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

Miquel Ribot¹, Eugènia Martí², Daniel von Schiller³, Francesc Sabater⁴, Holger Daims⁵, Tom J. Battin⁶,⁷

¹ Biogeodynamics and Biodiversity Group, Centre d’Estudis Avançats de Blanes, CSIC, Accés a la Cala St. Francesc 14, 17300, Blanes, Spain. E-mail: mribot@ceab.csic.es. Telf:+34972336101, Fax:+34972337806
² Biogeodynamics and Biodiversity Group, Centre d’Estudis Avançats de Blanes, CSIC, Accés a la Cala St. Francesc 14, 17300, Blanes, Spain. E-mail: eugenia@ceab.csic.es. Telf:+34972336101, Fax:+34972337806
³ Catalan Institute for Water Research, Emili Grahit 101, Edifici H2O, Parc Científic i Tecnològic de la Universitat de Girona, 17003, Girona, Spain. E-mail: dvonschiller@icra.cat. Telf: +34972183380, Fax:+34972183248
⁴ Department of Ecology, Faculty of Biology, University of Barcelona, Avinguda Diagonal 645, 08028 Barcelona, Spain. E-mail: fsabater@ub.edu. Telf: +34934021516, Fax: +34934111438
⁵ Department of Microbial Ecology, Ecology Centre, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria. E-mail: daims@microbial-ecology.net. Telf.+431427754392, Fax:+431427754389
⁶ Department of Freshwater Ecology and Hydrobotany, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria. E-mail: tom.battin@univie.ac.at. Telf:+431427757200, Fax:+431427757209
⁷ Interuniversity Center for Aquatic Ecosystem Research, WasserCluster Lunz, Dr. Carl Kupelwieser Promenade, A-3293 Lunz am See, Austria. Telf: +43074862006030
Abstract

In this study, we investigated how dissolved inorganic nitrogen (DIN) inputs from a waste water treatment plant (WWTP) effluent are biogeochemically processed by the receiving stream. We examined longitudinal patterns of ammonium and nitrate concentrations and their $^{15}$N signatures along a stream reach located downstream of a WWTP effluent. To determine the role of stream biofilms in N processing, we compared the $\delta^{15}$N signatures of epilithic biofilms with those of DIN. We analyzed the $\delta^{15}$N signatures of biofilms coating the light-side and the dark-side surfaces of cobbles separately to test if light constrains may influence the functioning of biofilm communities. We sampled during two contrasting periods of the year (winter and summer) to explore whether changes in environmental conditions had an effect on N biogeochemical processes. Results indicated that the study reach had a remarkable capacity for transformation and removal of DIN; however, 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, which suggested that nitrification was mostly associated with biofilms coating the dark-side of cobbles. In summer, N processing was intensified and denitrification became an important N removal pathway. The $\delta^{15}$N signature of the light- and dark-side biofilms was similar, suggesting a lower spatial segregation of N cycling processes at this microhabitat scale. Collectively, results from this study highlight the capacity of WWTP-influenced streams to transform and remove WWTP-derived N inputs and also indicate the active role of biofilms in these in-stream processes.

