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Nitrogen processing and the role of epilithic biofilms downstream of a wastewater treatment plant

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Abstract. We investigated how dissolved inorganic N (DIN) inputs from a wastewater treatment plant (WWTP) effluent are processed biogeochemically by the receiving stream. We examined longitudinal patterns of NH_4^+ and NO_3^- concentrations and their ^{15}N signatures along a stream reach downstream of a WWTP. We compared the $\delta^{15}N$ signatures of epilithic biofilms with those of DIN to assess the role of stream biofilms in N processing. We analyzed the $\delta^{15}N$ signatures of biofilms coating light- and dark-side surfaces of cobbles separately to test whether light constrains functioning of biofilm communities. We sampled during 2 contrasting periods of the year (winter and summer) to explore whether changes in environmental conditions affected N biogeochemical processes. The study reach had a remarkable capacity for transformation and removal of DIN, but the magnitude and relevance of different biogeochemical pathways of N processing differed between seasons. In winter, assimilation and nitrification influenced downstream N fluxes. These processes were spatially segregated at the microhabitat scale, as indicated by a significant difference in the $\delta^{15}N$ signature of light- and dark-side biofilms, a result suggesting that nitrification was mostly associated with dark-side biofilms. In summer, N processing was intensified, and denitrification became an important N removal pathway. The $\delta^{15}N$ signatures of the light- and dark-side biofilms were similar, a result suggesting less spatial segregation of N cycling processes at this microhabitat scale. Collectively, our results highlight the capacity of WWTP-influenced streams to transform and remove WWTP-derived N inputs and indicate the active role of biofilms in these in-stream processes.

Key words: nitrogen, wastewater treatment plant, stream, biofilm, stable isotopes, nitrification, denitrification.

Assimilation, nitrification, and denitrification are the predominant biological processes undergone by in-stream dissolved inorganic N (DIN) compounds during downstream transport (Bernot and Dodds 2005). Assimilation is biological removal of N from the water column during biosynthetic processes (Kendall et al. 2007). Nitrification is oxidation of NH_4^+ to NO_3^- via NO_2^- and is mediated by several specialized chemolithotrophic bacteria and Archaea

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(Lin et al. 2009, Daims and Wagner 2010). Nitrification decreases the effects of NH_4^+-rich wastewater treatment plant (WWTP) effluents by reducing high concentrations of NH_4^+ that are potentially lethal to resident biota and by converting NH_4^+ to NO_3^-, which can be removed from the stream via denitrification. Denitrification is dissimilatory reduction of NO_3^- to gaseous products, such as N_2, N_2O, or NO and usually occurs at low dissolved O_2 concentrations (Seitzinger 1988, Seitzinger et al. 2006, Lin et al. 2009). These in-stream DIN transformation and removal processes are largely driven by microbial communities (biofilms) that develop on stream substrata and hyporheic sediments (Pusch et al. 1998, Battin et al. 2003).

The ecological relevance of these in-stream N removal and transformation processes is well documented for various pristine and impacted headwaters (Peterson et al. 2001, Mulholland et al. 2008, Beaulieu et al. 2011). Fewer investigators have examined the importance of N removal and transformation in recipient streams with high loads of N from WWTPs (Martí et al. 2010). WWTP effluents are prominent sources of nutrients and microorganisms to recipient streams (Montuelle et al. 1996, Brion and Billen 2000, Gray 2004). WWTP inputs can cause deterioration of water quality and can adversely affect structure and function of stream communities (Miltner and Rankin 1998, Ra et al. 2007, Beyene et al. 2009). Nevertheless, nutrients from the WWTP may be transformed and removed, at least in part, by biofilms in the recipient stream before reaching downstream ecosystems and coastal waters (Howarth et al. 1996, Alexander et al. 2000). However, these processes have not been well characterized and their underlying mechanisms are not well understood.

WWTP-recipient streams have a high capacity for N assimilation, nitrification, and denitrification (Martí et al. 2004, Haggard et al. 2005, Merseburger et al. 2005). In these studies, net N uptake was derived from longitudinal changes in the concentration of DIN species, a measure that integrates removal and release processes along the stream. Longitudinal patterns of stable N isotopes have been used in conjunction with measured concentrations of N compounds to assess processes that drive N cycling in WWTP-recipient streams (De Brabandere et al. 2007, Lofton et al. 2007, Gammons et al. 2011). Nitrification, denitrification, and N assimilation cause isotopic fractionation because bacteria preferentially use the lighter N isotope (^{14}N; Kendall et al. 2007). Ultimately, these processes modify the relative proportion of ^{15}N in the substrate and the product, resulting in an enrichment or depletion of ^{15}N relative to ^{14}N. Therefore, ^{15}N signatures are good indicators of dominance of specific biogeochemical processes associated with DIN cycling. In addition, ^{15}N signatures in biofilms can be used to trace N sources. For instance, N sources, mostly NH_4^+, from WWTPs tend to be highly enriched in ^{15}N (high proportion of ^{15}N to ^{14}N) compared to N from the recipient natural waters because of the preferential use of ^{14}N during biological wastewater treatment (Heaton 1986, Vivian 1986, Cabana and Rasmussen 1996). Together with concentration measurements of the DIN compounds, this differential influence on the ^{15}N signature offers opportunities to trace the fate of N from the WWTP effluent along the recipient stream. Nitrification, as the dominant process in these types of streams (Merseburger et al. 2005), should decrease NH_4^+ concentration and increase NO_3^- concentration, with a concomitant increase in $^{15}NH_4^+$ and decrease in $^{15}NO_3^-$ along the reach (Gammons et al. 2011). Denitrification should decrease NO_3^- and DIN concentrations, with a concomitant increase in $^{15}NO_3^-$ along the reach, regardless of the concentration and ^{15}N signature of NH_4^+ (Lofton et al. 2007). In both scenarios, the ^{15}N signatures of stream biofilms and $^{15}NH_4^+$ in the water should be strongly correlated because NH_4^+ is preferred over NO_3^- as an N-source for assimilation (Dudley et al. 2001, Naldi and Wheeler 2002, Cohen and Fong 2004).

