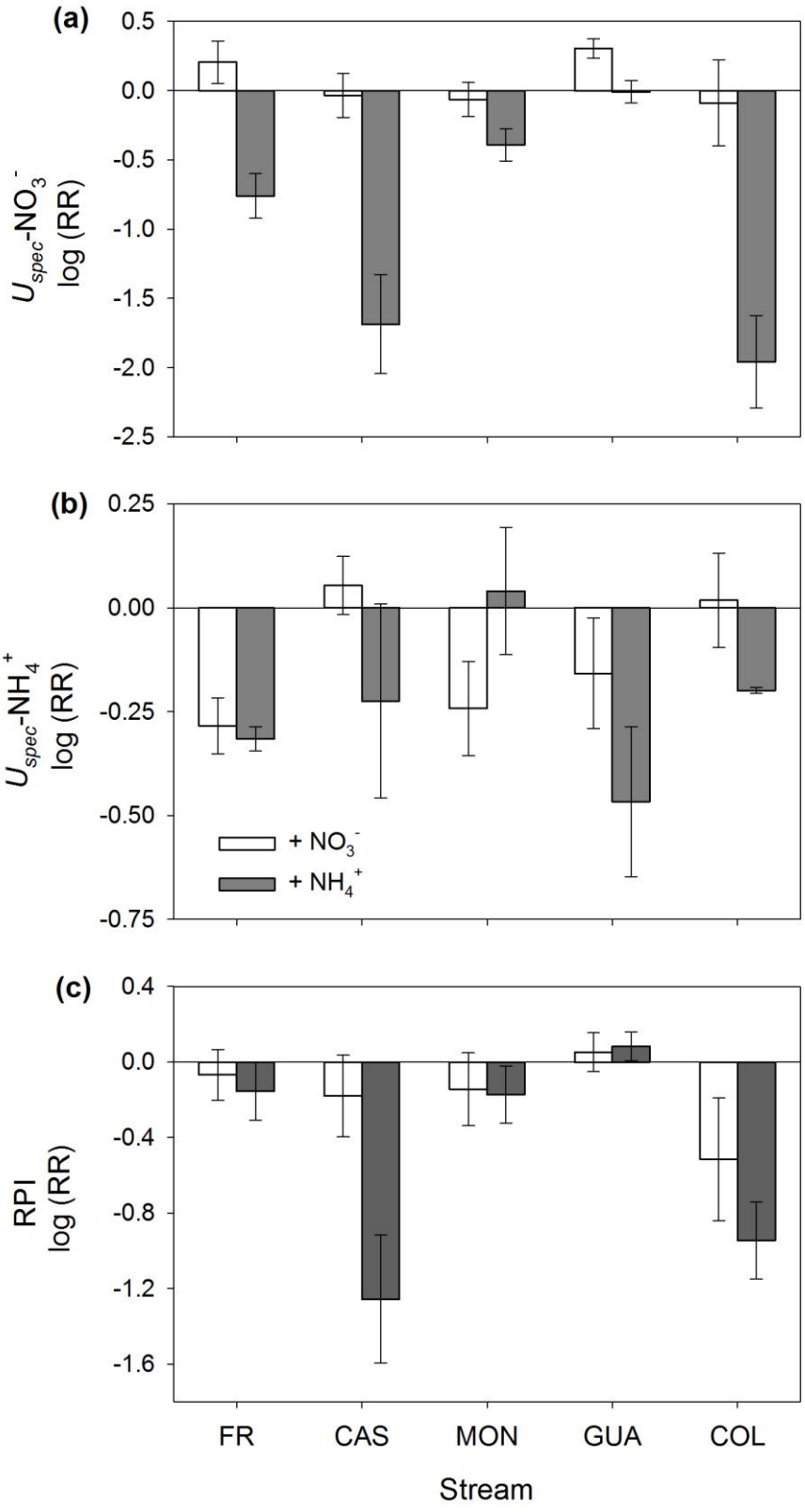

(a)
Biomass accrual rate
log (RR)
0.15
0.00
-0.15
-0.30
-0.45
+ NO3-
+ NH4+
(b)
Algal accrual rate
log (RR)
0.50
0.25
0.00
-0.25
-0.50
-0.75
-1.00
-1.25
(c)
C:N molar ratio
log (RR)
0.05
0.00
-0.05
-0.10
-0.15
FR
CAS
MON
GUA
COL
Stream

Figure 5


(a)
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
U_{spec}-NO_3^-
log (RR)
(b)
0.25
0.00
-0.25
-0.50
-0.75
U_{spec}-NH_4^+
log (RR)
+ NO_3^-
+ NH_4^+
(c)
0.4
0.0
-0.4
-0.8
-1.2
-1.6
RPI
log (RR)
FR
CAS
MON
GUA
COL
Stream