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

Assimilation, nitrification and denitrification are the predominant biological processes that in-stream dissolved inorganic nitrogen (DIN) compounds undergo during downstream transport (Bernot and Dodds 2005). Assimilation refers to biological nitrogen (N) removal from the water column during biosynthetic processes (Kendall et al. 2007). Nitrification is the two-step pathway of oxidation of ammonium (NH₄⁺) to nitrate (NO₃⁻) via nitrite, which is mediated by several specialized chemolithotrophic bacteria and archaea (Lin et al. 2009, Daims and Wagner 2010). Nitrification plays an important role in reducing the effects of NH₄⁺-rich waste water treatment plant (WWTP) effluents; both by reducing high concentrations of NH₄⁺ that are potentially lethal to resident biota, and by converting NH₄⁺ to NO₃⁻, which can further be removed from the stream via denitrification. The latter process refers to the dissimilatory reduction of NO₃⁻ to gaseous products such as N₂, N₂O or NO and usually occurs at low dissolved oxygen concentrations (Seitzinger 1988, Seitzinger et al. 2006, Lin et al. 2009). These in-stream transformation and removal processes associated with DIN are largely driven by microbial communities (hereafter referred to as 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 studies 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 constitute prominent sources of nutrients and microorganisms to recipient streams (Montuelle et al. 1996, Brion and Billen 2000, Gray 2004). Ultimately, WWTP inputs can deteriorate water quality and adversely affect both structure and function of the 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 even coastal waters (Howarth et al. 1996, Alexander et al. 2000). Yet, the characterization of these processes and the underlying mechanisms are not well understood.
Past research showed that WWTP-recipient streams have a high capacity for N assimilation, nitrification, and denitrification (Marti 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 both removal and release processes along the stream. More recently, 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 involve isotopic fractionation by preferentially using the lighter N isotope (i.e., $^{14}$N; Kendall et al. 2007). Ultimately, these processes modify the relative proportion of $^{15}$N of both 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 different biogeochemical processes associated with cycling of DIN. In addition, $^{15}$N signatures in biofilms can help tracing distinct N sources. For instance, N sources, mostly NH$_4^+$, from WWTPs tend to be highly enriched in $^{15}$N (i.e., high proportion of $^{15}$N to $^{14}$N) compared to N from the recipient natural waters due to 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 for tracing the fate of N from the WWTP effluent along the recipient stream.

Nitrification, as the previously reported dominant process in these type of streams (Merseburger et al. 2005), is expected to result in a decrease in NH$_4^+$ concentration coupled with an increase in NO$_3^-$ concentration, with a concomitant increase in $^{15}$NH$_4^+$ and a decrease in $^{15}$NO$_3^-$ along the reach (Gammons et al. 2011). In contrast, denitrification is expected to lead to a decrease in 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 either scenario, a strong correlation between the $^{15}$N signature of stream biofilms and the $^{15}$NH$_4^+$ in the water is expected, 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).
This study aimed to investigate the capacity of a recipient stream to process the inputs of DIN from the WWTP effluent and the biogeochemical processes involved. We measured the longitudinal patterns of ambient concentrations of DIN species in conjunction with the patterns of their $^{15}$N signatures along a stream reach located downstream of a municipal WWTP input. In addition, we assessed the role of benthic biofilms on in-stream N processing by comparing the longitudinal patterns of the $^{15}$N signatures of biofilms to those of DIN. For this purpose, we sampled biofilms from the upper part of cobbles exposed to light (hereafter referred to as light-side) and from the lower part of cobbles not exposed to light (hereafter referred to as dark-side). The study was done under two 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 located in the main course of La Tordera river, immediately downstream of the WWTP outlet of the village of Santa Maria de Palautordera (41º 41’ 7’’N, 2º 27’ 33’’E; Catalonia, NE Spain). This WWTP treats 11,747 population equivalents; where 1 population equivalent is the biodegradable organic matter load corresponding to a biological oxygen demand (BOD$_5$) of 60 g O$_2$ day$^{-1}$. The WWTP performs biological secondary treatment with activated sludge, but does not include tertiary treatment for N and phosphorus removal. Discharge of the 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 the stream hydrological conditions and can range from 3% to 100% (Merserburger et al. 2005). The WWTP effluent has a high concentration of DIN, but it can be highly variable among seasons mainly due to changes in the biologic activity of the WWTP activated sludge (Merserburger et al. 2006). Most DIN (>90%) in the WWTP effluent is in the form of NH$_4^+$ (Merserburger et al. 2005).

Eleven sampling sites were defined along a 850-m long reach located downstream of the WWTP outlet with no lateral surface water inputs. These sites were used to examine net longitudinal changes in nutrient concentrations and to characterize the $^{15}$N signature of NH$_4^+$ and NO$_3^-$ as well as that of the
biofilms. An additional sampling site upstream of the WWTP served as control to assess the impact of
the 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 (February,
11th 2008) and summer (September, 9th 2008) to account for possible seasonal changes in WWTP
effects on the recipient stream. In winter, we did not sample the site located 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 stream water discharge. In
summer, the stream was dry upstream of the WWTP input; and thus this site could not be sampled.