We investigated the capacity of a recipient stream to process DIN inputs from the WWTP effluent and the biogeochemical processes involved. We measured longitudinal patterns of ambient concentrations of DIN species and the patterns of their ^{15}N signatures along a stream reach downstream of a municipal WWTP input. We assessed the role of benthic biofilms in in-stream N processing by comparing longitudinal patterns of biofilm ^{15}N signatures to those of DIN. We sampled biofilms from the upper part of cobbles exposed to light (light-side) and from the lower part of cobbles not exposed to light (dark-side). We conducted our study during 2 contrasting seasonal conditions to assess the effect of changes in environmental conditions on the variability of longitudinal patterns.

Methods

Study site

The study site was in the main course of La Tordera River, immediately downstream of the WWTP outlet of the village of Santa Maria de Palautordera (lat 41°41′7″N, long 2°27′33″E; Catalonia, northeastern Spain). This WWTP treats 11,747 population equivalents, where 1 population equivalent is the biodegradable organic-matter load corresponding to a biological O_2 demand (BOD_5) of 60 g O_2/d. The WWTP provides biological secondary treatment with activated sludge, but not tertiary treatment for N and P removal. Discharge of WWTP effluent is relatively constant over the year (mean = 27.4 L/s), but its contribution to the discharge of the receiving stream depends on hydrological conditions and can range from 3% to 100% (Merseburger et al. 2005). The WWTP effluent has a high concentration of DIN, but the concentration can be highly variable among seasons mainly because of changes in the biologic activity of the WWTP activated sludge (Merseburger 2006). Most DIN (>90%) in the WWTP effluent is in the form of NH_4^+ (Merseburger et al. 2005).

We defined 11 sampling sites along an 850-m-long reach downstream of the WWTP outlet with no lateral surface-water inputs. We used these sites to examine net longitudinal changes in nutrient concentrations and to characterize the ^{15}N signature of NH_4^+, NO_3^-, and biofilms. A sampling site upstream of the WWTP served as control to assess the effect of WWTP input. Channel morphology of the selected reach was characterized by a low sinuosity, a run–riffle sequence with a few shallow pools, and a slope close to 1%. Streambed substrata were dominated by cobbles (34%), pebbles (22%), and boulders (22%). We sampled in winter (11 February 2008) and summer (9 September 2008) to account for possible seasonal changes in WWTP effects on the recipient stream. In winter, we did not sample the site 25 m downstream of the WWTP because cross-sectional measurements of electrical conductivity indicated that at this site, the water coming from the WWTP effluent was not completely mixed with streamwater discharge. In summer, we were unable to sample the site upstream of the WWTP input because it was dry.

Field sampling

We collected surface-water samples for analysis of nutrient concentrations (3 replicates/site) and $\delta^{15}N$ signatures (1 replicate/site) from the mid-channel area. We filtered samples in the field through precombusted Albet (Barcelona, Spain) FVF glass-fiber filters (0.7-µm pore size) into plastic containers and stored them on ice for transport to the laboratory. We processed samples for $^{15}NH_4^+$ analysis immediately (see below) and froze samples for nutrient and $^{15}NO_3^-$ analyses until further processing. We recorded electrical conductivity, water temperature, and dissolved O_2 concentration in the field at each site with WTW (Weilheim, Germany) 340i portable sensors.

We collected composite samples for epilithic-biofilm ^{15}N analysis at each site from 3 randomly selected cobbles by scraping and filtering the biomass onto precombusted and preweighed FVF glass-fiber filters. We sampled the light and dark sides of the same cobbles separately and stored samples on ice for transport to the laboratory.

We calculated stream discharge based on NaCl slug additions at the uppermost site downstream of the WWTP input and at the bottom of the study reach (Gordon et al. 1992).

Laboratory analyses

We analyzed NO_3^- + NO_2^- (hereafter NO_3^- because NO_2^- generally accounts for only 0.5% of DIN in our study stream; Merseburger 2006) and NH_4^+ concentrations in stream-water samples with standard colorimetric methods (APHA 1995) on a Bran+Luebbe (Nordersted, Germany) TRAACS 2000 Autoanalyzer. We calculated DIN concentration as the sum of NO_3^- and NH_4^+ concentrations.

We used the NH_3 diffusion technique (Sigman et al. 1997, Holmes et al. 1998) to process water samples for stable-isotope ($^{15}NH_4^+$ and $^{15}NO_3^-$) analyses. For $^{15}NH_4^+$, we amended samples with 3 g/L of MgO and 50 g/L of NaCl and used a Teflon filter packet containing an acidified glass fiber to trap the diffusing NH_3. For $^{15}NO_3^-$, we removed dissolved NH_4^+ by boiling the samples with 3 g of MgO and 5 g of NaCl and then reduced NO_3^- to NH_4^+ with Devarda's alloy. We treated the remaining sample as for $^{15}NH_4^+$. We diffused a set of standards of known volume and NH_4^+ concentration along with the water samples for volume-related fractionation corrections. We dried (60°C) biofilm samples for ^{15}N signature and weighed subsamples to the nearest 0.001 mg on a Mettler–Toledo MX5 microbalance (Greifensee, Switzerland). All ^{15}N samples were encapsulated in tins and analyzed at the University of California Stable Isotope Facility (Davis, California). We measured N content (as % dry mass) and the abundance of the heavier isotope (expressed as the ^{14}N:^{15}N ratio relative to that of a standard, i.e., N_2 from the atmosphere, $\delta^{15}N$ in units of ‰) by continuous-flow isotope-ratio mass spectrometry (20–20 mass spectrometer; PDZ Europa, Northwich, UK) after sample combustion in an online elemental analyzer (PDZ Europa ANCA-GSL).

Data analysis

We used the longitudinal patterns of ambient nutrient concentrations downstream of the WWTP effluent input to estimate the net nutrient uptake length ($S_{W\text{-}net}$) (Martí et al. 2004), in which the net

Table 1. Physical and chemical characteristics of the study reach in winter and summer. Data from downstream correspond to the 1st site (25 m and 75 m downstream of wastewater treatment plant [WWTP] effluent in summer and winter, respectively). Absence of upstream data in summer is because the stream was dry above the WWTP effluent. Data for nutrient concentrations are mean ± SE of 3 replicate samples.

