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

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Keywords: nitrogen, biofilm, uptake, ammonium, nitrate, stream

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^-

100 or NH_4^+ in a single location to measure their capacity for DIN assimilation of the two
101 species as well as their relative preference for the uptake of the two DIN species.
102 Comparison of assimilation rates between biofilms under control and DIN amended
103 conditions allowed us to estimate the effect of DIN species enrichments on N
104 assimilation rates of biofilms. We expected that biofilms in streams with higher ambient
105 DIN concentration would have higher growth rates and higher N demand (i.e., higher
106 DIN uptake rates) than those developed in low DIN concentrations if biofilms were not
107 limited by any other environmental factor. In addition, we expected that responses of
108 biofilms to NH_4^+ enrichments would be higher than those to NO_3^- enrichments because
109 of greater energetic cost of NO_3^- assimilation.
110
111 **Methods**
112 **Study sites**
113 La Tordera catchment (Catalonia, NE Spain) has an area of 868.5 km^2 dominated by
114 siliceous geology, and covers a 1700-m altitudinal gradient from the headwaters to the
115 sea level within a 35 km distance. Climate in this region is typically Mediterranean,
116 with warm, dry summers, and mild, humid winters. Although most of the catchment is
117 forested, agricultural, urban and industrial areas tend to concentrate in the river valley,
118 resulting in a heterogeneous land use template along the lowlands of the river network,
119 which affects stream N concentrations (von Schiller et al. 2008). Within this catchment,
120 we selected five streams draining sub-catchments with different land uses. Three sites
121 have forested land-use 99 % of the watersheds, and the other two sites have human
122 land-use (i.e., agriculture + urban) of 2.7 and 7.1 % (Table 1) mostly adjacent to the
123 stream. These streams were selected to cover a wide range of DIN concentration based
124 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

175 measured water conductivity and water temperature with a portable WTW conductivity
176 meter (Weilheim, Germany). In addition, we determined discharge on a single cross-
177 sectional transect by measuring mean wetted width, mean depth and mean water
178 velocity (Gordon et al. 1992).
179
180 **^{15}N constant rate additions**
181 In COL stream, we selected a 250-m reach to run the two ^{15}N additions. In this reach,
182 and prior to the ^{15}N additions, we randomly distributed all NDS from the other four sites
183 along a cross-section located 50 m downstream of the ^{15}N addition point. For each ^{15}N
184 addition (i.e., $^{15}NO_3^-$ and $^{15}NH_4^+$) we prepared a solution amended with either $^{15}NO_3^-$
185 (as 99 % enriched $K^{15}NO_3$) or $^{15}NH_4^+$ (as 99 % enriched $^{15}NH_4Cl$) in conjunction with
186 NaCl, as a conservative tracer. The amount of $K^{15}NO_3$ and $^{15}NH_4Cl$ and the pump flow
187 rate were set to achieve a target $\delta^{15}N$ enrichment of 10,000 ‰ for each DIN species in
188 the water column. We released the ^{15}N solutions at the top of the reach at a constant rate
189 using a Masterflex (Vernon Hills, Illinois, USA) L/S battery-powered peristaltic pump.
190 The two ^{15}N additions started at midnight (00:00) and lasted for 12 h. The $^{15}NO_3^-$
191 addition was run on July 12th and the $^{15}NH_4^+$ addition was run on August 1st.
192 We collected stream water samples at the NDS location for the analysis of the ^{15}N
193 isotopic signature of both DIN species ($^{15}NO_3^-$ and $^{15}NH_4^+$) 24 h prior to the start of the
194 ^{15}N tracer additions and at plateau conditions. To verify plateau conditions during each
195 ^{15}N addition, we automatically recorded conductivity every 10 s at the end of the stream
196 reach using a portable WTW conductivity meter connected to a Campbell Scientific
197 (Logan, Utah, USA) data logger. 24 h after the end of each ^{15}N addition, coinciding
198 with the water collection described above, we also collected the NDS filters, cut them in
199 half and kept them on ice in the field until further laboratory analyses.