Field sampling

Surface water samples for the analysis of nutrient concentrations (3 replicates per site) and \( \delta^{15}N \)
signatures (1 replicate per site) were collected from the mid-channel area, filtered in the field through
precombusted Albet (Barcelona, Spain) FVF glass fiber filters (0.7 \( \mu \)m pore size) into plastic
containers, stored on ice, and brought to the laboratory. Samples for \( ^{15}NH_4^+ \) analysis were immediately
processed (see below). The samples for nutrient analyses and \( ^{15}NO_3^- \) were stored frozen until further
processing. Electrical conductivity, water temperature, and dissolved oxygen concentration were
recorded in the field at each site with WTW (Weilheim, Germany) 340i portable sensors.

Composite samples for epilithic biofilm \( ^{15}N \)-analysis were collected at each site from 3 randomly
selected cobbles by scraping and filtering the biomass onto precombusted and pre-weighted FVF glass
fiber filters. Samples were taken separately from the light- and the dark-side of the same cobbles,
stored on ice in the field and brought to the laboratory for further processing.

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

Laboratory analyses

Stream water samples were analyzed for \( NO_3^- + NO_2^- \) and \( NH_4^+ \) concentrations following
standard colorimetric methods (APHA 1995) on a Bran+Luebbe (Nordersted, Germany) TRAACS
2000 Autoanalyzer. Concentration of \( NO_3^- \) is used hereafter to refer to the concentration of \( NO_3^- + \)
NO$_2^-$, because NO$_2^-$ generally accounts for only 0.5% of DIN in our study stream (Merseburger 2006). DIN concentration was calculated as the sum of NO$_3^-$ and NH$_4^+$ concentrations.

Water samples for stable isotope (i.e., $^{15}$NH$_4^+$ and $^{15}$NO$_3^-$) analyses were processed using the ammonia diffusion technique (Sigman et al. 1997, Holmes et al. 1998). For $^{15}$NH$_4^+$, samples were amended with 3 g/L of MgO and 50 g/L of NaCl and a Teflon filter packet containing an acidified glass fiber to trap the diffusing NH$_3$. For $^{15}$NO$_3^-$, dissolved NH$_4^+$ was removed first by boiling the samples with 3 g of MgO and 5 g of NaCl, followed by the reduction of NO$_3^-$ to NH$_4^+$ with Devarda’s alloy. The remaining sample was then treated as for $^{15}$NH$_4^+$. A set of standards of known volume and NH$_4^+$ concentration were diffused along with the water samples for volume-related fractionation corrections. Biofilm samples for $^{15}$N signature were dried (60 ºC) and subsamples were weighted 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, USA). The N content (as a percent of dry mass) and the abundance of the heavier isotope, expressed as the $^{14}$N:$^{15}$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; PDZEuropa, Northwich, UK) after sample combustion in an on-line elemental analyzer (PDZEuropa 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 ($S_{\text{W-net}}$) (Martí et al. 2004), in which the net variation of nutrient concentration along the reach can be described as:

$$ N_x = N_1 \left( C_x / C_1 \right) e^{-K_c x} $$

[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 downstream site located $x$ m downstream of site 1, respectively. $K_c$ is the net nutrient uptake coefficient per unit of reach length (m$^{-1}$); and the negative inverse of $K_c$ equals $S_{\text{W-net}}$. Positive values of $S_{\text{W-net}}$ indicate that the reach acts as a net nutrient sink (i.e. nutrient uptake > nutrient
release), whereas negative values of $S_{W,\text{net}}$ indicate that the reach acts as a net nutrient source (i.e. nutrient uptake < nutrient release). Regardless of the sign, this metric indicates the efficiency at which nutrients are either removed from or released to the water column. Longitudinal patterns in $\text{NH}_4^+$ or $\text{NO}_3^-$ concentrations along the reach, and thus the $K_c$ values, were assumed to differ from zero when the fit of ambient values with the Eq. 1 was significant ($p<0.05$; von Schiller et al. 2011). Longitudinal patterns in $\delta^{15}\text{NH}_4^+$, $\delta^{15}\text{NO}_3^-$ and $\delta^{15}\text{N}$ of the biofilm along the downstream reach were examined using linear regression analysis. To assess the relevance of denitrification or nitrification along the reach, we examined the correlation between the concentration of the different DIN species and their $\delta^{15}\text{N}$ values using Spearman rank correlations. To compare the $\delta^{15}\text{N}$ values of the light- and dark- side biofilms downstream of the WWTP, we used a Wilcoxon matched pair test. This test was also used to compare biofilm $\delta^{15}\text{N}$ values to those of the DIN species. Finally, we examined the relationship between $\delta^{15}\text{N}$ values of biofilm and of DIN species using Spearman rank correlations with data from both biofilm types separately. Statistical analyses were done with the software PASW Statistics 18 (v18.0.0/SPSS Inc). Statistical results were evaluated at the $\alpha=0.05$ significance level.

**Results**

**Influence of the WWTP effluent on stream physical and chemical parameters**

The WWTP effluent modified physical and chemical parameters in the recipient stream, with noticeable differences between both seasons (Table 1). In winter, the WWTP effluent accounted for 26% of downstream discharge. On this date, electrical conductivity, $\text{NH}_4^+$ and DIN concentrations increased considerably downstream of the WWTP effluent, whereas comparably irrelevant changes in water temperature and $\text{NO}_3^-$ concentration were observed. In summer, the WWTP effluent accounted for 100% of downstream discharge; and thus, completely determined downstream water chemistry.

Electrical conductivity and water temperature downstream of the WWTP were lower in winter than in summer, whereas dissolved oxygen 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 seven times 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. Values of $\delta^{15}$NH$_4^+$ downstream of the WWTP were higher in summer than in winter, whereas values of $\delta^{15}$NO$_3^-$ were similar between sampling dates and lower than those of $\delta^{15}$NH$_4^+$.  

**Longitudinal patterns of N downstream of the WWTP effluent**  

Longitudinal patterns of NH$_4^+$ and NO$_3^-$ concentrations downstream of the WWTP differed between both seasons (Fig. 1A and B). In winter, the high NH$_4^+$ concentration downstream of the WWTP effluent decreased gradually along the study reach, resulting in a $S_{W-net}$ for NH$_4^+$ of 4219 m (Fig. 1A). Conversely, the relatively low NO$_3^-$ concentration downstream of the WWTP effluent increased gradually along the study reach, resulting in a $S_{W-net}$ for NO$_3^-$ of -3212 m (Fig. 1A). As a result of the contrasted longitudinal patterns in NH$_4^+$ and NO$_3^-$ concentrations, DIN concentration remained relatively constant along the reach (i.e., $S_{W-net}$ for DIN was not significant, $p=0.753$; Fig. 1A). In summer, the NH$_4^+$ concentration decreased sharply along the reach (Fig. 1B) resulting in a relatively short (157 m) $S_{W-net}$. In contrast, NO$_3^-$ concentration showed a hump-shaped longitudinal pattern (Fig. 1B). Over the first 600 m of the reach, $S_{W-net}$ for NO$_3^-$ 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$_3^-$. $S_{W-net}$ 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 and D). In winter, the values of $\delta^{15}$NH$_4^+$ increased along the study reach (linear regression, $p<0.001$; Fig. 1C), whereas the $\delta^{15}$NO$_3^-$ values decreased (Fig. 1C; linear regression, $p=0.001$). In summer, values of $\delta^{15}$NH$_4^+$ 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). Values of $\delta^{15}$NO$_3^-$ gradually increased along the entire reach in summer (linear regression, $p<0.001$). In both seasons, values of $\delta^{15}$NO$_3^-$ were consistently lower than those of $\delta^{15}$NH$_4^+$.  