	Winter		Summer	
Variable	Upstream	Downstream	Upstream	Downstream
Discharge (L/s)	54.2	73.3	–	13.6
Effluent contribution (%)		26		100
Temperature (°C)	10.1	10.9	–	24.8
Electrical conductivity (μS/cm)	182.5	408	–	708
O_2 (mg/L)	9.92	9.92	–	6.17
O_2 saturation (%)	100	100	–	71.8
NO_3^- (μg N/L)	2203 ± 6	1773 ± 16	–	456 ± 53
NH_4^+ (μg N/L)	38 ± 10	4298 ± 19	–	1298 ± 33
DIN (μg N/L)	2241 ± 16	6071 ± 3	–	1701 ± 74
NO_3^-:NH_4^+	58.4	0.4	–	0.3
$\delta^{15}NH_4^+$ (‰)	−7.1	12.9	–	29.7
$\delta^{15}NO_3^-$ (‰)	8.0	9.5	–	11.1

variation of nutrient concentration along the reach can be described as:

$$N_x = N_1 (C_x/C_1) e^{-K_C x} \qquad [1]$$

where N_1 and C_1 are the nutrient concentration and electrical conductivity at the first site downstream of the WWTP input, respectively, and N_x and C_x are the nutrient concentration and electrical conductivity at the site x m downstream of site 1, respectively. K_c is the net nutrient uptake coefficient per unit of reach length (/m); and the negative inverse of K_c equals $S_{W\text{-}net}$. Positive values of $S_{W\text{-}net}$ indicate that the reach acts as a net nutrient sink (nutrient uptake > nutrient release), whereas negative values of $S_{W\text{-}net}$ indicate that the reach acts as a net nutrient source (nutrient uptake < nutrient release). Regardless of the sign, this metric indicates the efficiency with which nutrients are removed from or released to the water column. Longitudinal patterns in NH_4^+ or NO_3^- concentrations along the reach, and thus the K_c values, were assumed to differ from 0 when the fit of ambient values with the Eq. 1 was significant ($p < 0.05$; von Schiller et al. 2011).

We examined longitudinal patterns in $\delta^{15}NH_4^+$, $\delta^{15}NO_3^-$, and $\delta^{15}N$ of the biofilm along the downstream reach with linear regression analysis. To assess the relevance of denitrification or nitrification along the reach, we used Spearman rank correlations to examine the correlation between the concentrations of different DIN species and their $\delta^{15}N$ values. We used a Wilcoxon matched pair test to compare the $\delta^{15}N$ values of the light- and dark-side biofilms downstream of the WWTP. We also used this test to compare biofilm $\delta^{15}N$ values to those of DIN species. Last, we used Spearman rank correlations to examine the relationship between $\delta^{15}N$ values of biofilm and of DIN species with data from both biofilm types separately. We ran statistical analyses with the software PASW Statistics 18 (version 18.0.0; SPSS Inc, Chicago). We evaluated statistical results at the $\alpha = 0.05$ significance level.

Results

Influence of the WWTP effluent on stream physical and chemical variables

The WWTP effluent modified physical and chemical variables in the recipient stream, with noticeable differences between seasons (Table 1). In winter, WWTP effluent accounted for 26% of downstream discharge. Electrical conductivity, NH_4^+, and DIN concentrations increased considerably downstream of the WWTP effluent, whereas relatively small changes were observed in water temperature and NO_3^- concentration. In summer, WWTP effluent accounted for 100% of downstream discharge, and thus, completely defined downstream water chemistry.

Electrical conductivity and water temperature downstream of the WWTP were lower in winter than in summer, whereas dissolved O_2 showed the opposite pattern. Concentration of DIN downstream of the WWTP was higher in winter than in summer because DIN concentration in the effluent was 7× higher in winter than in summer (mean ± SE, 12.6 ± 0.2 and 1.7 ± 0.2 mg/L, respectively). The NO_3^-:NH_4^+ ratio was <1 on both dates. $\delta^{15}NH_4^+$ values downstream of the WWTP were higher in summer than in winter, whereas $\delta^{15}NO_3^-$ values were similar between sampling dates and lower than $\delta^{15}NH_4^+$ values.

Longitudinal patterns of N downstream of the WWTP effluent

Longitudinal patterns of NH_4^+ and NO_3^- concentrations downstream of the WWTP differed between seasons (Fig. 1A, B). In winter, high NH_4^+ concentration downstream of the WWTP effluent decreased gradually along the study reach to yield $S_{W\text{-}net}$ = 4219 m (Fig. 1A). In contrast, the relatively low NO_3^- concentration downstream of the WWTP effluent increased gradually along the study reach to yield $S_{W\text{-}net} = -3212$ m (Fig. 1A). As a result of the opposite longitudinal patterns in NH_4^+ and NO_3^- concentrations, DIN concentration was relatively constant along the reach ($S_{W\text{-}net}$ for DIN was not significant, $p = 0.753$; Fig. 1A). In summer, the NH_4^+ concentration decreased sharply along the reach to yield a relatively short $S_{W\text{-}net}$ (157 m; Fig. 1B). In contrast, NO_3^- concentration showed a hump-shaped longitudinal pattern (Fig. 1B). Over the first 600 m of the reach, $S_{W\text{-}net}$ was −303 m, whereas it was 625 m over the last 250 m of the reach. DIN concentration also showed a hump-shaped pattern similar to that of NO_3^-. $S_{W\text{-}net}$ for DIN was −833 m over the first 600 m, whereas it was 625 m over the last 250 m (Fig. 1B).

The magnitude and longitudinal patterns of the $\delta^{15}N$ values also differed between seasons (Fig. 1C, D). In winter, $\delta^{15}NH_4^+$ values increased along the study reach (linear regression, $p < 0.001$; Fig. 1C), whereas $\delta\ ^{15}NO_3^-$ values decreased (linear regression, $p = 0.001$; Fig. 1C). In summer, $\delta^{15}NH_4^+$ values downstream of the WWTP showed a hump-shaped longitudinal pattern, increasing along the first 600 m (linear regression, $p = 0.001$) and then decreasing over the last 250 m (Fig. 1D). $\delta^{15}NO_3^-$ values gradually increased along the entire reach (linear regression, $p < 0.001$). In both seasons, $\delta^{15}NO_3^-$ values were consistently lower than $\delta^{15}NH_4^+$ values.

