Effects of human activities on nitrogen cycling in mediterranean streams: contrasts between nitrate and ammonium dynamics

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Tesis doctoral

Universitat de Barcelona

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Effects of human activities on nitrogen cycling in Mediterranean streams: contrasts between nitrate and ammonium dynamics

Memòria presentada per Sr. Miquel Ribot Bermejo
per optar al grau de doctor per la Universitat de Barcelona

Centre d'Estudis Avançats de Blanes (CEAB)
Consejo Superior de Investigaciones Científicas (CSIC)

Miquel Ribot Bermejo

Vist i plau dels directors i tutor de la tesis

La directora de la tesi	*El codirector de la tesi*	*El tutor de la tesi*
Dra. Eugènia Martí i Roca	**Dr. Daniel von Schiller Calle**	**Dr. Francesc Sabater i Comas**
Científica titular al CSIC	Investigador a la Universidad del País Vasco (UPV-EHU)	Professor al Departament d'Ecologia (UB)

Als meus pares

Agraïments

Arribats a aquest punt, un mira enrere i es dona compte de la quantitat de gent que ha col·laborat de manera directa/indirecta, voluntària/involuntària o fins conscient/inconscientment per què avui, aquesta Tesi sigui una realitat. En el meu cas, podríem dir que ha estat un llarg viatge que va començar l'any 2004 quan per primera vegada vaig posar els peus en aquesta casa i sobretot en aquest grup. Poc que m'ho podia pensar llavors que això acabaria així, però bé, ja se sap, la vida hijo...

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Dani: joder tronco, que difícil sem fa donar-te les gràcies per tot al que has fet per mi. Vam començar essent companys de feina compartint aventures amb la C-15, després vam compartir pis durant uns quants anys i finalment has acabat essent el meu director de tesis. Crec sincerament que ets una d'aquestes persones tocades per una vareta màgica. Per sobre de tot però, et considero un d'aquells amics que diuen que es poden comptar amb els dits d'una mà.

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Steffi, Clara, Edu, Elliot, Susana (Bernis), Lollo, Sandra, Paula, Alba Argerich: Amb alguns hem compartit el passat més passat, amb d'altres el més recent i amb d'altres el present. Vull pensar que les persones s'acaben ajuntant amb gent similar i això voldria dir que sóc una bona persona i algú que val la pena conservar per tota la vida. Ha estat un plaer haver compartit tantes aventures científiques, extraescolars i nocturnes. No sé que serà de tots nosaltres en el futur, però espero que els nostres camins es segueixin creuant de manera recurrent.

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Per últim agraïr als diferents projectes d'investigació i institucions que m'han permès realitzar aquesta tesis doctoral:

- Contracte de Personal técnico de apoyo del Ministerio de Ciencia e Innovación associat al projecte d'investigació GL2008-05504-C02-02/BOS: Isótopos estables de nitrógeno en ecosistemas fluviales, papel de los componentes bióticos como indicadores de fuentes y procesos del nitrógeno (ISONEF).
- Projecte CGL2005-7362: Influence of nitrogen concentrations on stream nutrient dynamics: contrast among sources and biogeochemical processes associated with ammonium and nitrate (NICON). Plan Nacional de I+D+I del MCI
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- Generalitat de Catalunya per concedir-me l'ajuda BE (2012 BE1-00379) que em va permetre realitzar l'estada a Arizona, EUA.

Miquel Ribot Bermejo

Blanes, 22 de Gener de 2015

Informe del directors

La Dra. Eugènia Martí Roca del Centre d'Estudis Avançats de Blanes (CEAB-CSIC) i el Dr. Daniel von Schiller Calle de la Universidad del País Vasco (UPV-EHU), directors de la Tesi Doctoral elaborada pel Sr. Miquel Ribot Bermejo i que porta per títol *"Effects of human activities on nitrogen cycling in Mediterranean streams: contrasts between nitrate and ammonium dynamics"*.

