Differences in ammonium oxidizer abundance and N uptake capacity between epilithic and epipsammic biofilms in an urban stream

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Abstract: The capacity of stream biofilms to transform and assimilate N in highly N-loaded streams is essential to guarantee the water quality of freshwater resources in urbanized areas. However, the degree of N saturation experienced by urban streams and their response to acute increases in N concentration are largely unknown. We measured changes in the rates of NH_4^+ uptake (U_{NH4}) and oxidation (U_{AO}) resulting from experimental increases in NH_4^+-N concentration in mature biofilms growing downstream of a wastewater treatment plant (WWTP) and, thus, naturally exposed to high N concentration. We investigated the responses of U_{NH4} and U_{AO} to NH_4^+-N increases and the abundance of NH_4^+ oxidizing bacteria and archaea (AOB and AOA) in epilithic and epipsammic biofilms. U_{NH4} and U_{AO} increased with increasing NH_4^+-N concentration for the 2 biofilm types, suggesting no N saturation under ambient levels of NH_4^+-N. Thus, these biofilms can contribute to mitigating N excesses and the variability of NH_4^+-N concentrations from WWTP effluent inputs. The 2 biofilm types exhibited different Michaelis–Menten kinetics, indicating different capacity to respond to acute increases in NH_4^+-N concentration. Mean U_{NH4} and U_{AO} were 5× higher in epilithic than epipsammic biofilms, coinciding with a higher abundance of AOA+AOB in the former than in the later (76×10^4 vs 14×10^4 copies/cm^2). AOB derived from active sludge dominated in epilithic biofilms, so our results suggest that WWTP effluents can strongly influence in-stream NH_4^+ processing rates by increasing N inputs and by supplying AOA+AOB that are able to colonize some stream habitats.

Key words: stream biofilms, uptake kinetics, nitrogen saturation, ammonium uptake, ammonium oxidation, ammonia oxidizing bacteria and archaea, waste water treatment plant input

The increase in urbanization and associated human activities is foreseen as one of the major environmental threats for maintaining stream water quality and the integrity of freshwater ecosystems (Schlesinger et al. 2006, Grimm et al. 2008). The effluents from wastewater treatment plants (WWTPs) supply large amounts of nutrients, in particular inorganic N, to receiving streams (Martí et al. 2004). Moreover, microorganisms from active sludge supplied to the stream can colonize stream biofilms, thereby altering the composition and function of microbial stream communities downstream of WWTP effluent inputs (Mussmann et al. 2013, Sonthiphand et al. 2013, Merbt et al. 2015).

Streams receiving effluent from WWTPs may become hot spots of nitrification because of biofilm colonization by allochthonous nitrifiers from WWTP active sludge and stimulation of biogeochemical activity by high NH_4^+-N concentrations (Merseburger et al. 2005, Mussmann et al. 2013). Ammonium oxidizing archaea (AOA) and bacteria (AOB) and the recently discovered comammox bacteria, which carry out the whole nitrification process, all encode for

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NH_3 monooxygenase, the enzyme responsible for the conversion of NH_4^+ to NO_2^- (Fernández-Guerra and Casamayor 2012, Prosser and Nicol 2012, Daims et al. 2015). High abundance of NH_4^+ oxidizers and chronic exposure to high N loadings can strongly affect N uptake in WWTP-receiving streams, which can affect their capacity to mitigate N pollution (Martí et al. 2004, Bunch and Bernot 2012). However, studies exploring the degree of N saturation experienced by urban streams affected by WWTP effluents and their capability to respond to acute increases in N availability are scarce in the literature.

Prevailing theories predict that biological nutrient uptake tends to saturate at high levels of nutrient concentration because other factors eventually start to limit nutrient transformation rates (Earl et al. 2006). Previous investigators have described the saturation of N uptake under increasing N concentrations, which usually follows a Michaelis–Menten (M-M) model, at both reach and mesocosm scales (Bernot and Dodds 2005, O'Brien and Dodds 2008, Ribot et al. 2013). However, other investigators have found no N saturation, i.e., no changes or even steady increases in N uptake with increasing N concentration (Dodds et al. 2002, Kemp and Dodds 2002, Ribot et al. 2013). Several factors have been invoked to explain these differences in N uptake kinetics among streams, or even among habitats within the same stream. These include differences in the composition of microbial assemblages, acclimatization of stream microbes to increases in nutrient concentrations (Bunch and Bernot 2012), or changes in the physical diffusion of solutes through sediments and biofilm structures (Earl et al. 2006, Johnson et al. 2015). However, a good understanding of the factors contributing to saturation of N uptake in stream biofilms is lacking. Moreover, most of these manipulative studies are based on ranges of NH_4^+-N concentration (0.01–1.5 mg N/L) well below those naturally observed in WWTP-influenced streams (0.1–>10 mg N/L), which limits our capability to predict the extent to which stream biofilms can mitigate N pollution in aquatic systems exposed to high N loads. Filling this knowledge gap is particularly important in regions with water scarcity, where WWTP-receiving streams have a small dilution capacity and, thus, can show high variability and acute increases in N concentrations (Martí et al. 2004, Merbt et al. 2015).