The relationships between the concentrations of DIN species and their $\delta^{15}N$ signatures differed between seasons (Fig. 2A–D). In winter, NH_4^+ concentrations and $\delta^{15}NH_4^+$ values were not correlated (Spearman rank correlation, $r = -0.52$, $p = 0.128$; Fig. 2A), whereas NO_3^- concentrations and $\delta^{15}NO_3^-$ were significantly correlated (Spearman rank correlation, $r = -0.67$, $p = 0.03$; Fig. 2B). In summer, concentrations of both DIN species were significantly correlated with their respective $\delta^{15}N$ signatures (Spearman rank correlation, $r = -0.99$, $p < 0.001$; $r = 0.88$, $p = 0.002$ for NH_4^+ and NO_3^-, respectively; Fig. 2C, D).

$\delta^{15}N$ signature of epilithic biofilms

In winter, $\delta^{15}N$ values of light- and dark-side biofilms upstream of the WWTP effluent were similar, whereas $\delta^{15}N$ values of the 2 biofilm types differed significantly downstream (Wilcoxon matched pair test, $p < 0.001$; Fig. 3A). Dark-side biofilms were depleted in $\delta^{15}N$ (mean ± SD = 2.8 ± 1.2‰, range =1.7–5.2‰) compared to light-side biofilms (mean ± SD = 11 ± 2.7‰, range = 6.2–14.9‰). Despite this difference, $\delta^{15}N$ values of both biofilm types increased along the reach downstream of the WWTP (linear regression, $p = 0.034$, $p = 0.005$ for light- and dark-side biofilms, respectively; Fig. 3A). In summer, $\delta^{15}N$ values did not differ between biofilm types (Wilcoxon matched pair test, $p = 0.213$; Fig. 3B), and $\delta^{15}N$ values of both biofilm types increased along the reach downstream of the WWTP (linear regression, $p < 0.001$; Fig. 3B).

In winter, $\delta^{15}N$ and $\delta^{15}NH_4^+$ values of light-side biofilms downstream of the WWTP were similar, but slightly higher than those of $\delta^{15}NO_3^-$. In contrast, $\delta^{15}N$ values of dark-side biofilms were significantly depleted by an average of 10.7‰ and 5.9‰ relative to $\delta^{15}NH_4^+$ and $\delta^{15}NO_3^-$, respectively. $\delta^{15}N$ of both biofilm types were correlated with $\delta^{15}NH_4^+$ (Spearman rank correlation, $r = 0.74$, $p = 0.01$, $r = 0.77$, $p = 0.016$ for light- and dark-side biofilms, respectively; Fig. 4A), but not with $\delta^{15}NO_3^-$ ($r = -0.406$, $p = 0.244$; $r = -0.45$, $p = 0.244$ for light- and dark-side biofilms, respectively, Fig. 4B).

In summer, $\delta^{15}N$ of light- and dark-side biofilms was depleted relative to $\delta^{15}NH_4^+$ by an average of 20.7‰ and 22.2‰, respectively, and it was enriched relative to $\delta^{15}NO_3^-$ by an average of 6.9‰ and 5.7‰, respectively. $\delta^{15}N$ values of light- and dark-side biofilms were not correlated with $\delta^{15}NH_4^+$ (Spearman rank correlation, $r = 0.32$, $p = 0.365$; $r = -0.006$, $p = 0.987$ for light- and dark-side biofilms, respectively; Fig. 4C). In contrast, $\delta^{15}N$ of light- and dark-side biofilms was significantly correlated with $\delta^{15}NO_3^-$ ($r = 0.82$, $p = 0.002$; $r = 0.936$, $p < 0.001$ for light- and dark-side biofilms, respectively; Fig. 4D).

Discussion

N cycling processes in a WWTP-influenced stream

Our results show that the recipient stream was capable of processing a relevant fraction of WWTP-derived N over a relatively short distance. The observed patterns in DIN concentration and $\delta^{15}N$ values were the net result of the interaction of in-stream N removal (e.g., assimilation, denitrification) and release (e.g., nitrification, mineralization) and the