The relationships between the concentration of the different DIN species and their $\delta^{15}$N signature differed between seasons (Fig. 2). In winter, there was no significant correlation between NH$_4^+$ and $\delta^{15}$NH$_4^+$ (Spearman rank correlation, $r=-0.52$, $p=0.128$; Fig. 2A), whereas NO$_3^-$ and $\delta^{15}$NO$_3^-$
were significantly correlated (Spearman rank correlation, r= -0.67, p=0.03; Fig. 2B). In summer, both DIN species were significantly correlated with their $\delta^{15}N$ signatures (Spearman rank correlation; r=-0.99, p<0.001 and r=0.88, p=0.002 for NH$_4^+$ and NO$_3^-$, respectively; Fig. 2C and D).

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

In winter, the $\delta^{15}N$ values of light- and dark-side biofilms located upstream of the WWTP effluent were similar, whereas the $\delta^{15}N$ values of the two 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 to 5.2‰) compared to light-side biofilms (mean±SD=11±2.7‰, range=6.2 to 14.9‰). Despite this difference, the $\delta^{15}N$ values of both biofilm types increased along the reach downstream of the WWTP (linear regression, p=0.034 and p=0.005 for light- and dark-side respectively; Fig. 3A). In summer, there were no significant differences in the $\delta^{15}N$ values between the two biofilm types (Wilcoxon matched pair test, p=0.213; Fig. 3B). However, as in winter, $\delta^{15}N$ values of both biofilm types increased along the reach downstream of the WWTP (linear regression, p<0.001; Fig. 3B).

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

In summer, the $\delta^{15}N$ of both the 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. There were no significant relationships between the $\delta^{15}N$ of biofilms from both cobble sides and the $\delta^{15}NH_4^+$ (Spearman rank correlation, r=0.32, p=0.365 for light-side biofilms and r=0.006, p=0.987 for dark-side biofilms, Fig. 4C). In contrast, the $\delta^{15}N$ of light- and dark-side biofilms was significantly correlated with the $\delta^{15}NO_3^-$ (r= 0.82, p=0.002 for light-side biofilms and r= 0.936, p<0.001 for dark-side biofilms Fig. 4D).
Discussion

*N cycling processes in a WWTP-influenced stream*

Results from this study 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 δ¹⁵N values are the net result of the interaction between in-stream N removal (e.g., assimilation, denitrification) and release (e.g., nitrification, mineralization) as well as the differential ¹⁵N fractionation involved in each process (Kendal et al. 2007). As a consequence, concomitant processes may mask the pattern derived solely by a unique process. With this in mind, observed patterns suggest differences in the dominance of N cycling processes between the two sampling dates.