INFORMEN

Que els treballs de recerca portats a terme pel Sr. Miquel Ribot Bermejo com a part de la seva formació pre-doctoral i inclosos a la seva Tesi Doctoral han donat lloc a dos articles publicats, un article enviat que està en procés de revisió, i un manuscrit que està a punt de ser enviat a una revista d'àmbit internacional. A continuació, es detalla la llista d'articles així com els índexs d'impacte (segons el SCI de la ISI Web of Knowledge) de les revistes on han estat publicats o bé s'han enviat els treballs.

1. Ribot, M., E. Martí, D. von Schiller, F. Sabater, H. Daims, and T. J. Battin. 2012. Nitrogen processing and the role of epilithic biofilms downstream of a wastewater treatment plant. Freshwater Science. **31:** 1057-1069.

A l'any de publicació d'aquest treball, la revista Journal of the North American Benthological Society (JNABS) va canviar el seu nom a Freshwater Science. Per tant, s'han utilitzat els indicadors per l'any 2012 quan encara tenia el nom de JNABS. L'índex d'impacte del JNABS al 2012 va ser de 2.957. Aquesta revista està inclosa a la categoria "Marine and Freshwater Biology". Aquesta categoria té una mediana d'índex d'impacte de 1.411 i inclou un total de 100 revistes. Tenint en compte l'índex d'impacte de la

revista Freshwater Science a l'any de la publicació de l'article, aquesta ocupa el 9è lloc de la seva categoria, quedant inclosa en les revistes del 1er quartil.

2. Ribot, M., D. von Schiller, M. Peipoch, F. Sabater, N. B. Grimm, and E. Martí. 2013. Influence of nitrate and ammonium availability on uptake kinetics of stream biofilms. Freshwater Science. **32:** 1155-1167.

A l'any 2013, la revista Freshwater Science ja té un índex d'impacte propi, essent de 1.423. Aquesta revista està inclosa a la categoria "Marine and Freshwater Biology" i inclou un total de 103 revistes. Aquesta categoria té una mediana d'índex d'impacte de 1.423. Tenint en compte l'índex d'impacte de la revista Freshwater Science a l'any de la publicació de l'article, aquesta ocupa el lloc 52 de la seva categoria , quedant inclosa en les revistes del 3er quartil.

3. Ribot, M., D. von Schiller, F. Sabater, and E. Martí. Biofilm growth and nitrogen uptake responses to increases in nitrate and ammonium availability. Aquatic Sciences (en revisió).

L'índex d'impacte de la revista Aquatic Sciences l'any 2013 va ser de 2.712. Aquest revista està inclosa a la categoria "Marine and Freshwater Biology" i inclou un total de 103 revistes. Aquesta categoria té una mediana d'índex d'impacte de 1.423. Tenint en compte l'índex d'impacte de la revista Aquatic Sciences a l'any de la publicació de l'article, aquesta ocupa el lloc 13 de la seva categoria, quedant inclosa en les revistes del 1er quartil.

Alhora, CERTIFIQUEN

Que el Sr. Miquel Ribot Bermejo ha participat activament en el desenvolupament del treball de recerca associat a cadascun d'aquests articles

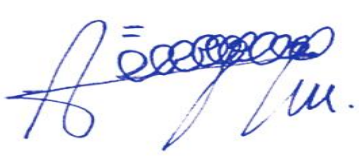
així com en la seva elaboració. En concret, la seva participació en cadascun dels articles ha estat la següent:

- Participació en el plantejament inicial dels objectius de cadascun dels treballs els quals estaven emmarcats en dos projectes del Plan Nacional del Ministerio de Ciencia e Innovación (ISONEF i MED_FORESTREAM) i en un projecte aprovat per la Comissió Europea i finançat per la European Science Fundation (Coupling biofilm diversity and ecosystem functioning: the role of communication and mixing in microbial landscapes; COMIX).
- Plantejament i realització de la part experimental de cada estudi, i posada a punt de les metodologies de camp i de laboratori associades a cadascun dels experiments.
- Processat i anàlisi de totes les mostres obtingudes. Càlcul de resultats i anàlisi estadístic de les dades.
- Redacció dels articles i seguiment del procés de revisió dels mateixos.