The objective of our study was to examine the N uptake response to acute increases in NH_4^+-N concentration of biofilms grown naturally in a WWTP-influenced stream and, thus, acclimated to relatively high N ambient concentrations. We also examined whether the response to NH_4^+-N spike additions varied between the 2 dominant biofilm types in streams: epilithon (developed on cobbles) and epipsammon (developed on hyporheic sediments) for which we quantified AOA and AOB abundance. Last, we assessed the contribution of NH_4^+ oxidation to NH_4^+ uptake across the increased range of NH_4^+-N concentration because this process can account for a large fraction of NH_4^+ uptake in WWTP-receiving streams (Bernal et al. 2017). Given the broad range in experimental NH_4^+-N concentrations (>1 order of magnitude), we expected that biofilm N uptake would show a saturation response and follow M-M kinetics. However, we expected differences in the affinity for NH_4^+ between epipsammic and epilithic biofilms because of differences in biological (e.g., microbial assemblages) and physical factors (e.g., solute diffusion). Last, we expected no changes in the contribution of NH_4^+ oxidation to NH_4^+ uptake if high levels of this nutrient did not affect the activity of AO.

METHODS

Sampling site and biofilm collection

We selected a stream site in La Tordera River (northeastern Spain) situated 850 m downstream of the incoming effluent from the WWTP of Santa Maria de Palautordera (lat 41°41′3.47″N, long 2°27′33.19″W). The contribution of the WWTP effluent to stream discharge can range from 60% in winter to 100% in summer when the stream dries upstream of the WWTP (Merseburger et al. 2005). The WWTP effluent has high NH_4^+-N concentrations (0.6–> 20 mg NH_4^+-N/L), and consequently stream NH_4^+-N concentrations are many-fold higher down- than upstream of the WWTP (Merseburger et al. 2011, Merbt et al. 2015). Thus, we were confident that mature biofilms collected at the selected downstream site were already acclimated to high NH_4^+-N concentrations. The stream bed had cobbles and sandy loam sediment with 80% gravels and sands and 20% silt and clays.

We gently collected fist-size cobbles and stream sediments from 0 to 3 cm deep in spring 2014. We used a small trowel to transfer the stream sediments carefully to a metallic-mesh basket (10 × 15 × 5 cm) especially designed to facilitate manipulation and transport of sediments to the laboratory for the experiments. To ensure minimal disturbance of the epipsammic biofilms, we kept sediment baskets in the stream for 5 d before transportation to the laboratory. We transported cobbles and sediment baskets to the laboratory submerged in stream water from the same location on the day before the incubation experiment. We ran experiments with cobbles and sediment baskets on different days, and on each day of collection we took 1 streamwater sample from the thalweg with an acid-washed polyethylene bottle. Moreover, we measured stream water temperature (T, in °C) and dissolved O_2 concentrations (DO, in mg/L) with an O_2 meter (HQ 30d; HACH, Loveland, Colorado) at each collection site.

Experimental setting

We carried out a set of replicated experiments in recirculating incubation chambers separately for biofilms on cobbles and sediments to evaluate the N-uptake response of mature stream biofilms to increasing levels of NH_4^+-N concentration. For each experimental setting,

we placed either 3 to 4 cobbles or 2 sediment baskets in methacrylate chambers (30 × 30 × 10 cm) filled with 8 L of stream water from the site at which stream substrates were collected. Water was recirculated continuously with a submerged peristaltic pump (12 V) and water temperature and DO were constant during the experiments (21 ± 1.6°C, 8.6 ± 0.6 mg O_2/L). We ran the incubations under dark conditions to ensure optimal conditions for nitrification and to avoid confounding effects associated with photoinhibition (Merbt et al. 2017). Running the experiments in darkness might have led us to underestimate NH_4^+ uptake to some extent because we did not account for photoautotrophic NH_4^+ assimilation. However, the degree to which algae contributes to NH_4^+ uptake in this type of experiment depends on the specific light conditions set during the incubations. For instance, in a previous study, assimilatory NH_4^+ uptake increased when epilithic biofilms were exposed to experimental dark and light alternation cycles, though no changes were detected under full light conditions, probably because some other element became limiting (Merbt et al. 2017).

The experiments were run 6 times, each targeting a different NH_4^+-N concentration. The target NH_4^+-N concentrations ranged from 0.2 to 11.7 mg NH_4^+-N/L (0.2, 0.4, 0.8, 1.7, 4.7, and 11.7 mg NH_4^+-N/L), a range that largely encompassed the variability in stream NH_4^+-N concentration measured at this location during the last decade (0.2–13 mg N/L, $n = 19$) (SB, unpublished data). For each NH_4^+-N concentration, we used fresh biofilms collected from the stream on the previous day and held overnight in the recirculating chambers to ensure acclimatization to laboratory conditions. We ran each NH_4^+-N concentration in triplicate (3 independent chambers) for each type of biofilm. We also conducted incubations in stream water without an NH_4^+-N spike (2 independent chambers for each type of biofilm). We used these incubations to evaluate the magnitude of net changes in NH_4^+-N concentration and to ensure that N uptake responses measured in the experimental chambers was associated with the NH_4^+-N spike additions.

Biofilm NH_4^+ uptake and oxidation rates

We estimated NH_4^+ uptake and oxidation rates for each of the 6 NH_4^+-N concentrations after adding the spikes of NH_4Cl. In each case, the NH_4^+-N concentration of the spike addition was tailored to achieve the target concentration in the recirculating chamber. We collected 40-mL water samples 3, 15, 30, 60, 150, and 300 min after adding the spike.

All water samples (including those collected in situ) were filtered immediately, stored at −20°C, and analyzed for NH_4^+-N, NO_2^--N, and NO_3^--N concentrations with standard colorimetric methods (APHA 1995) on an autoanalyzer (FUTURA, Frepillon, France).