In winter, we found that the longitudinal decrease of the NH₄⁺ concentration downstream of the WWTP was counterbalanced by the increase in NO₃⁻ concentration, resulting in a relatively constant DIN concentration along the reach. These patterns, together with a longitudinal increase in δ¹⁵NH₄⁺ and a decrease in δ¹⁵NO₃⁻, suggest that nitrification was relevant in winter. The negative relationship between NO₃⁻ concentration and δ¹⁵NO₃⁻ further corroborates this observation. Previous studies have suggested that nitrification is an important process in streams receiving high NH₄⁺ loads from WWTPs (Gammons et al. 2010, Martí et al. 2010). Results from N stable isotopes in this study further support this finding. However, there was no significant relationship between NH₄⁺ concentration and δ¹⁵NH₄⁺, which would also be expected as a result of nitrification. This suggests that despite nitrification was a dominant process, its rate was not high enough to influence the pattern of δ¹⁵NH₄⁺. This argument is supported by the relatively long net uptake length of NH₄⁺ (i.e., in the range of km) measured in winter indicative of reduced NH₄⁺ removal efficiency. This uptake length is long compared to values from forested streams of similar size (Ensign and Doyle 2006), but it is well 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 NH₄⁺ transformation and NO₃⁻ uptake were strongly coupled over a remarkably short stream distance. The longitudinal patterns of NH₄⁺ and NO₃⁻ over the first 600 m of the reach were similar to those observed in winter, but more pronounced. These results, together with a sharp increase in δ¹⁵NH₄⁺, ultimately indicate high...
nitrification rates in summer. This finding agrees with previous studies from the same stream (Merseburger et al. 2005) and others showing high nitrification rates downstream of WWTP effluents in summer when water temperature and residence time are elevated (Cebron et al. 2003). However, in summer we also observed an increase in DIN concentration, mainly as NO$_3^-$, along the first 600m of the reach, suggesting that other sources of N were contributing to this increase. Because groundwater inputs were unlikely during dry summer conditions in this losing stream, the observed DIN increases could be due to nitrification of NH$_4^+$ produced by in-stream mineralization of organic matter as suggested in previous studies (Haggard et al. 2005). The low dissolved oxygen values observed in summer suggest high rates of heterotrophic activity probably favored by elevated water temperatures. This 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 contrasted with the expected pattern if it was solely driven by nitrification, especially considering that NH$_4^+$ concentration was sharply depleted 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. In fact, Chérnier et al. (2006) showed a 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 $\delta^{15}$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 oxygen concentration, such as those
observed in summer in the study stream, which are mostly favored within the oxic/anoxic interfaces of both epilithic biofilms and hyporheic sediments (Seitzinger et al. 2006, Lin et al. 2009). Furthermore, denitrification during summer could have been enhanced by the high water temperature (Chénier et al. 2003, Boulêtreau et al. 2012). Supporting these observations, previous studies have reported the relevance 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 are most influenced by the WWTP. Other processes, such as anammox and DNRA, may further have contributed to the highly efficient N cycling in summer. However, these processes seem to be more relevant in lentic systems (Op Den Camp et al. 2006, Burgin and Hamilton 2007, Zhu et al. 2010) and our data do not allow assessing their relative importance. Ammonia volatilization, as an alternative explanation for the observed patterns, was unlikely 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). Although we did not directly measure pH in our study, pH values just downstream from the WWTP effluent were probably even lower than in the nearby monitoring station do to enhanced heterotrophic respiration (Merseburger et al. 2006). In addition, in both seasons a decrease in NH$_4^+$ concentration was counterbalanced by an increase of NO$_3^-$ 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$_4^+$. Interestingly, $\delta^{15}$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, could also contribute to whole-reach DIN patterns. However, in this study we focused
on the role of epilithic biofilms that grow on cobbles, because these were the microbial communities
coating most of the dominant stream bed substrata.

The $\delta^{15}$N of biofilms clearly varied with time in accordance with the changes of the $\delta^{15}$N of DIN
species, and especially of NH$_4^+$. The biofilm $\delta^{15}$N signature is a net result of the isotope fractionation
during N assimilatory and dissimilatory processes (Sulzman et al. 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 both communities. This is evidence towards a fine-scale spatial segregation of
biogeochemical processes. In winter, light was not a limiting factor for phototrophic organisms
growing on the upper side of cobbles because riparian canopy cover was leaf-less. This clearly
contrasted with light-limited conditions on the dark-side of cobbles, which most likely lead to
differences in microbial communities between dark- and light-side biofilms. This segregation at
microhabitat scale may be due to the general light intolerance of nitrifying organism (Prosser 1989,
Merbt et al. 2012) or to poor competition capacity for NH$_4^+$ with respect to phototrophic organisms
(Risgaard-Petersen 2004). Ammonia-oxidizing bacteria grow slower and have lower N uptake rates
than photoautotrophs (Risgaard-Petersen 2003, Risgaard-Petersen 2004), which may favor their
development on dark-side environments. However, findings by Teissier et al. (2007) showed that
ammonia-oxidizing bacteria growing on light-exposed biofilms could compete successfully with algae
for NH$_4^+$, which would discard the previous argument. Finally, nitrifying bacteria supplied by the
WWTP may be less competitive for NH$_4^+$ than the autochthonous bacteria and consequently, they may
be forced to the dark-side environment where there is no competition by phototrophs (Cebron et al.
2003). In fact, in the same study reach during winter, Merbt et al. (2011) found that ammonia-
oxidizing archaea developed on both sides of the cobbles, whereas ammonia-oxidizing bacteria were
only found below the WWTP input and were restricted to the dark-side of cobbles. These results
would support previous findings by Cebron et al. (2003) and may explain the observed differences 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, the δ¹⁵N enrichment of the light-side biofilms was decoupled from that of δ¹⁵NO₃⁻, which suggests that these biofilm communities displayed a higher preference to assimilate NH₄⁺ than NO₃⁻.