200 **Laboratory analyses**

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

206 The other half of the filter was kept frozen until the measurement of chlorophyll-
207 a (chla) content following McIntire et al. (1996). We submerged the frozen half-filters
208 in a known volume of 90 % v/v acetone and kept them in the dark at 4 °C overnight. We
209 then sonicated the filters for 5 min and centrifuged them for 10 min at 4000 rpm. We
210 measured the absorbance of the resultant supernatant at 664, 665 and 750 nm before and
211 after acidification using a Shimadzu (Tokyo, Japan) UV spectrometer.

212 We analyzed water samples for the concentrations of NO_3^-, NH_4^+, and soluble
213 reactive phosphorus (SRP) on a Bran + Luebbe (Norderstedt, Germany) TRAACS 2000
214 autoanalyzer following standard colorimetric methods (APHA 1995). We processed
215 water samples for the analysis of $^{15}NH_4^+$ and $^{15}NO_3^-$ as described in Holmes et al.
216 (1998) and Sigman et al. (1997), respectively. Briefly, for $^{15}NH_4^+$ determination, we
217 amended a known volume of sample with 3 g L^{-1} of MgO and 50 g L^{-1} of NaCl and a
218 Teflon filter packet containing an acidified 1-cm-diameter ashed Whatman GF/D fiber
219 glass filter to trap the volatilized NH_3, and incubated it on a shaker at 40 °C for 4 weeks.
220 For $^{15}NO_3^-$ determination, we amended a known volume of the sample with 3 g of MgO
221 and 5 g of NaCl and boiled it to remove the NH_4^+. We then added 0.5 mg of MgO and
222 0.5 mg Devarda's alloy to reduce the NO_3^- to NH_4^+, and treated the remaining sample
223 as for $^{15}NH_4^+$. We also diffused a set of standards of known volume for volume-related
224 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).
370
371
372

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.

398 The RRs of the biofilm C:N molar ratio were consistently negative across the
399 streams and for both +NH_4^+ and +NO_3^- treatments. Thus, biofilms exposed to DIN
400 enrichments increased their N content relative to their C content. Differences in RRs of
401 C:N were significant among streams, but not between +NO_3^- and +NH_4^+ treatments
402 (Fig. 4c; Table 3). The responses to DIN enrichments were more negative in biofilms
403 developed in GUA (Tukey HSD tests, $p < 0.005$).
404 The RRs of U_{spec}-NO_3^- for biofilms and DIN species enrichments differed
405 significantly depending on the stream in which the biofilms had developed and between
406 +NO_3^- and +NH_4^+ treatments (Fig. 5a; Table 4). The interaction between the two factors
407 was also significant (Table 4). The reason for the interaction was the RR of U_{spec}-NO_3^-
408 was null in biofilms grown in +NO_3^- treatments and particularly negative for biofilms
409 grown in +NH_4^+ treatments in 4 of the 5 sites (Fig. 5a). However, the pattern was
410 different in GUA. Overall patterns suggest lower biomass-specific uptake of NO_3^- when
411 biofilms are exposed to NH_4^+ enrichment.
412 The RRs of U_{spec}-NH_4^+ for biofilms developed in different streams and DIN
413 species enrichments were similar regardless of the stream considered and the NDS
414 treatment at which they developed (Fig. 5b; Table 4). In general, the RRs of U_{spec}- NH_4^+
415 were negative, but lower than the RRs of U_{spec}-NO_3^-, indicating a lower effect of DIN
416 enrichments on U_{spec}- NH_4^+ than on U_{spec}-NO_3^-.
417 The RRs of biofilm RPI differed significantly depending on the stream in which
418 the NDS were incubated and between +NO_3^- and +NH_4^+ treatments, with no significant
419 interaction between factors (Fig. 5c; Table 4). The exceptions were COL (both solutes)
420 and CAS (NH_4^+). However, despite these differences, the RRs of RPI were not different
421 from 0 in 7 out of 10 cases (Fig. 5c), indicating no overall preference for any of the two
422 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^-