Finalment, certifiquem que cap dels co-autors dels articles abans esmentats i que formen part de la Tesi Doctoral del Sr. Miquel Ribot Bermejo, ha utilitzat o bé té present utilitzar implícita o explícitament aquests treballs per a l'elaboració d'una altra Tesi Doctoral.

Atentament,

Blanes, 22 de Gener 2015



Eugènia Martí Roca Daniel von Schiller Calle

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Chapter 1

General Introduction

Cover: river surrounded by agricultural fields in the American northeast. Photograph by Miquel Ribot

1.1 Stream and river networks in human-impacted landscapes

Stream and rivers networks are hierarchically organized in a set of subsystems, which interact from large to small spatial scales (Frissell et al. 1986). Due to their inherent characteristic of unidirectional flow, physical and chemical variables in fluvial networks present a gradient from headwaters to river mouth that shapes in-stream biological communities (Vannote et al. 1980). Climate, geology and vegetation of the catchment exert a strong influence on stream and river ecosystems. Therefore, it was early assumed that a holistic view of the stream and its catchment was fundamental to understand in-stream patterns and processes (Hynes 1975).

During the last decades, humans have caused a worldwide transition in the landscape from undeveloped land to agricultural and urban areas (Foley et al. 2005). Land transformations cause multiple morphological, hydrological and chemical alterations in the catchments as well as in the stream ecosystems; including the modification of water courses via channelization or damming, changes in the hydrological regime mainly due to water abstraction, and a general increase in the availability of bioreactive elements such as nitrogen (N) and phosphorous (P; Malmqvist and Rundle, 2002; Allan, 2004).

The expansion and intensification of agricultural practices to supply the food demand of an increasing human population are major contributors to the human-driven land transformations worldwide (Matson et al. 1997). Over the last decades, generalized use of high-yielding crop varieties, fertilizers and pesticides as well as technological advances related to irrigation and mechanization have resulted in a large increase of worldwide food production (Matson et al. 1997). Agricultural practices, however, have negatively affected natural ecosystems, including nutrient enrichments of ground and surface water bodies through diffuse pathways (Carpenter et al. 1998; Withers and Lord, 2002; Monteagudo, Moreno and Picazo, 2012; Ballantine and Davies-Colley, 2014). Studies conducted across European freshwater ecosystems have revealed that diffuse sources, mostly derived from

agriculture, can account for up to 60% and 25% of the total N and P loads, respectively (DEFRA 2007; EEA, 2012).

On the other hand, during the last decade and for the first time in human history, half of the human population is concentrated in urban areas (Grimm et al. 2008a). Urbanization has increased the human pressure on fluvial ecosystems (Grimm et al. 2008b; Paul and Meyer, 2008). The array of biological, chemical and hydrogeomorphological alterations that usually affect urban streams has been commonly denominated as the "urban stream syndrome" (Walsh et al. 2005; Paul and Meyer, 2008). In developed countries, the implementation of sewer systems and waste water treatment plants (WWTP) in urban areas has contributed to reduce the point source inputs of nutrients and other pollutants to streams derived from urban activity (Martí, Riera and Sabater, 2010). However, despite relevant technological advances during the last decades, WWTP effluents still represent important point sources of nutrients and microorganisms to recipient streams, which can ultimately cause deterioration of the water quality and the ecological status of these ecosystems (Montuelle et al. 1996; Brion and Billen, 2000; Gray, 2004; Mussmann et al. 2013). In terms of nutrient enrichments, point sources may account for >50% of stream and river N and P loads (Martí, Riera and Sabater, 2010).