Similar results have been reported in comparative studies of NH₄⁺ and NO₃⁻ uptake by primary producers (Dudley et al. 2001, Naldi and Wheeler 2002, Cohen and Fong 2004). This contrasts with the clearly depleted δ¹⁵N signatures of the dark-side biofilms, which could be explained by a 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 ¹⁵N content. However, this hypothesis cannot be verified in the present study because we lack data from DIN sources other than the water column, such as hyporheic water.

Contrastingly, the similar δ¹⁵N signatures of the light- and dark-side biofilm communities found in summer suggest a lower spatial segregation of N cycling processes at microhabitat scale during this season. In summer, riparian canopy was completely closed, thereby reducing light availability in the stream. Therefore, differences between the light- and dark-side biofilms in terms of light availability were smaller than in winter, and the development of photoautotrophs in light-side biofilms was probably limited (von Schiller et al. 2007). This is supported by results from Ortiz et al. (2005) who found that chlorophyll a was one order of magnitude lower in summer (mean= 11.3 mg Chl a/m²) than in winter (mean= 572 mg Chl a/m²) in this study reach. In addition, a recent study by Merbt et al. (2012) suggests that under low light conditions, nitrifiers could be more active and may not be restricted just to the dark-side of the cobbles. Thus, the compositions of light- and dark-side communities may be more homogeneous in summer than in winter, resulting in similar δ¹⁵N signatures. Presence of nitrifiers on both sides of the cobbles in summer may be further supported by the clear ¹⁵N-depletion of biofilms relative to δ¹⁵NH₄⁺ as a result of the high isotopic fractionation associated with nitrification. Alternatively, the similar δ¹⁵N signature of biofilms to that of δ¹⁵NO₃⁻ may indicate a preferential uptake of NO₃⁻ during summer conditions, at least over the last 200 m of the reach where the concentration of NH₄⁺ was very low. Regardless of the mechanisms underlying N cycling at the biofilm scale, δ¹⁵N results indicate that the biogeochemical role of epilithic biofilms on
N cycling at reach scale changes seasonally and at microhabitat scale. This agrees with Chénier et al. 2006 who observed that both the microbial component of river biofilms and its activity vary seasonally, with higher activity as well as a tighter linkage with the phototrophic component of the biofilm during summer.

Overall, our study revealed that the longitudinal patterns of stream DIN concentrations together with the $\delta^{15}$N signatures downstream of the WWTP effluent were useful 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 $\delta^{15}$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, could have also contributed to the observed whole-reach patterns in DIN concentrations. Our results also 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. Collectively, our results highlight the capacity of WWTP-influenced streams to process the additional N released from point-source urban-related activities along the adjacent landscape.
Acknowledgements

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Naldi, M., and P. A. Wheeler. 2002. $^{15}$N measurements of ammonium and nitrate by Ulva fenestrata (Clorophyta) and Gracilaria pacifica (Rhodophyta): comparison of net nutrient disappearance, release of ammonium and nitrate, and $^{15}$N accumulaion in algal tissue. Journal of Phycology 38:135-144.