473 (Bernhardt et al. 2002; Bechtold et al. 2012). It is worth noting that the streams where
474 the NDS were incubated were heavily shaded by riparian vegetation, which may have
475 limited N demand, especially by algae in biofilms (Hill et al. 1995; Sabater et al. 2000;
476 von Schiller et al. 2007). Therefore, it is possible that light-limitation may have masked
477 the effects of other factors such as variation in DIN concentration or relative availability
478 between DIN and SRP among streams, on algal uptake (von Schiller et al. 2007).
479
480 **Biofilm responses to enrichments in NO_3^- or NH_4^+**
481 We expected a positive response of biofilms to NO_3^- and NH_4^+ enrichments if these
482 DIN species were below saturation under ambient conditions within each stream and if
483 other environmental conditions were favorable. In addition, we expected that the
484 biofilm responses would be more positively pronounced for NH_4^+ than for NO_3^-
485 enrichments because biofilms have a higher preference for the former DIN species.
486 However, we found that biofilm response to either NO_3^- or NH_4^+ enrichments was in
487 general either null or negative for most of the investigated variables, suggesting that
488 biofilms were either above DIN saturation at the ambient conditions at which they
489 developed or that the experimental enrichments affected the structure or the species
490 composition of the biofilms leading to lower biomass accrual rates. Furthermore, algal
491 accrual, U_{spec}-NO_3^- and RPI response ratios were consistently more negative in those
492 biofilms that developed under NH_4^+ enriched conditions compared to NO_3^- enriched
493 conditions, suggesting a differential effect of the two DIN species on biofilm
494 development and biogeochemical activity.
495 The negative response to DIN enrichments was more pronounced for algal
496 accrual than for bulk biomass accrual. This may be explained by the low light
497 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

548 clear decrease in U_{spec}-NO_3^-. Knowledge on these responses provides a better
549 understanding of the effects of elevated DIN availability on biofilm development and
550 contribution to in-stream N uptake. We suggest that biofilms developing in streams with
551 high NO_3^- concentration, such as those draining agricultural catchments (Stanley and
552 Maxted 2008; Lassaletta et al. 2009) may have a limited capacity to retain excess NO_3^-.
553 On the other hand, biofilms developing in streams with low NO_3^-:NH_4^+ ratios due to
554 inputs of NH_4^+-rich sources, such as streams receiving wastewater treatment plant
555 effluents (Marti et al. 2004; Martí et al. 2010), may show decreases in the capacity for
556 NO_3^- uptake. Biofilm responses to increases in the concentration of the DIN species,
557 which can be driven by land use changes, may have relevant implications for the export
558 of DIN to downstream ecosystems.
559

Acknowledgments

We thank M. Martí and S. Pla for their field and laboratory assistance. We are also grateful to the to the Font del Regàs landowners, Massaneda Garden and the Direcció del Parc Natural del Montseny (Diputació de Barcelona) for allowing access to the study sites during the experiments. This study was funded by the Spanish Ministry of Education and Science through NICON project (ref: CGL2005-7362). MR was supported by a contract with the Spanish Ministry of Science and Innovation through the MED_FORSTREAM project (CGL2011-30590-C02-02). DvS's work was also funded by a Juan de la Cierva postdoctoral contract (JCI-2010-06397) from the Spanish Ministry of Science and Innovation.

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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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758

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 ($p < 0.05$).