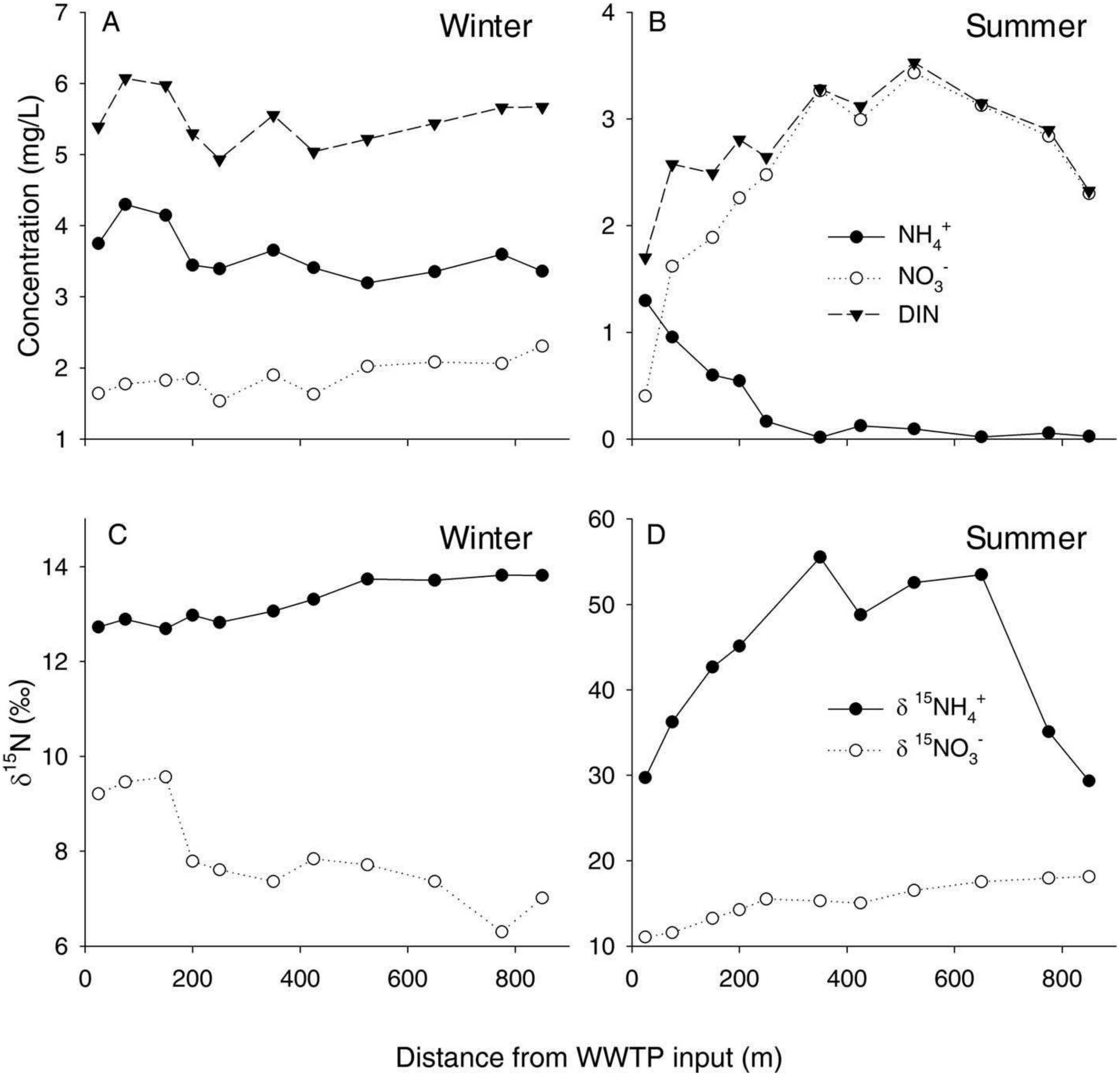

FIG. 1. Variation of ambient concentrations (A, B) and δ^{15}N signatures (C, D) of dissolved N species along the study reach in winter (A, C) and summer (B, D). WWTP = wastewater treatment plant.

differential ^{15}N fractionation involved in each process (Kendall et al. 2007). Thus, concomitant processes may mask patterns for individual processes. Given this observation, the observed patterns suggest differences in the dominance of N cycling processes between the 2 sampling dates. In winter, the longitudinal decrease of the NH_4^+ concentration downstream of the WWTP was counterbalanced by the increase in NO_3^- concentration, resulting in a relatively constant DIN concentration along the reach. These patterns, together with a longitudinal increase in $\delta^{15}NH_4^+$ and a decrease in $\delta^{15}NO_3^-$, suggest that nitrification was important in winter. The negative relationship between NO_3^- concentration and $\delta^{15}NO_3^-$ further corroborates this conclusion. Authors of previous studies have suggested that nitrification is an important process in streams receiving high NH_4^+ loads from WWTPs (Gammons et al. 2011,

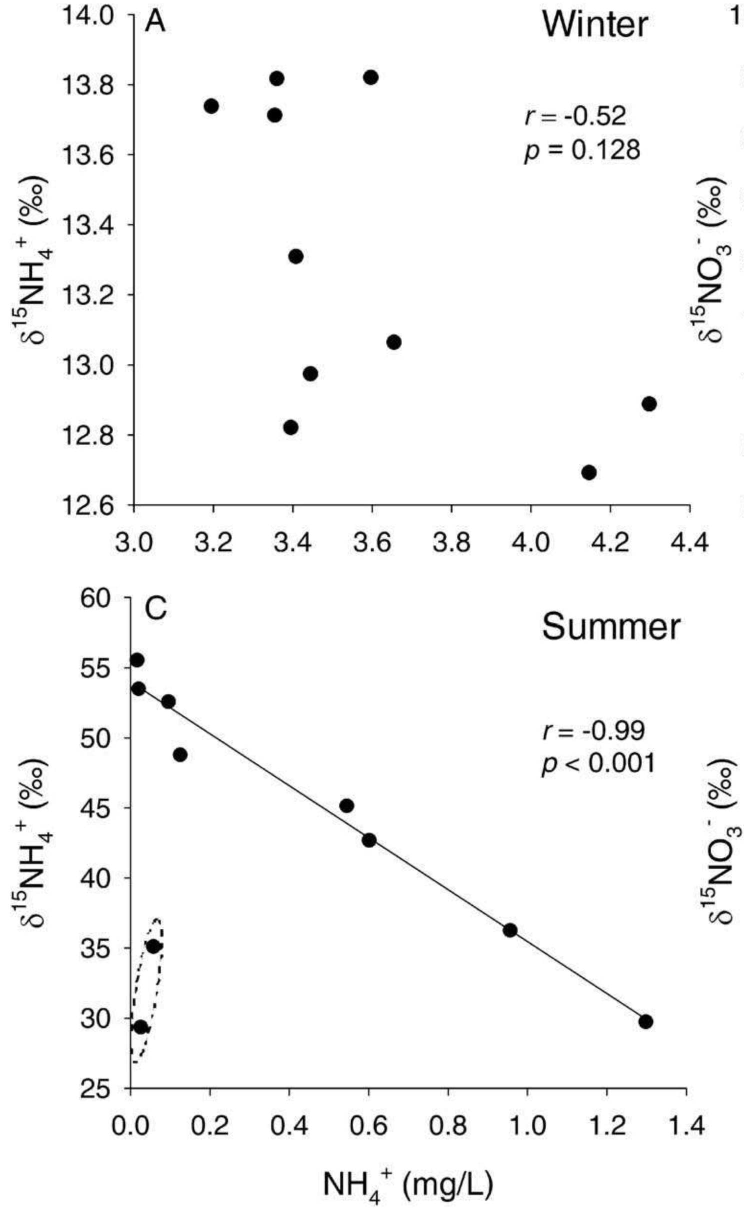

FIG. 2. Relationships between the concentrations of NH_4^+ (A, C) and NO_3^- (B, D) and their respective $\delta^{15}N$ signatures in winter (A, B) and summer (C, D). The dashed ellipse in C indicates 2 outliers of the correlation corresponding with the last 2 sampling sites. Results are for Spearman rank correlations.