Table 1 Physical and chemical characteristics of the study reach on the two seasons (winter and summer). Data from downstream correspond to the first site (25m and 75m downstream of the WWTP effluent in summer and winter, respectively). Lack of upstream data in summer is due to the fact that the stream was dry above the WWTP effluent. Data for nutrient concentrations is the mean±SE of 3 replicate samples.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Winter</th>
<th></th>
<th>Summer</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Upstream</td>
<td>Downstream</td>
<td>Upstream</td>
<td>Downstream</td>
</tr>
<tr>
<td>Discharge (L/s)</td>
<td>54.2</td>
<td>73.3</td>
<td>-</td>
<td>13.6</td>
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<tr>
<td>Effluent contribution (%)</td>
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<td>100</td>
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<td></td>
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<tr>
<td>Temperature (°C)</td>
<td>10.1</td>
<td>10.9</td>
<td>-</td>
<td>24.8</td>
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<tr>
<td>Electrical conductivity (µS/cm)</td>
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<td>408</td>
<td>-</td>
<td>708</td>
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<tr>
<td>Oxygen (mg/L)</td>
<td>9.92</td>
<td>9.92</td>
<td>-</td>
<td>6.17</td>
</tr>
<tr>
<td>Oxygen saturation (%)</td>
<td>100</td>
<td>100</td>
<td>-</td>
<td>71.8</td>
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<tr>
<td>NO$_3^-$ (µg N/L)</td>
<td>2203±6</td>
<td>1773±16</td>
<td>-</td>
<td>456±53</td>
</tr>
<tr>
<td>NH$_4^+$ (µg N/L)</td>
<td>38±10</td>
<td>4298±19</td>
<td>-</td>
<td>1298±33</td>
</tr>
<tr>
<td>DIN (µg N/L)</td>
<td>2241±16</td>
<td>6071±3</td>
<td>-</td>
<td>1701±74</td>
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<td>NO$_3^-$: NH$_4^+$</td>
<td>58.4</td>
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<td>-</td>
<td>0.3</td>
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<td>δ$^{15}$NH$_4^+$ (%)</td>
<td>-7.1</td>
<td>12.9</td>
<td>-</td>
<td>29.7</td>
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<tr>
<td>δ$^{15}$NO$_3^-$ (%)</td>
<td>8.0</td>
<td>9.5</td>
<td>-</td>
<td>11.1</td>
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</tbody>
</table>
Figure legends

Fig. 1. Variation of ambient concentrations and $\delta^{15}$N signatures of dissolved N species along the study reach in winter (A, C) and summer (B, D).

Fig. 2. Relationships between the concentrations of NH$_4^+$ and NO$_3^-$ and their respective $\delta^{15}$N signatures in winter (A, C) and summer (B, D). The dashed ellipse in C indicates the two outliers of the correlation corresponding with the last two sampling sites. Results of Spearman rank correlations are shown.

Fig. 3. Variation along the study reach of the $\delta^{15}$N values of biofilm types from the light-side (white bars) and dark-side (black bars) of cobbles measured in winter (A) and summer (B). Negative distance values denote the location of the upstream site in relation to the WWTP input.

Fig. 4. Relationships between the longitudinal variation of the $\delta^{15}$N signature of dissolved N species and the $\delta^{15}$N signature of the biofilm from the light-side (white circles) and dark-side (black circles) in winter (A, C) and summer (B, D). Significant Spearman rank correlations ($p<0.05$) are shown. Dashed lines denote 1:1 relationships.
Fig. 1

(A) Winter
Concentration (mg/L)

(B) Summer
- $\text{NH}_4^+$
- $\text{NO}_3^-$
- $\text{DIN}$

(C) Winter
$\delta^{15}\text{N}$ (%o)

(D) Summer
- $\delta^{15}\text{NH}_4^+$
- $\delta^{15}\text{NO}_3^-$

Distance from WWTP input (m)
Fig. 3

(A) Winter

(B) Summer

Biofilm $\delta^{15}$N (‰)

Distance from WWTP input (m)

Light-side biofilm
Dark-side biofilm