For each type of biofilm and NH_4^+-N concentration, we estimated the NH_4^+ uptake rate coefficient (k_{NH4}, in 1/min) by fitting the decrease in NH_4^+-N concentration over time to a 1st-order exponential function:

$$C_t = C_0 e^{-kt}, \quad \text{(Eq. 1)}$$

where t is time (min) and C_0 and C_t are the concentrations of NH_4^+-N (mg/L) at time 0 (3 min after the spike) and at consecutive incubation times, respectively (Stream Solute Workshop 1990). We used a similar approach to estimate the NH_4^+ oxidation rate coefficient (k_{AO}, 1/min) but, in this case, we fitted the exponential function (Eq. 1) to the increase in NO_x-N (NO_3^--N + NO_2^--N) concentration over time (Bernal et al. 2017). For a given incubation experiment, values of k_{NH4} and k_{AO} were not considered for further analysis if the regression fit was not significant ($p > 0.05$). If the regression fit was not significant for k_{NH4} but significant for k_{AO}, then k_{NH4} was excluded for further analysis. In this case, we did not set k_{NH4} to 0 because the existence of NO_x-N production (i.e., $k_{AO} > 0$) implies that at least a fraction of the added NH_4^+-N was taken up by nitrifiers, even if we were unable to detect it. We calculated NH_4^+ uptake rates per unit colonized area of each substrate considered for each biofilm type (U_{NH4}, in mg NH_4^+-N m^{-2} min^{-1}) with

$$U_{NH4} = C_o \times V \times k_{NH4}/A, \quad \text{(Eq. 2)}$$

where A is the total substrate colonized area (m^2) and V is the water volume in the chamber, which was assumed to be 8 L despite a slight decrease that occurred each time a water sample was taken during the incubation experiment. To estimate the NH_4^+ oxidation rate (U_{AO}, in mg NO_x^--N m^{-2} min^{-1}), we used the initial concentration of NO_x^--N and substituted k_{AO} for k_{NH4} in Eq. 2. We expressed the N fluxes, U_{NH4} and U_{AO}, by colonized area (see below) to make N processing rates comparable between epilithic and epipsammic biofilms.

The relative contribution of NH_4^+ oxidation to NH_4^+ uptake was calculated as a percentage of the ratio between U_{AO} and U_{NH4} for each biofilm type and target NH_4^+-N concentration. Values of U_{AO}:U_{NH4} close to 100% indicate that the contribution of NH_4^+ oxidation to NH_4^+ uptake is high, whereas values close to 0 indicate the opposite.

Biofilm characterization

Once each incubation period ended, we measured ash-free dry mass (AFDM). For epilithic biofilms, we scraped the cobble surface with a sterile metallic brush. The total area scraped was estimated by a mass-to-area relationship after covering the cobbles' surface with Al foil (Merbt et al. 2011). A known volume of the biofilm slurry was filtered onto 0.7-μm-pore-size glass-fiber filters (Albet, Barcelona, Spain). We obtained 1 biofilm composite per chamber and treated it as an independent replicate. For epipsammic biofilms, we placed a 30 g subsample in an aluminium tray after mixing well. For types of biofilm, we weighed samples (~0.1 mg) on an analytical balance (model MC1; Sartorius,

Göttingen, Germany) after drying them at 60°C until constant mass, and then reweighed them after combustion at 500°C for 5 h. AFDM was the difference between dry and combusted mass and was considered a proxy for biomass and detritus content.

We also estimated the abundance of both AOB and AOA in the 2 types of biofilm. AOB and AOA are responsible for the 1st step of the nitrification process (i.e., NH_4^+ oxidation; Könneke et al. 2005, Martens-Habbena et al. 2009), and thus, contribute to the dissimilatory fraction of the NH_4^+ uptake. For epilithic biofilms, we filtered 5 mL of well-mixed biofilm sludge through a 0.2-mm-pore-size polycarbonate membrane (Millipore, Billerica, Massachusetts), air-dried the filter and placed it in lysis buffer (40 mmol/L ethylenediaminetetra-acetic acid; 50 mmol/L Tris, pH 8.3; and 0.75 mol/L sucrose). For epipsammic biofilms, we weighed ~1 g of wet sediment and placed it in a similar lysis buffer. We extracted DNA after incubation with lysozyme, proteinase K, and sodium dodecyl sulfate and phenol-chloroform (Hervàs and Casamayor 2009). Abundances of AOA and AOB were estimated by quantitative polymerase chain reaction (qPCR) with primers CrenamoA23f (5′-ATGGTCTGGCTWAGACG-3′)-CrenamoA616r (5′-GCCATCCATCTGTATGTCCA-3′; Tourna et al. 2008) for AOA and amoA1F (5′-GGGTTTCTACTGGTGGT-3′)-amoA2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′; Rotthauwe et al. 1997) for AOB (Merbt et al. 2011, 2015).

For comparison purposes, we reported AFDM and the abundance of AO in the 2 types of biofilms per unit of colonized area (g AFDM/m^2 and number of copies/cm^2, respectively). For epilithic biofilms, the colonized area was equivalent to the scraped surface area of the cobble. For epipsammic biofilm, we estimated the colonized area by summing the surface areas of different grain-size fractions of a previously weighed sediment sample (~100 g). We separated grain-size fractions >63 μm by sieving and assumed sphericity to calculate surface area (Horowitz 1991). We obtained the surface area of grain-size fractions <63 μm by analyzing sediment samples with a particle-size counter (MasterSizer 2000; Malvern, Herrenberg, Germany).