764

765

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

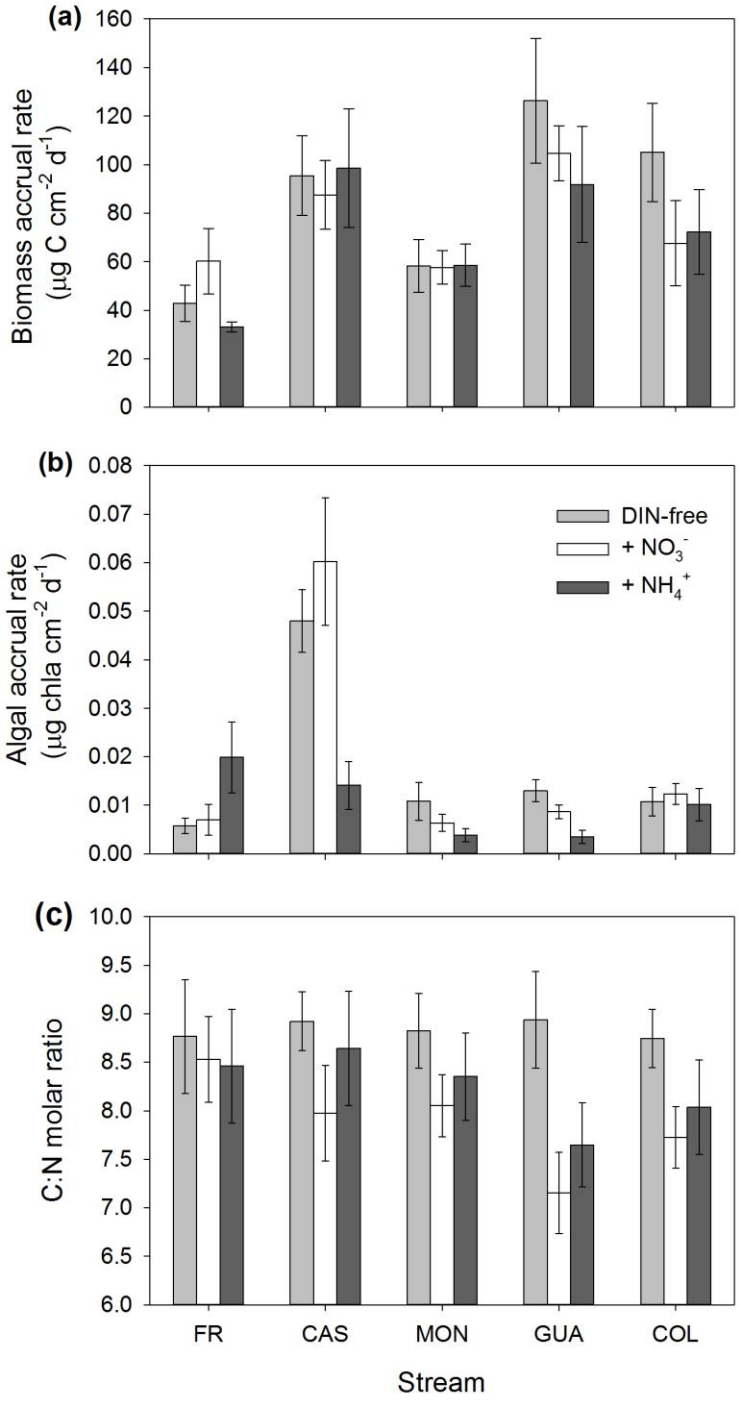

(a)
Biomass accrual rate
(µg C cm-2 d-1)
160
140
120
100
80
60
40
20
0
(b)
Algal accrual rate
(µg chla cm-2 d-1)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
DIN-free
+ NO3-
+ NH4+
(c)
C:N molar ratio
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
FR
CAS
MON
GUA
COL
Stream