Martí et al. 2010). Our N stable-isotope results further support this finding. NH_4^+ concentration and $\delta^{15}NH_4^+$ were not correlated, a result that would be caused by nitrification. Despite its dominance, nitrification rate was not high enough to influence the pattern of $\delta^{15}NH_4^+$. This argument is supported by the relatively long $S_{W\text{-}net}$ of NH_4^+ (in the range of km) in winter, a result indicative of reduced efficiency of NH_4^+ removal. This $S_{W\text{-}net}$ value is long compared to values from forested streams of similar size (Ensign and Doyle 2006), but it is bracketed by values reported from similar WWTP-recipient streams (Martí et al. 2010).

Our results from summer indicate that N cycling was intense and that NH_4^+ transformation and NO_3^- uptake were strongly coupled over a remarkably short stream distance. Longitudinal patterns of NH_4^+ and NO_3^- over the first 600 m of the reach were similar to those observed in winter, but more pronounced. These results and the sharp increase in $\delta^{15}NH_4^+$ indicate high nitrification rates in summer. This finding agrees with those of a previous study in

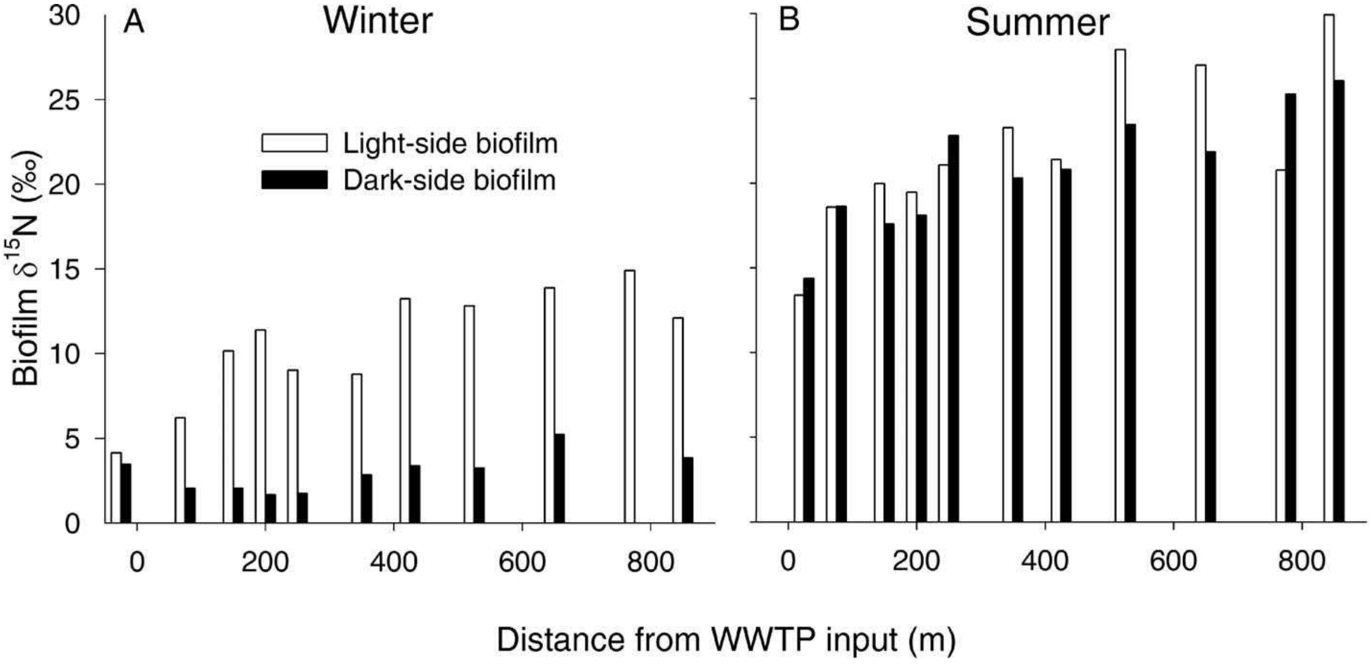

Fig. 3. Variation along the study reach in δ^{15}N values of biofilm types from the light and dark sides of cobbles measured in winter (A) and summer (B). Negative values for distance indicate the site upstream of the wastewater treatment plant (WWTP) input (0 m).

the same stream (Merseburger et al. 2005) and in others showing high nitrification rates downstream of WWTP effluents in summer when water temperature and residence time are elevated (Cebron et al. 2003). However, we also observed an increase in DIN concentration, mainly as NO_3^-, along the first 600 m of the reach, a result suggesting that other sources of N were contributing to this increase. Groundwater inputs were unlikely during dry summer conditions in this losing stream, but the observed DIN increases could have been caused by nitrification of NH_4^+ produced by in-stream mineralization of organic matter, as suggested in a previous study (Haggard et al. 2005). The low dissolved O_2 values in summer suggest high rates of heterotrophic activity, which probably was favored by elevated water temperatures. This activity, in turn, could have resulted in high rates of organic matter mineralization tightly coupled with high nitrification rates (Starry et al. 2005, Teissier et al. 2007).

Nevertheless, the consistent increase in $\delta^{15}NO_3^-$ along the reach in summer clearly differed from the pattern expected had it been driven solely by nitrification, especially considering that NH_4^+ concentration was sharply lower along the upper section of the reach. Possible explanations for this longitudinal $\delta^{15}NO_3^-$ enrichment could be related to processes associated with NO_3^- uptake, such as NO_3^- assimilatory uptake or anaerobic N dissimilatory uptake (i.e., denitrification), which involve isotopic fractionation. The hump-shaped pattern of NO_3^- concentration along the reach provides further support for these explanations. In addition, it suggests a shift along the reach in the relative dominance of nitrification and NO_3^- uptake processes (i.e., assimilation or denitrification, as discussed above). The relevance of nitrification seemed to decrease along the reach concomitantly with the decrease in NH_4^+ concentration. Both denitrification and assimilatory NO_3^- uptake could have contributed to the observed longitudinal decline of NO_3^- concentration over the last section of the reach. Chénier et al. (2006) showed close coupling between photoautotrophic assimilatory NO_3^- uptake and denitrification in river biofilms exposed to high nutrient concentrations. Occurrence of NO_3^- assimilatory uptake by biofilms along the reach in summer is supported by similar δ^{15}N values in biofilms and NO_3^- and a significant correlation between them. In addition, denitrification occurs under conditions of high NO_3^- concentration and low dissolved O_2 concentration, such as those observed in summer in our study stream, which are most favored at oxic/anoxic interfaces of epilithic biofilms and hyporheic sediments (Seitzinger et al. 2006, Lin et al. 2009). Furthermore, denitrification could have been enhanced by the high water temperature during