It is worth noting that the effects of human activity on running waters, especially point sources from urban activity, may be exacerbated in regions with water scarcity, such as the Mediterranean region, due to the low dilution capacity of anthropogenic inputs from the catchments. This is the case of the study streams where this Thesis has been conducted. Mediterranean climate is characterized by warm, dry summers, and mild, humid winters. These conditions dictate the overall seasonal hydrologic regime of these streams, which can become intermittent during dry summers. In addition, events of intense precipitation during the year are common with episodic events of extreme flooding. Drying and flooding events ultimately shape the natural structure and function of these streams (Acuña et al. 2005; von Schiller et al.,

2011; Bernal et al. 2013; Bonada and Resh, 2013). We need to take into account that on top of this hydrologic variability, streams on this region are subjected to episodic inputs from diffuse and point sources and constant inputs from urban WWTP point sources, which ultimately determine the chemistry of these streams. These conditions are likely to become intensified under the climate change predictions for the Mediterranean region, which point to an overall decrease in precipitation, but an increase in episodic heavy rain events (Giorgi and Lionello, 2008). Last but not least, the Mediterranean area has been densely populated for centuries, with large areas occupied by agricultural land, of which a large part have recently been transformed to urban land use (Gasith and Resh, 1999; Gurluk, 2009). This change increases the demand of freshwater at specific points in the catchment, which further reduces stream flow and increases the relevance of urban stream inputs to the receiving streams. Therefore, streams from this region are particularly susceptible to the influence of urban activity.

1.2 Nitrogen enrichment in human-impacted streams

Considering elemental cycling at global scale, that of N is probably the most altered by human activities. This is exemplified by a 70% increase in bioreactive N inputs into fluvial ecosystems since the 19th century (Galloway et al. 2004, Seitzinger et al. 1998). Understanding the effects of in-stream N enrichment is critical because N is a key element for organisms and its availability can either limit ecosystem production or favor eutrophication (Dodds and Welch, 2000; Francoeur, 2001; Smith, Tilman and Nekola, 1999). Moreover, there is a growing body of evidence showing that changes in land use driven by human activity not only increase in-stream N concentrations, but also modify the relative availability of the two major dissolved inorganic nitrogen (DIN) species: nitrate (NO_3^-) and ammonium (NH_4). Agriculture causes N enrichment of the draining streams mainly in the form of NO_3^- (Stanley and Maxted, 2008; Von Schiller et al. 2008b; Lassaletta et al. 2009; Ballantine and Davies-Colley, 2014; Wang et al.

2014). On the other hand, urban storm water runoff and WWTP effluents tend to cause N enrichments in receiving streams in the form of NH_4^+ (Marti et al. 2004; Merseburger, Martí and Sabater, 2005; von Schiller et al. 2008b; Martí, Riera and Sabater, 2010). As a consequence, despite overall increases in DIN concentration, the in-stream NO_3^-:NH_4^+ ratio may vary according to the proportion of the different land uses within the catchment (Fig. 1.1) , which may have distinct influences on the way DIN is processed in the streams.