Statistical analysis

We tested differences in AFDM, U_{AO}, U_{NH4}, U_{AO}:U_{NH4}, and the abundances of AOA and AOB between the 2 types of biofilms with Kruskal–Wallis tests. We used nonparametric tests because some variables were not normally distributed (Shapiro–Wilk's test, $p < 0.05$) (Zar 2010). For each type of biofilm, we explored whether U_{AO}:U_{NH4} changed with increasing NH_4^+-N concentration by fitting a linear model.

We tested differences in U_{NH4} and U_{AO} between treatments with an analysis of covariance (ANCOVA) and used biofilm type (biofilm) as a factor and target NH_4^+-N concentration (concentration) as a covariate. We used a post hoc Tukey's test to identify which groups differed from each other (Zar 2010). We used Shapiro–Wilk's tests to test for normality of the residuals and $\log_{10}(x)$-transformed variables to fulfill normality requirements if needed.

To explore the relationship between U_{NH4} and U_{AO} and different levels of NH_4^+-N concentration, we used M-M kinetics, a mathematical framework previously used in nutrient addition experiments (Ribot et al. 2013). The M-M model follows the equation:

$$U = \frac{U_{max} \times C}{K_S + C}, \quad \text{(Eq. 3)}$$

where C is NH_4^+-N concentration, U_{max} is the maximum U, and K_s is the ½-saturation constant, which is the value of NH_4^+-N concentration at which U is ½ of U_{max}. U_{max} expresses the maximum uptake across the study range of NH_4^+-N concentrations by the studied biofilms, and K_s indicates the biofilm affinity for NH_4^+. In the case of K_s, lower values denote higher affinity than higher values. We calculated these 2 metrics by nonlinear least squares regression based on the Gauss–Newton algorithm. We also calculated 95% confidence intervals (CIs) for each metric.

We conducted all statistical analyses in R using the *stats* and *multcomp* packages (version 3.2.2; R Project for Statistical Computing, Vienna, Austria).

RESULTS

In situ environmental conditions and characterization of stream biofilms

During the experimental period, stream water temperature and DO concentration averaged 17.3 ± 0.6°C and 8.3 ± 0.2 mg/L, respectively. Mean dissolved inorganic N (DIN) concentration in stream water was 1.8 ± 0.2 mg N/L ($n = 5$). Stream NO_3^--N concentration made up 81% of total DIN-N, whereas NH_4^+-N and NO_2^--N accounted for 14 and 5%, respectively.

AFDM differed consistently between the 2 biofilm types and was 2× lower in epilithic than in epipsammic biofilms (Table 1). The abundance of AOA was similar between the 2 biofilm types, whereas AOB were more abundant in epilithic than in epipsammic biofilms (Table 1). In epipsammic biofilms, AOA and AOB accounted for 60 and 40% of total AO abundance, respectively. In epilithic biofilms, AOA accounted for 11% of total AO abundance, whereas AOB were the predominant AO type (89%).

Biofilm NH_4^+ uptake and oxidation rates

The background concentrations of NH_4^+-N, NO_2^--N, and NO_3^--N in the water column of the chambers without NH_4^+-N spikes averaged 0.01 ± 0.001, 0.003 ± 0.0001, and 1.6 ± 0.1 mg N/L, respectively. These concentrations showed small changes during the incubation time. For the 2 chambers incubated with stream cobbles, an increase in NH_4^+-N concentration was detected in 1 chamber ($F =$

Table 1. Mean (±SE) ash-free dry mass (AFDM) expressed per unit of colonized area and abundance of archaea ammonia oxidizers (AOA) and bacterial ammonia oxidizers (AOB) in epilithic and epipsammic biofilms collected downstream of the wastewater treatment plant (WWTP) effluent input. The number of cases (n) is shown in parenthesis. The p-value and χ^2 statistic of the Kruskal–Wallis test are shown for each variable.

	Epilithon	Epipsammon	p	χ^2
AFDM (g/m^2)	3.0 ± 0.2 (21)	5.8 ± 0.7 (21)	<0.0001	26.9
AOA (10^4 copies/cm^2)	5.7 ± 4.4 (3)	8.4 ± 1.1 (3)	0.5	0.43
AOB (10^4 copies/cm^2)	70 ± 21.3(3)	5.6 ± 0.9 (3)	0.049	3.86

6.97, df = 6, $p = 0.046$) and a decrease in NO_3^--N concentration was detected in the other ($F = 7.4$, df = 6, $p = 0.042$). For the 2 chambers incubated with sediments, no significant trends in NH_4^+-N, NO_2^--N, or NO_3^--N concentration were detected ($F < 1$, $p > 0.05$ for the 6 regression fits).

NH_4^+ spikes induced changes in the N concentration of the water of the chambers. After the NH_4^+ spike, the concentration of NH_4^+-N tended to decrease over time, whereas NO_2^--N and NO_3^--N showed the opposite pattern (Fig. 1A, B). Decreases in NH_4^+-N concentration were statistically significant in 75% of the cases (27 of 36), with mean U_{NH4} 57.4 ± 14.6 μg N m^{-2} min^{-1} for all incubations including the 2 types of biofilms. All chambers with no significant decrease in NH_4^+-N ($n = 9$) contained epilithic biofilms, and 6 of those were treated with NH_4^+-N spikes that resulted in the highest levels of NH_4^+-N concentration (>2 mg N/L). Increases in NO_x^--N concentration were statistically significant in all the incubations ($n = 36$). Mean U_{AO} was 35.3 ± 10.4 μg N m^{-2} min^{-1} for all incubations including the 2 types of biofilms.