Figure 2

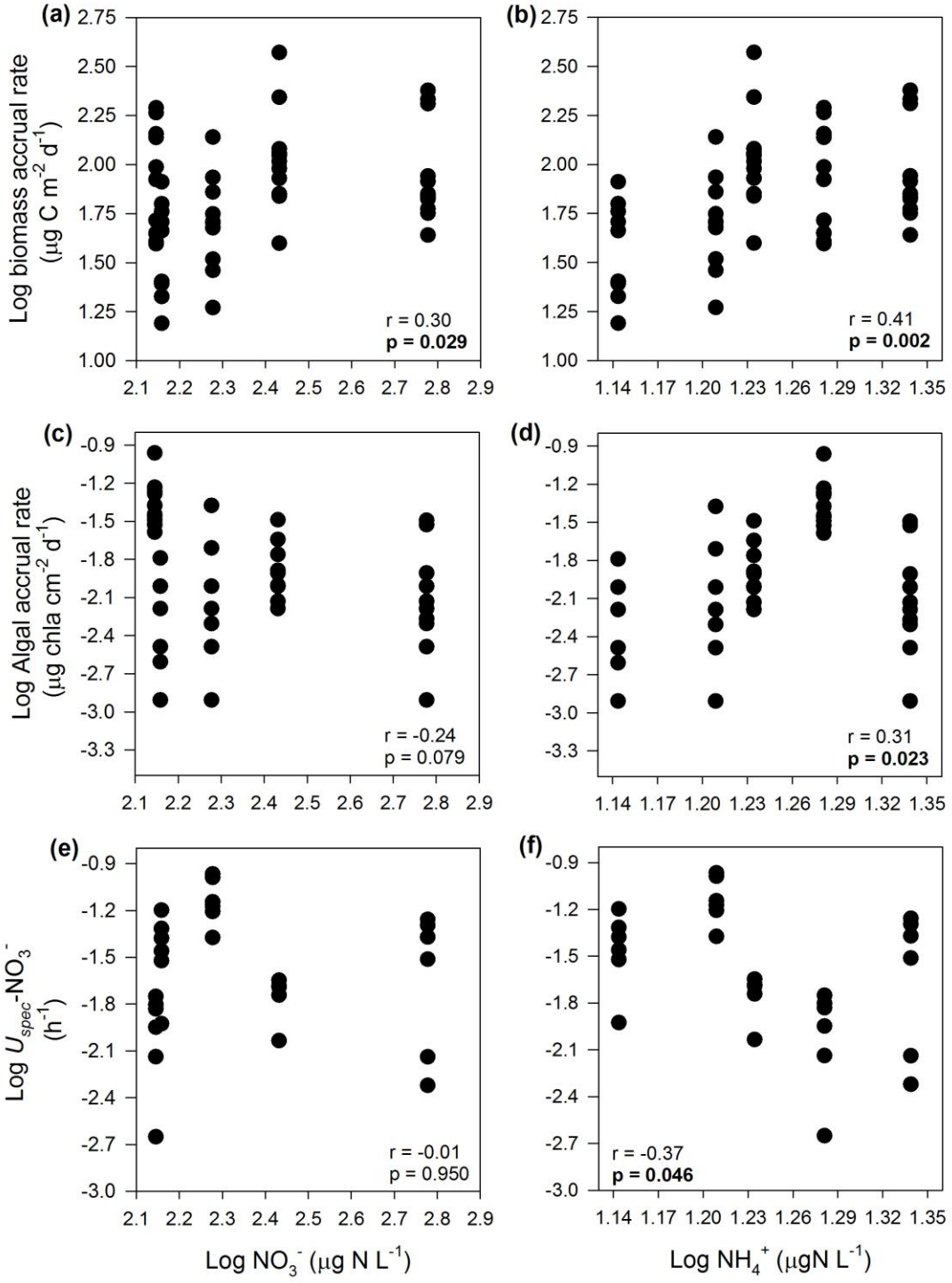

(a)
(b)
(c)
(d)
(e)
(f)
Log biomass accrual rate (µg C m-2 d-1)
Log Algal accrual rate (µg chla cm-2 d-1)
Log U spec -NO3- (h-1)
r = 0.30
p = 0.029
r = 0.41
p = 0.002
r = -0.24
p = 0.079
r = 0.31
p = 0.023
r = -0.01
p = 0.950
r = -0.37
p = 0.046
Log NO3- (µg N L-1)
Log NH4+ (µgN L-1)