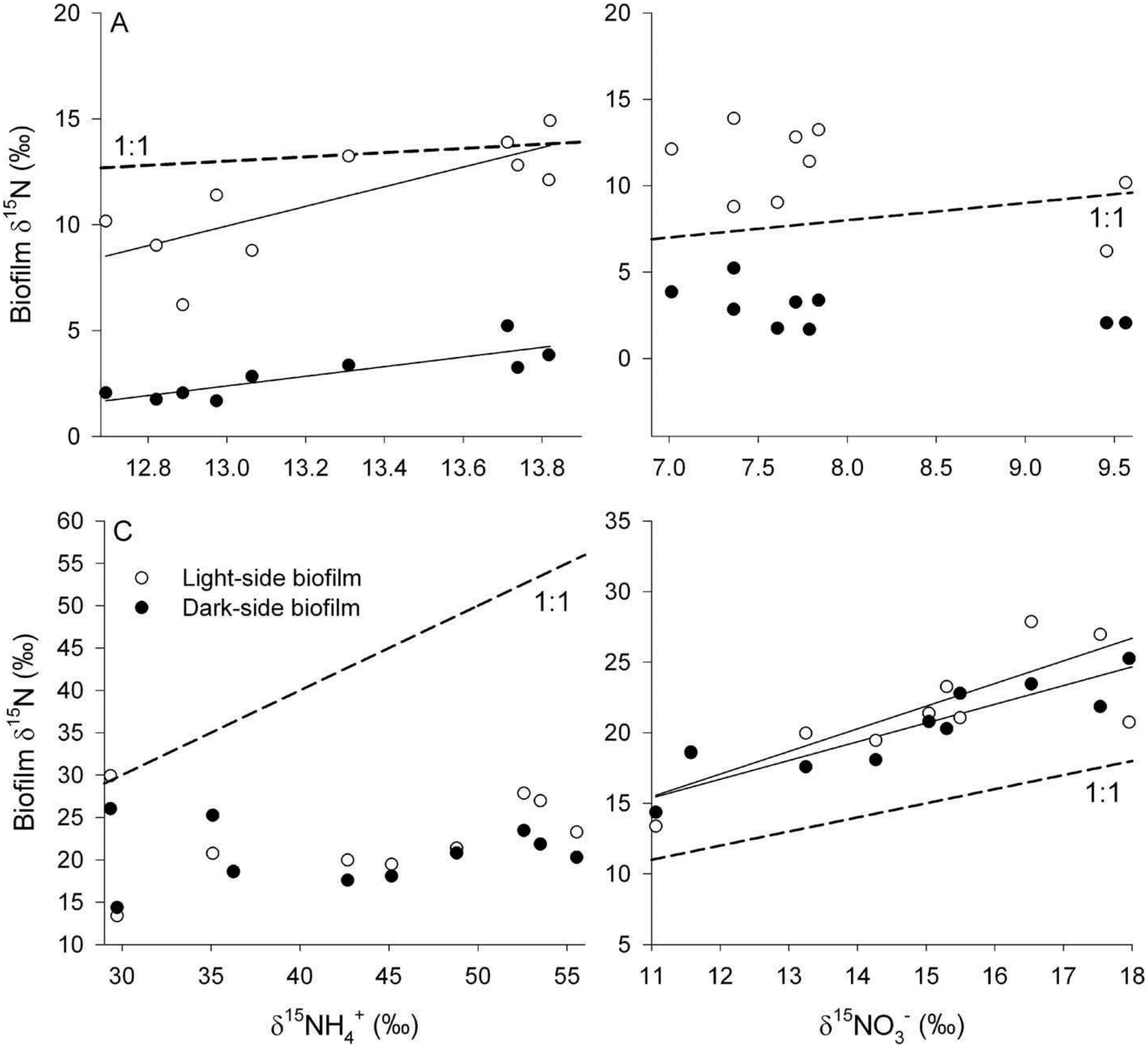

FIG. 4. Relationships between $\delta^{15}N$ signature of NH_4^+ (A, C) and NO_3^- (B, D) and $\delta^{15}N$ signature of the biofilm from the light and dark sides of cobbles in winter (A, B) and summer (C, D). Significant Spearman rank correlations ($p < 0.05$) are indicated by lines. Dashed lines denote 1:1 relationships.

summer (Chénier et al. 2003, Boulêtreau et al. 2012). Supporting these observations, authors of previous studies have reported the importance of in-stream denitrification in WWTP-influenced streams based on trends in stable isotopes (Lofton et al. 2007) or in microbial communities (Wakelin et al. 2008). Regardless of the relative importance of the different processes, our results indicate active N cycling in this recipient stream, especially in summer when streamwater discharge and chemistry were most influenced by the WWTP.

Other processes, such as anammox and dissimilatory nitrate reduction to ammonium (DNRA), may have further contributed to the highly efficient N cycling in summer. However, these processes seem to be more important in lentic than in lotic systems (Op den Camp et al. 2006, Burgin and Hamilton 2007, Zhu et al. 2010), and our data do not allow us to assess their relative importance. NH_3 volatilization, as an alternative explanation for the observed patterns, was unlikely to be an important N removal process in the study reach because pH values in this stream during both study periods were <8 (data from nearby water-quality monitoring station from the Catalan Water Agency; http://aca-web.gencat.cat). We did not directly measure pH in our study, but pH values probably were even lower just downstream from the WWTP effluent than in the nearby monitoring station because of enhanced heterotrophic respiration (Merseburger 2006). In addition, in both seasons the decrease in NH_4^+ concentration was counterbalanced by an increase of NO_3^-, results suggesting no net loss of NH_4^+ along the study reach.