Values of U_{NH4} and U_{AO} differed significantly between biofilm types and among levels of added NH_4^+-N. Both U_{NH4} and U_{AO} were higher for epilithic than for epipsammic biofilms (biofilm, $F = 209.3$ and 402.2 for U_{NH4} and U_{AO}, respectively; in both cases df = 1 and $p < 0.001$). The 2 N fluxes increased with increasing the level of added NH_4^+-N (concentration, $F = 33.1$ and 9.6 for U_{NH4} and U_{AO}, respectively, df = 5, $p < 0.001$) (Table 2).

The mean contribution of NH_4^+ oxidation to NH_4^+ uptake was higher for epilithic than for epipsammic biofilms (53.6 ± 5.8 vs 39.3 ± 4.2%) (Kruskal–Wallis test, $\chi^2 = 5.5$, df = 1, $p = 0.019$; Fig. 2). U_{AO}:U_{NH4} did not change with increasing NH_4^+-N concentration in epilithic biofilms, whereas a consistent decrease in the ratio was observed for epipsammic biofilms (linear regression, $R^2 = 0.71$, df = 4, $p = 0.034$; Fig. 2).

M-M kinetics and N saturation in stream biofilms

In the 2 types of biofilm, U_{NH4} and U_{AO} levelled off with increasing NH_4^+-N concentration, following an M-M pattern (Fig. 3A–D). However, M-M parameters differed substantially between biofilm types and showed that epilithic biofilms had a higher affinity for NH_4^+ than epipsammic biofilms. For U_{NH4}, K_s was 4× lower and U_{max} was 4× higher in epilithic than in epipsammic biofilms (Table 3). U_{AO} showed a similar pattern, and K_s was 4× lower and U_{max} 7× higher in epilithic than in epipsammic biofilms

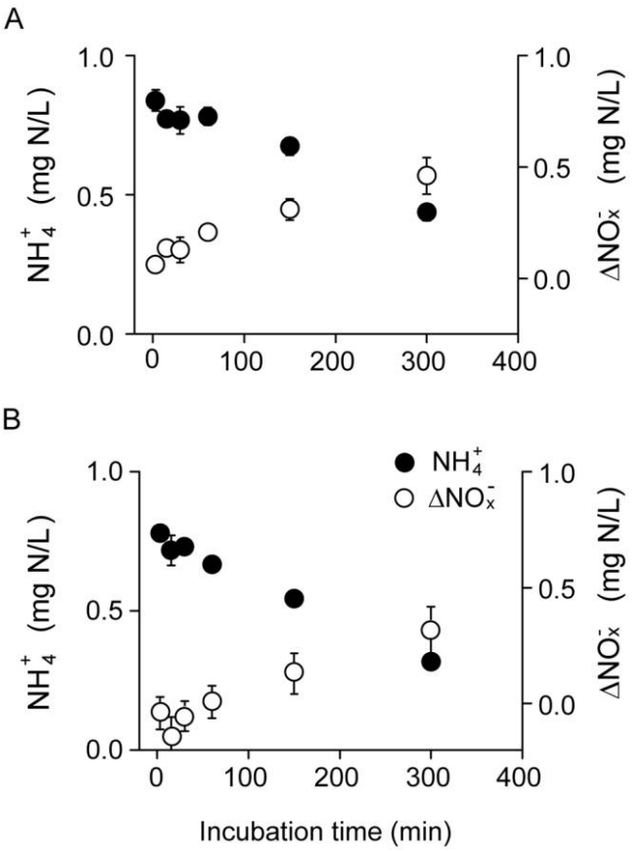

Figure 1. Example of temporal changes in mean (±SE, $n = 3$) NH_4^+-N and NO_x^--N (N-NO_2^- + NO_3^--N) concentrations after the NH_4^+-N spike in recirculating chambers containing epilithon (A) and epipsammon (B) during the incubation experiments. The example shown corresponds to the experiment for which the target increase in NH_4^+-N concentration was set to result in a concentration of 0.8 mg NH_4^+-N/L. For illustration purposes, NO_x^- is expressed as the concentration at each sampling time minus initial concentration (i.e., Δ NO_x^--N). Concentrations were $\log_{10}(x)$-transformed to calculate NH_4^+ uptake and NH_4^+ oxidation rate coefficients.

Table 2. Mean (±SE) rates of NH_4^+ uptake (U_{NH4}) and NH_4^+ oxidation (U_{AO}) for the 2 types of biofilm (epilithic and epipsammic) and the 6 levels of added NH_4^+-N. For each N processing rate, treatments with the same uppercase letter are not significantly different after conducting an analysis of covariance (factor: biofilm, covariate: concentration) followed by post hoc Tukey's tests. $n = 3$ for each treatment, except when indicated. ns = not significant.