Figure 3

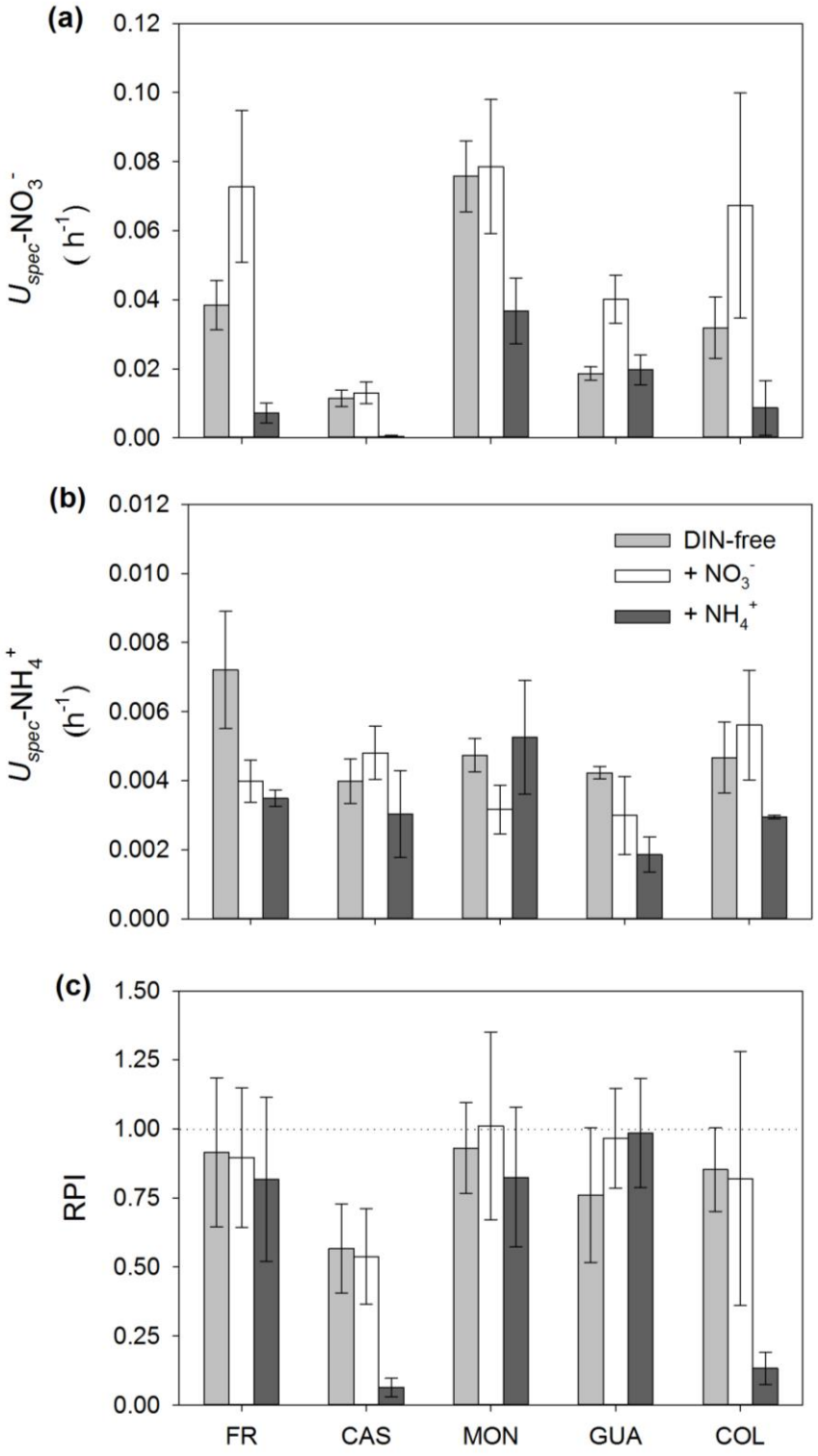

(a)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
U_{spec}-NO_3^- (h^{-1})
(b)
0.012
0.010
0.008
0.006
0.004
0.002
0.000
U_{spec}-NH_4^+ (h^{-1})
DIN-free
+ NO_3^-
+ NH_4^+
(c)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
RPI
FR
CAS
MON
GUA
COL