The role of biofilms in N cycling

The WWTP effluent increased both the concentration and $\delta^{15}N$ signature of DIN in the recipient stream, especially for NH_4^+. $\delta^{15}N$ of epilithic biofilms downstream of the WWTP traced the increases of $\delta^{15}N$-DIN. These results suggest that epilithic biofilms were an active compartment in N uptake, contributing to some extent to the observed longitudinal DIN patterns. Nevertheless, we acknowledge that biofilms developed in other stream compartments, such as the hyporheic zone, also could contribute to whole-reach DIN patterns. However, we focused on the role of epilithic biofilms that grow on cobbles because these were the microbial communities coating most of the dominant streambed substrata.

The $\delta^{15}N$ of biofilms varied with time in accordance with the changes of the $\delta^{15}N$ of DIN species, particularly NH_4^+. The biofilm $\delta^{15}N$ signature is a net result of isotope fractionation during N assimilatory and dissimilatory processes (Sulzman 2007). The differences between the $\delta^{15}N$ signatures of light- and dark-side biofilms in winter suggest that processes involved in N cycling differ between communities and provide evidence of fine-scale spatial segregation of biogeochemical processes. In winter, when the riparian canopy was leafless, light-side phototrophic organisms were not light limited, but dark-side organisms were. The difference in available light probably led to differences between dark- and light-side microbial assemblages. Segregation at the microhabitat scale may be the result of the general light intolerance of nitrifying organisms (Prosser 1989, Merbt et al. 2012) or of their poor ability to compete with photosynthetic organisms for NH_4^+ (Risgaard-Petersen et al. 2004). NH_4^+-oxidizing bacteria grow more slowly and have lower N uptake rates than photoautotrophs (Risgaard-Petersen 2003, Risgaard-Petersen et al. 2004), which may favor their development in dark-side environments. However, Teissier et al. (2007) showed that NH_4^+-oxidizing bacteria growing in light-exposed biofilms could compete successfully with algae for NH_4^+, a result that would lead to rejection of the previous argument. Last, nitrifying bacteria from the WWTP may be less competitive for NH_4^+ than autochthonous bacteria, and consequently, they may be forced to the dark-side environment where competition from phototrophs is absent (Cebron et al. 2003). During winter in our study reach, Merbt et al. (2011) found that NH_4^+-oxidizing Archaea developed on both sides of the cobbles, whereas NH_4^+-oxidizing bacteria were found only below the WWTP input and were restricted to the dark-side of cobbles. These results would support findings by Cebron et al. (2003) and may explain the differences we found in $\delta^{15}N$ signature of biofilms coating the light- and dark-sides of cobbles during winter.

In winter, the similar $\delta^{15}N$ signatures between NH_4^+ and light-side biofilms suggest that NH_4^+ from the effluent was partly assimilated by these biofilms without undergoing substantial fractionation. Moreover, $\delta^{15}N$ enrichment of the light-side biofilms was uncoupled from $\delta^{15}NO_3^-$ enrichment, a result suggesting that these biofilm communities preferentially assimilated NH_4^+ over NO_3^-. Similar results have been reported in comparative studies of NH_4^+ and NO_3^- uptake by primary producers (Dudley et al. 2001, Naldi and Wheeler 2002, Cohen and Fong 2004). The enriched $\delta^{15}N$ signature of light-side biofilms contrasts with the depleted $\delta^{15}N$ signatures of the dark-side biofilms, which could be explained by high isotopic fractionation associated with nitrification, in agreement with previous studies (Mariotti et al. 1981, Casciotti et al. 2003). An alternative explanation could be that dark-side biofilms used a different source of N with lower ^{15}N content. However, we could not test this hypothesis because we lack data from DIN sources other than the water column, such as hyporheic water.

The similar $\delta^{15}N$ signatures of the light- and dark-side biofilms in summer suggest less spatial segregation of N cycling processes at the microhabitat scale during this season. In summer, the riparian canopy was completely closed, and light availability in the stream was lower than in winter. Therefore, differences in light availability between the light- and dark-side biofilms were smaller than in winter, and development of photoautotrophs in light-side biofilms probably was limited (von Schiller et al. 2007). This explanation is supported by results obtained by Ortiz (2005), who found that chlorophyll *a* (chl *a*) was an order of magnitude lower in summer (mean =

11.3 mg chl a/m^2) than in winter (mean = 572 mg chl a/m^2) in our study reach. In addition, results of a recent study by Merbt et al. (2012) suggest that nitrifiers could be more active under low-light than under high-light conditions and may not be restricted to the dark side of cobbles. Thus, the compositions of light- and dark-side communities may be more similar in summer than in winter, resulting in similar δ^{15}N signatures. The idea that nitrifiers might be present on both sides of the cobbles in summer may be further supported by the clear ^{15}N-depletion of biofilms relative to $\delta^{15}NH_4^+$ resulting from high isotopic fractionation associated with nitrification. Alternatively, the similar δ^{15}N signature of biofilms to that of $\delta^{15}NO_3^-$ may indicate preferential uptake of NO_3^- during summer conditions, at least over the last 200 m of the reach where the concentration of NH_4^+ was very low. Regardless of the mechanisms underlying N cycling at the biofilm scale, δ^{15}N results indicate that the biogeochemical role of epilithic biofilms in N cycling changes seasonally at both reach and microhabitat scales. Chénier et al. (2006) also observed that the microbial component of river biofilms and its activity vary seasonally, with higher activity and tighter linkage with the phototrophic component of the biofilm in summer than in winter.

Overall, our study revealed that the longitudinal patterns of stream DIN concentrations and δ^{15}N signatures downstream of the WWTP effluent could be used to infer the magnitude and relative dominance of in-stream N cycling processes (e.g., assimilation, nitrification, denitrification) in this N-enriched stream. The observed linkage between the δ^{15}N signal of DIN sources and the biofilm demonstrates the influence of epilithic biofilms on in-stream N cycling in these WWTP-influenced streams. Nonetheless, microbial activity in other stream compartments, such as the hyporheic zone, also could have contributed to the observed whole-reach patterns in DIN concentrations. Our results show clear seasonal differences in the capacity of receiving streams to cycle excess of N from WWTPs and in the dominance of different N cycling processes. Our results highlight the capacity of WWTP-influenced streams to process additional N released from point-source urban-related activities in the adjacent landscape.

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