	U_{NH4} (μg N m^{-2} min^{-1})		U_{AO} (μg N m^{-2} min^{-1})	
Target NH_4^+-N (mg N/L)	Epilithon	Epipsammon	Epilithon	Epipsammon
0.2	51.9 ± 17.1^{AB}	4.7 ± 0.7^{D}	[b]48.9 ± 16.4^{A}	2.4 ± 0.2^{B}
0.4	[a]102.7^{BC}	13 ± 2.9^{DE}	50.9 ± 5.8^{A}	5.2 ± 0.7^{BC}
0.8	145.8 ± 27.6^{C}	11.5 ± 1.2^{E}	86.5 ± 17.5^{A}	4.8 ± 0.3^{BC}
1.7	[b]186.1 ± 36.5^{C}	24.1 ± 2.7^{AEF}	78.7 ± 16.6^{A}	10.1 ± 2.1^{C}
4.7	ns	39.3 ± 1.8^{BF}	109.4 ± 32.7^{A}	11.5 ± 2.04^{C}
11.7	ns	53 ± 0.4^{BF}	†89.5 ± 3.8^{A}	13.7 ± 1.5^{C}

[a] $n = 1$
[b] $n = 2$

(Table 3). For epilithic biofilms, we excluded values of U_{NH4} measured at target concentrations of 4.7 and 11.7 mg NH_4^+-N/L from the M-M analysis because k_{NH4} was not statistically significant and $k_{AO} > 0$ for all replicates. This pattern indicates that at least a part of the added NH_4^+-N was taken up by nitrifiers despite our inability to detect changes in NH_4^+-N concentration in the recirculating chambers.

DISCUSSION

We investigated how mature stream biofilms grown naturally under high N concentrations respond to acute increases in N concentration and whether this response varied between epilithic and epipsammic biofilms. In concordance with our expectation, the N uptake response to increases in NH_4^+-N concentration followed an M-M pattern in the 2 biofilm types. However, values of K_s were as high as 3 mg NH_4^+-N/L, a concentration 10× higher than that measured in stream water during the period of study (0.3 mg NH_4^+-N/L). Therefore, the biofilms were far from N saturation at ambient NH_4^+-N concentration and showed a high capacity to process additional N inputs despite being exposed to chronically high NH_4^+-N concentrations. These results are consistent with previous studies showing that the potential for N processing and retention in urban streams is high at reach and river-network scales and that these ecosystems can positively influence the quality of freshwater resources (e.g., Grimm et al. 2005, Kaushal et al. 2014).

The emergence of M-M patterns for both U_{NH4} and U_{AO} also indicated that the N processing capacity of these stream biofilms eventually saturates at very high N concentrations, as observed in previous studies (O'Brien et al. 2007, Mulholland et al. 2008). Understanding the capacity of biofilms to process such high N concentrations is important because, like many other semiarid streams, the study stream had intermittent flow and dried upstream of the WWTP in summer. During those periods, the stream's dilution capacity is nearly 0 and biogeochemical processing becomes the major pathway for buffering N inputs from the WWTP effluent (Martí et al. 2004). Measured K_s values (0.6–3 mg NH_4^+-N/L) were higher than the maximum concentration considered in previously published short-term NH_4^+ addition studies (<2 mg NH_4^+-N/L) (e.g., Dodds et al. 2002, Ribot et al. 2013) and higher than K_s values reported in less-polluted streams (K_s < 0.6 mg NH_4^+-N/L) (Kemp and Dodds 2002, O'Brien and Dodds 2008, Ribot et al. 2013). Thus, discrepancies regarding the existence of N saturation patterns could be explained, at least partially, by the fact that experimental concentration ranges are usually too narrow to detect saturation concentration levels. Moreover, the high K_s values measured in our study suggest that stream biofilm assemblages have the ability to cope with the prevailing environmental conditions, in particular

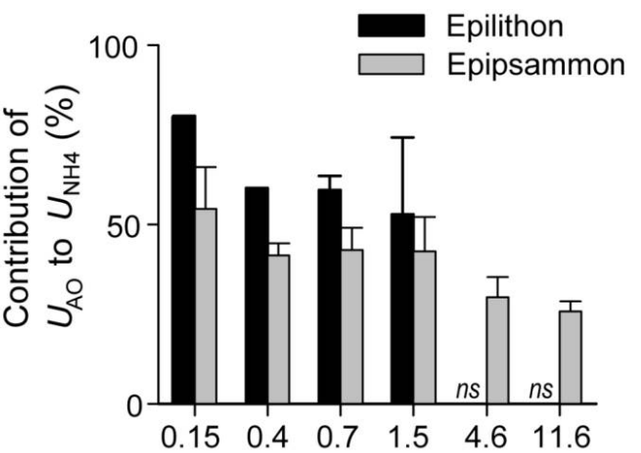

Figure 2. Mean (+SE) contribution of NH_4^+ oxidation (U_{AO}) to NH_4^+ uptake (U_{NH4}) measured in the incubation chambers with epilithic and epipsammic biofilms experimentally exposed to 6 different levels of NH_4^+-N concentration.