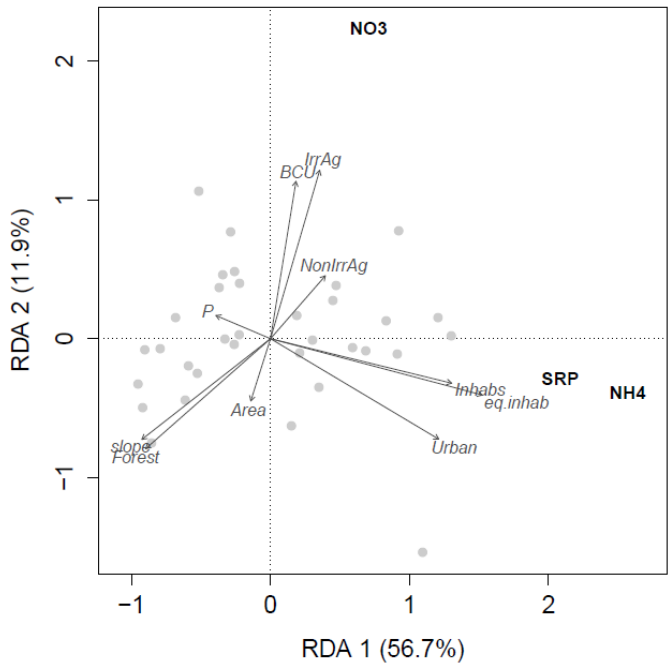

Figure 1.1 RDA analysis of in-stream nutrient concentrations vs. catchment characteristics in 31 headwater catchments in Catalonia (NE Spain). Note that nitrate (NO3) concentration is positively associated with irrigated agriculture (IrrAg) and bovine cattle units (BCU), and negatively associated with mean catchment slope (slope) and percent forest land (Forest), In contrast, phosphorus (SRP) and ammonium (NH4) concentration appear associated with urban point sources (Urban: percent urban land use, Inhabs: number of inhabitants, eq. inhab: inhabitant equivalents). These results illustrate the incidence of different land use activities on the relative proportion of the two DIN species in stream water. Other variables included in the analysis are: P: precipitation, Area: log area, NonIrrAg: non irrigated agriculture. Source: Joan Lluís Riera, modified from Martí, Riera and Sabater (2010).

1.3 Nutrient spiraling in streams

Before the 1980's, running waters were traditionally considered as mere conduits transporting materials originated at the adjacent terrestrial ecosystems to the ocean. However, studies conducted over the past decades have shown that streams and rivers have the capacity to take up dissolved nutrients, such as N and P (Marti and Sabater, 1996; Peterson et al. 2001; von Schiller et al. 2008a; Mulholland et al. 2009). Nutrient uptake in stream ecosystems not only occurs in the stream benthos, but also in saturated sediments from hyporheic and parafluvial zones as well as in the riparian zone (Fisher et al. 1998). The different in-stream assimilatory and dissimilatory uptake processes (see below) can lead to the retention, transformation and removal of nutrients form the water column during downstream transport. In this sense, the nutrient spiraling concept emerged in the late 1970's (Webster and Patten, 1979; Newbold et al. 1981) as a conceptual framework, which proposes to combine nutrient cycling and downstream transport to understand nutrient dynamics in streams. According to this concept, nutrient cycling in streams is best described by a spiral, and the tightness of the spiral indicates the degree at which nutrients are used within the streams (Webster and Patten, 1979).

The first studies investigating stream nutrient spiraling focused on P using tracer additions of the radioactive isotope ^{32}P (Newbold et al. 1983; Mulholland et al. 1985). Some years later, research on in-stream nutrient spiraling developed strongly through the increased use of additions of regular nutrient salts (Stream Solute Workshop 1990; Marti and Sabater, 1996; Valett et al. 1996; Butturini and Sabater, 1998) and of stable isotope ^{15}N tracers (Mulholland et al. 2001; Peterson et al. 2001; Mulholland et al. 2009). Most studies to date have been conducted in pristine or relatively low-impacted streams (Peterson et al. 2001; von Schiller et al. 2008a; Marti and Sabater, 1996; Ensign and Doyle, 2006). Results from these studies point out the ecological relevance of these streams in the removal and transformation of N and P. However, more recently, there has been an increasing effort to evaluate

nutrient spiraling in human-impacted streams, such as those receiving WWTP inputs (Marti et al. 2004; Merseburger, Martí and Sabater, 2005; Lofton, Hershey and Whalen, 2007) or those draining catchments with agricultural and urban activities (Mulholland et al. 2008; Von Schiller et al. 2008b; Bernot et al. 2006). Collectively, results from these studies suggest that despite human-impacted streams also have a bioreactive capacity to process nutrients, the efficiency at which nutrients are taken up is modified compared to that in undisturbed streams. Nevertheless, despite some studies have shown the in-stream uptake response to increases in DIN availability (Dodds et al. 2002, Newbold et al. 2006, Mulholland et al. 2008), few studies have compared the in-stream uptake response to the increases in the concentration of the two different species of DIN.

1.4 Biogeochemical pathways of in-stream N uptake.

N uptake in streams is mostly driven by microbial assemblages (i.e. biofilms), which develop on stream benthic substrata and hyphoreic sediments (Pusch et al. 1998; Teissier et al. 2007; Battin et al. 2008). Biofilms are complex structures composed of algae, bacteria and fungi embedded in a mucopolysaccharide matrix (Lock et al. 1984). In streams, benthic biofilms are ubiquitous since they develop on inorganic substrates (cobbles and finer sediments), which are also referred to as epilithic biofilms, and on organic detritus such as leaves, small wood and fine benthic organic matter (FBOM). Relative composition of the biofilms varies depending on each substrata and the environmental conditions. For instance, biofilm on FBOM mainly hosts microbial assemblages dominated by bacteria (Findlay et al. 2002), whereas in relatively un-shaded streams, biofilms on cobbles have a high dominance of diatoms and filamentous algae (Romani and Sabater, 1999). In addition, in open low land streams, macrophytes can substantially contribute to stream N uptake (Riis et al. 2012; Pastor et al. 2013; Peipoch et al. 2013).