Figure 4

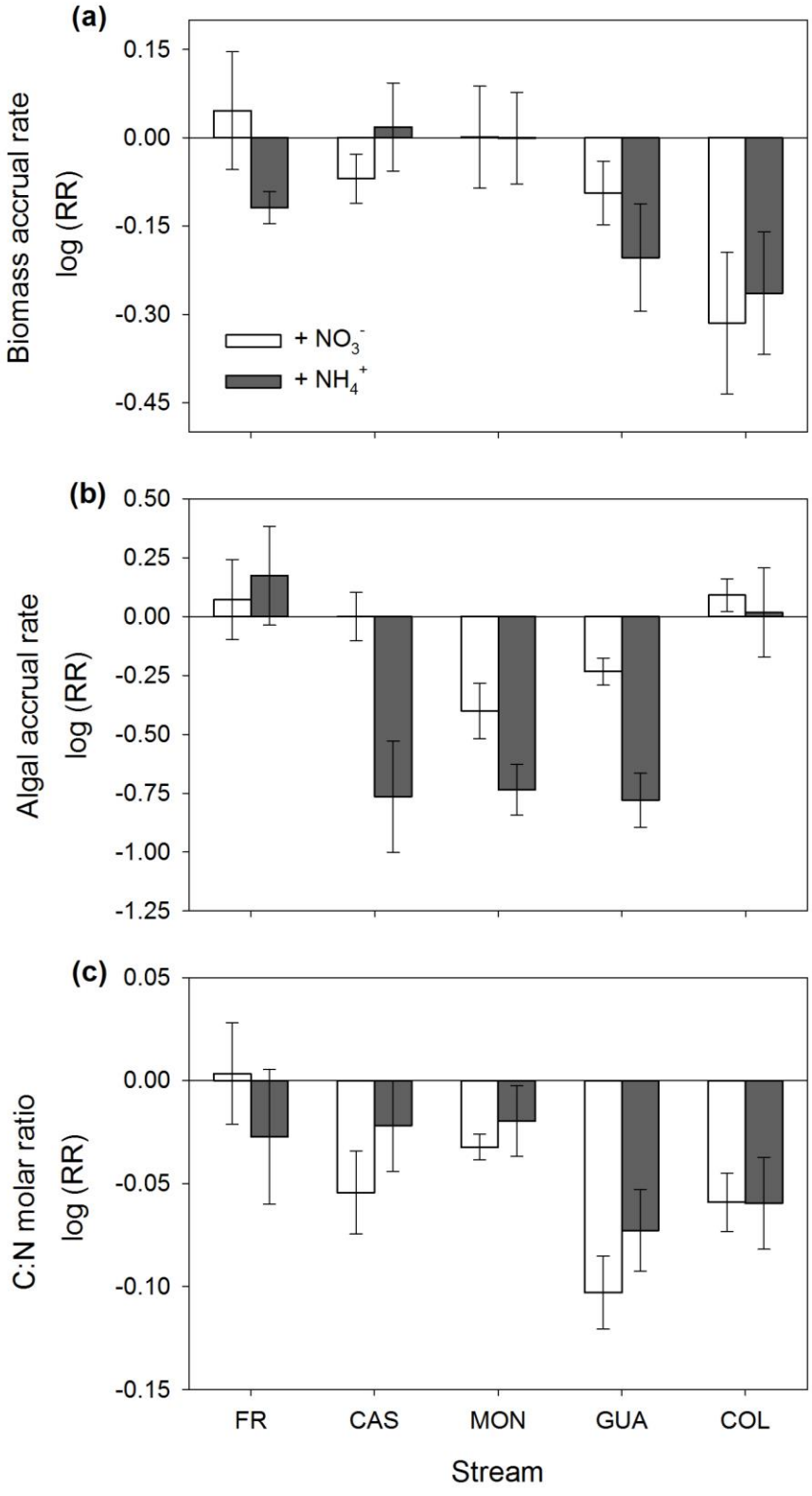
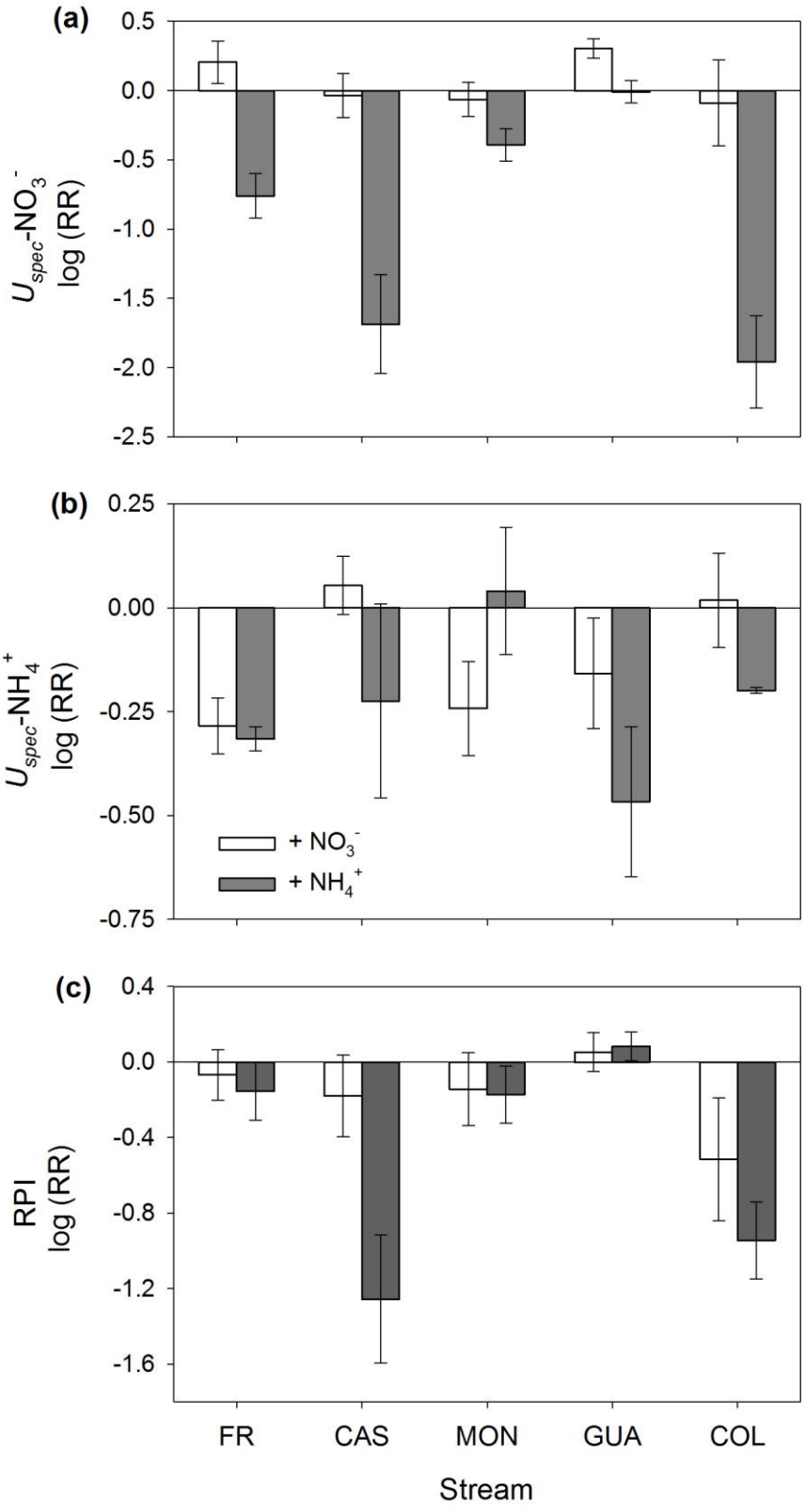

(a)
Biomass accrual rate
log (RR)
0.15
0.00
-0.15
-0.30
-0.45
+ NO3-
+ NH4+
(b)
Algal accrual rate
log (RR)
0.50
0.25
0.00
-0.25
-0.50
-0.75
-1.00
-1.25
(c)
C:N molar ratio
log (RR)
0.05
0.00
-0.05
-0.10
-0.15
FR
CAS
MON
GUA
COL
Stream

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