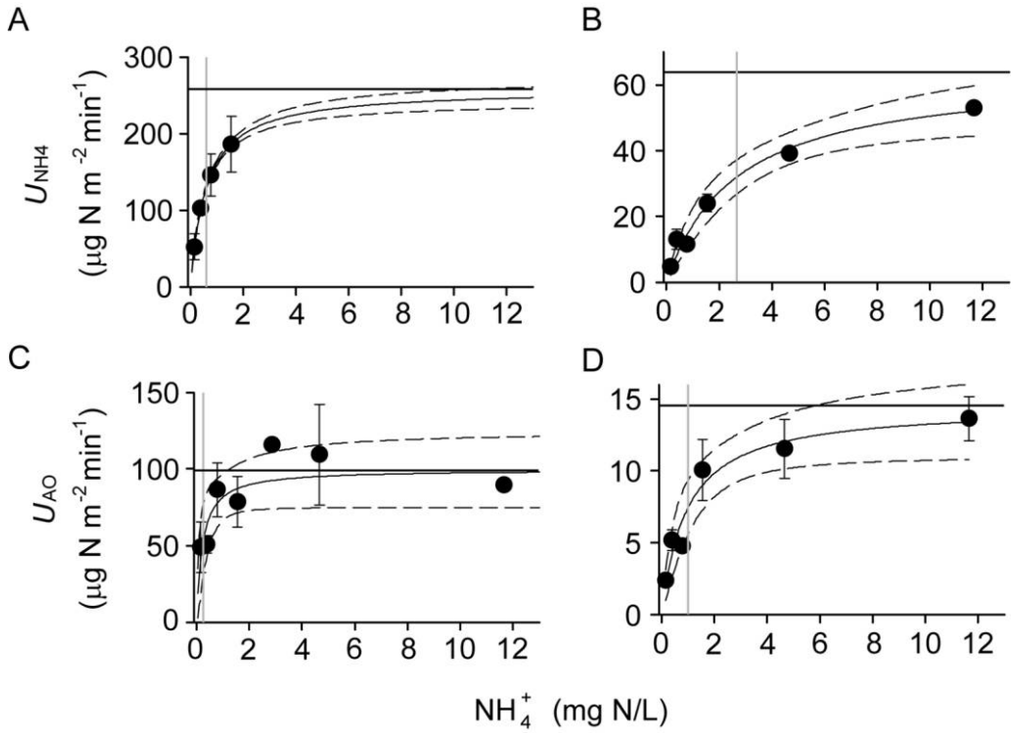

Figure 3. Relationship between mean (±SE) U_{NH4} (A, B) and U_{AO} (C, D) and NH_4^+-N concentration in the incubation chambers with epilithic (A, C) and epipsammic (B, D) biofilms. The solid line is the Michaelis–Menten fitted model and the dashed lines are the corresponding 95% confidence intervals. The U_{max} and K_s values obtained with the model are indicated with horizontal and vertical lines, respectively.

with chronically high N concentrations (Bunch and Bernot 2012, Ribot et al. 2013, Artigas et al. 2015).

The N saturation pattern at high levels of NH_4^+-N concentration suggests, first, that uptake was regulated by biota rather than by physical processes in both biofilm types. If slow diffusion had limited mass transfer through the liquid–solid interface, then we would have observed either constant or linear changes of U_{NH4} with increasing NH_4^+-N concentration (Earl et al. 2006). Second, biota may have a limited capacity to respond to acute increases in NH_4^+-N concentration during storms and episodes of WWTP malfunction, when NH_4^+-N concentration can be higher than K_s. Last, these biofilms may have difficulty coping with elevated NH_4^+-N concentrations like those that prevail during low-dilution periods. However, further studies are needed to assess how stream biofilms adapt to chronic (>2–3 wk) increases in N concentration because microbial communities can evolve and acclimate to environmental changes in relatively short periods (in the scale of few weeks) (Bunch and Bernot 2012, Artigas et al. 2015, Tlili et al. 2017).

Our results support the expectation that the capacity to take up N can differ substantially among substrate types in freshwater ecosystems (e.g., Kemp and Dodds 2002). In particular, we found that the NH_4^+ uptake (U_{NH4}) and the

Table 3. Best-fit parameters obtained after adjusting a Michaelis–Menten model to the variation of rates of NH_4^+ uptake (U_{NH4}) and NH_4^+ oxidation (U_{AO}) with increasing NH_4^+-N concentration. K_s is the ½-saturation constant and U_{max} is the maximum uptake. The 95% confidence interval and the p-value of the best-fit model are indicated in parenthesis in each case.

		Epilithon	Epipsammon
U_{NH4}	K_s (mg N/L)	0.6[a] ± 0.02 (0.001)	2.7 ± 0.6 (0.012)
	U_{max} (µg N m^{-2} min^{-1})	258.1 ± 3.7 (<0.001)	64 ± 5.5 (<0.001)
U_{AO}	K_s (mg N/L)	0.22 ± 0.1 (0.08)	0.95 ± 0.3 (0.025)
	U_{max} (µg N m^{-2} min^{-1})	99.65 ± 9 (<0.001)	14.5 ± 1.2 (<0.001)

[a] U values at target concentrations of 4.7 and 11.7 mg NH_4^+-N/L were not statistically significant and were not included in the Michaelis–Menten analysis

affinity for this nutrient (as indicated by K_s) were multiple-fold higher in epilithic than epipsammic biofilms. These findings agree with the idea that NH_4^+ uptake is higher in riffles than in pools if we assume that cobbles dominate in the former and sandy beds in the latter (O'Brien and Dodds 2008). However, our results contrast with those of other habitat-specific incubation experiments showing similar N uptake rates between sand and cobbles (O'Brien et al. 2012). Additional information on microbial community composition, water exchange through biofilm structures, and physicochemical characteristics would be necessary to explain differences (or similarities) in habitat-specific N uptake responses to increases in N availability. For instance, differences in U_{NH4} between the 2 biofilm types could be explained partially by differences in the diffusion of NH_4^+ throughout biofilm structures. Solutes probably flowed slowly throughout the sediment baskets (5 cm deep) compared to thin epilithic biofilms (thickness < 1 mm), which probably were more exposed to the overlying water velocity. The rapid exchange of NH_4^+ at the solid–liquid interface probably enhanced NH_4^+ uptake in epilithic biofilms (Arnon et al. 2013) and release of any potential NH_4^+ derived from mineralization or cell exudates to the water column. These differences in microscale hydrodynamics could explain why chambers with cobbles sometimes showed N-NO_x production but no changes in NH_4^+-N concentration, suggesting that part of the NH_4^+ taken up by nitrifiers was counterbalanced by internal NH_4^+ production.