During downstream transport, NO_3^- and NH_4^+ undergo different biogeochemical pathways (Fig. 1.2), which are mainly mediated by the biotic

components of the stream. The two DIN species can be taken up from the water column by in-stream biota for biosynthetic processes (i.e., assimilatory uptake; Kendall et al. 2007; Pastor et al. 2013). NH_4^+ diffuses passively through the cellular membrane; thus, it can be directly incorporated into biomass via anabolic pathways. Conversely, incorporation of NO_3^- into the cells requires an energy-consuming active pumping and a further reduction to NH_4^+ (McCarty, 1995; Geisseler et al. 2010). Therefore, it is often assumed that biota prefer NH_4^+ over NO_3^- due to the lower energetic cost to assimilate the former N species (Naldi and Wheeler, 2002; Hildebrand, 2005). The assimilated N is temporarily retained in the organic pool, since it can be converted to inorganic N that will be released to the water column during mineralization, another bacterial-mediated biogeochemical pathway that renders NH_4^+ (Teissier et al. 2007; O'Brien et al. 2012).

Besides assimilatory uptake, NO_3^- and NH_4^+ can also undergo different energy-yielding dissimilatory pathways (Fig. 1.2). NO_3^- serves as substrate for two bacterial-mediated pathways that occur under sub-oxic to anoxic conditions: denitrification and dissimilatory nitrate reduction to ammonium (DNRA), which results into the elimination of DIN from the system or in its transformation to another DIN source. In denitrification, NO_3^- is sequentially reduced to N_2O and N_2 gas in presence of organic matter, thereby it results in a permanent removal of NO_3^- from the ecosystem (Seitzinger et al. 2006; Lin et al. 2009). In DNRA, NO_3^- is reduced to NH_4^+ under anaerobic conditions (Silver, Herman and Firestone, 2001; Burgin and Hamilton, 2007). While denitrification may account for a significant proportion of total NO_3^- uptake in streams (Mulholland et al. 2008; von Schiller, Marti and Riera, 2009), the importance of DNRA in NO_3^- removal is still relatively unknown (Burgin and Hamilton, 2007).

NH_4^+ also serves as substrate for two other bacterial-mediated dissimilatory pathways: nitrification and anaerobic ammonium oxidation (anammox). Nitrification refers to the aerobic NH_4^+ oxidation to NO_3^- by which some specialized chemoautotrophic bacteria and archaea meet their

energy demand (Prosser 1989; Lin et al. 2009). Anammox refers to the anaerobic oxidation of NH_4^+ to N_2 using NO_2^- as the electron acceptor (Op den Camp et al. 2006). Nitrification may account for a large portion of total NH_4^+ uptake in streams since the oxygenized in-stream conditions favor this process (e.g. Peterson et al. 2001), while anammox has been less studied in lotic systems and its contribution to the DIN cycling is still under consideration (Burgin and Hamilton, 2007).