Regarding differences in microbial community composition, we found that AO were 5× more abundant in epilithic than in epipsammic biofilms. Moreover, epilithic biofilm was dominated by AOB, which in principle, tolerate higher NH_4^+-N concentrations than AOA, and thus, can be less sensitive to increased levels of NH_4^+-N concentration (Martens-Habbena et al. 2009, Herrmann et al. 2011, Verhamme et al. 2011). Our results confirm these previous observations because the contribution of NH_4^+ oxidation to NH_4^+ uptake was independent of N availability in epilithic biofilms (AOB dominated), but not in epipsammic biofilms. Thus, the predominance of AOB in epilithic biofilms could contribute to higher NH_4^+ uptake rates in epilithic than epipsammic biofilms and explain the higher contribution of NH_4^+ oxidation to NH_4^+ uptake in epilithic (54%) than episammic (40%) biofilms. Our study did not include comammox bacteria, a type of nitrifying organism discovered after we concluded our analysis (Daims et al. 2015). Further work is needed to shed light on the potential contribution of comammox to NH_4^+ uptake in these types of biofilm.

Previous studies showed that AOB lineages found downstream of WWTP effluent inputs differ from those upstream and are composed mostly of allochthonous bacteria derived from active sludge (e.g., *Nitrosomonas europea* and *Nitrosopira*) (Mussmann et al. 2013, Sonthiphand et al. 2013, Merbt et al. 2015). The prevalence of these AOB lineages suggests that they are able to colonize epilithic biofilms and that they probably out-compete autochthonous AO types that are found in much lower numbers (Merbt et al. 2015). This finding would explain why mean NH_4^+ oxidation rates can be 20× higher in epilithic biofilms growing down- than upstream of the WWTP (90 vs 4 μg N m^{-2} min^{-1}) (SB, unpublished data). Moreover, our results suggest that the shift in community composition experienced by WWTP-influenced streams can profoundly alter their N processing capacity by increasing the relative proportion of oxidized NH_4^+ from what is taken up globally by epilithic biofilms. Based on the measured rates, this type of biofilm could contribute to in-stream NH_4^+ oxidation as much or even more than epipsammic biofilms. This idea is in contrast to the idea proposed for less polluted streams that hyporheic zones are major drivers of nitrification (Jones et al. 1995, Bernot and Dodds 2005, Zarnetske et al. 2011). However, biofilms were incubated in the dark in our study, so the contribution of photoautotrophic assimilation to NH_4^+ uptake was underestimated. This process could be especially noticeable in epilithic biofilms where the presence of microalgae was conspicuous. The study stream is well shaded, but we have estimated that photoautotrophic assimilatory uptake could account for ~30% of whole-reach NH_4^+ uptake during daytime (Bernal et al. 2017). The potential for primary productivity could be even higher in urban streams with high nutrient availability where open reaches predominate (e.g., Grimm et al. 2005).

In the sediments, environmental conditions may prevent the establishment of AOB, while favoring the persistence of AOA. In the study stream, the hyporheic zone typically shows lower concentrations than surface water of both DO (2.6 ± 0.4 vs 5.4 ± 0.4 mg/L) and NH_4^+-N (0.9 ± 0.2 vs 1.8 ± 0.3 mg N/L) (SB, unpublished data). DO and NH_4^+ availability are key drivers of AO activity. Thus, the observed differences in AOA and AOB abundance partially could be responses to the different physicochemical conditions prevailing in surface water and hyporheic environments. Moreover, epipsammic biofilms showed the lowest NH_4^+ uptake rates, and the highest sensitivity to increases in NH_4^+-N concentration. These results are in line with data from laboratory cultures showing that inhibitory NH_4^+-N concentrations can be orders of magnitude lower for AOA than for AOB (Hatzenpichler 2012). Our findings suggest that streams or particular habitats in the stream dominated by AOA may have a limited capacity to deal with N excesses compared to those colonized mostly by AOB.

In conclusion, the study stream biofilms were able to respond to acute increases in NH_4^+ availability by substantially increasing both NH_4^+ uptake and NH_4^+ oxidation rates. This result suggests that the biofilms in the study stream were not N saturated despite exposure to chronic inputs of N from the WWTP effluent. Stream biofilms

were actively mitigating N pollution by taking up NH_4^+ via assimilatory and dissimilatory pathways. Epilithic and epipsammic biofilms showed a differential response to increases in NH_4^+-N concentration that could be related, at least partially, to large differences in the abundance and predominant type of AO in each biofilm type. Habitat heterogeneity is becoming increasingly important for understanding the magnitude and variability of whole-reach N-uptake patterns within and across streams (Peipoch et al. 2016). However, factors underlying these differences are rarely identified, a situation that complicates the interpretation of discrepancies among published studies and limits our ability to manage and restore altered streams. We propose that a good characterization of the most representative stream habitats in terms of physicochemical conditions, microbial community composition, and biogeochemical processing rates is essential for assessing the effect of human activities in polluted streams and for understanding how stream ecosystems contribute to improve stream water quality.

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Author contributions: SB and EM designed the study. SB and AS conducted the field work, AS conducted chamber incubations, and SNM conducted the molecular analysis. AS, EM, and SB analyzed data. SB wrote the manuscript with contributions from AS, SNM, and EM.

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