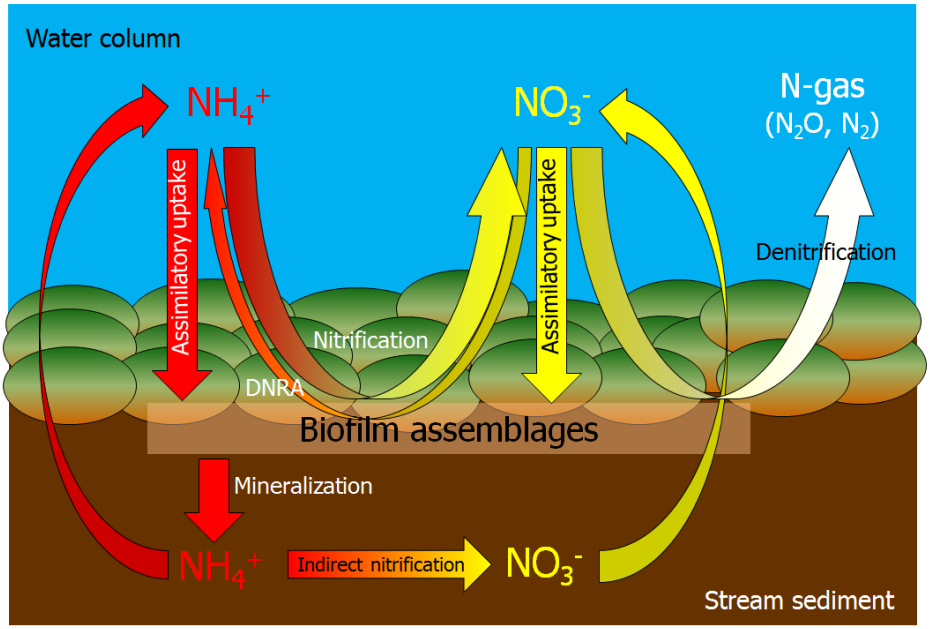

Figure 1.2. Main biogeochemical pathways driving DIN spiraling in streams. DIN concentration as NO_3^- and NH_4^+ are assimilated into organic forms during assimilatory uptake. In addition, in-stream uptake of the two DIN species can undergo differential specific energy-yielding dissimilatory pathways. During nitrification, NH_4^+ is oxidized to NO_3^- under oxic conditions. In addition, NO_3^- can be removed from the water column by either denitrification (transformation into N-gas compounds such as N_2O or N_2 under anaerobic conditions) or by DNRA which produces NH_4^+. Organic N can also be oxidized to NH_4^+ during mineralization. Indirect nitrification refers to the immediate nitrification of the NH_4^+ released from mineralization.

In-stream uptake pathways of NO_3^- and NH_4^+ can be sensitive to the concentration of the two DIN species. For instance, increases in NO_3^- or NH_4^+ concentration may enhance assimilatory uptake fluxes of in-stream biota, however excess of either DIN species usually produce the saturation of DIN uptake (Kemp and Dodds, 2002; Naldi and Wheeler, 2002; O'Brien and Dodds, 2008). Furthermore, microbial assimilation of NO_3^- is induced by the presence of NO_3^-, while it is suppressed by the presence of NH_4^+ (Geisseler et al. 2010; Gonzalez et al. 2006; Cresswell and Syrett, 1979). Therefore, uptake of the two DIN species is subjected to the availability of each specific DIN species; and thus, the relative proportion between them can ultimately affect the in-stream uptake of DIN. In addition, increases in NO_3^- availability may enhance in-stream denitrification, but with a concomitant decrease in the uptake efficiency (Mulholland et al. 2008). Other studies also point out that increases in NH_4^+ concentration may enhance in-stream nitrification fluxes, but excess of NH_4^+ may also produce saturation of this process (Kim, Lee and Keller, 2006; Vadivelu, Keller and Yuan, 2007). Furthermore, in-stream nitrification may also be favored by increases in NO_3^-. Increases in this DIN species may alleviate heterotrophic N demand, thus NH_4^+ may mostly fuel chemoautotrophic activity (Bernhardt, Hall and Likens, 2002). Therefore, increases in DIN availability and changes in the NO_3^-:NH_4^+ ratio may alter in-stream DIN uptake pathways, with potentially relevant but widely unexplored consequences on the downstream export of DIN (Bernot and Dodds, 2005; O'Brien et al. 2007).

1.5 The use of stable isotopes in stream DIN spiraling research

During the last 40 years, the ratio of abundance of the stable isotopes of N (^{14}N and ^{15}N) has been increasingly used in ecological studies (Fry, 2008). This is based on the fact that the natural abundance of the heavier isotope (i.e., ^{15}N) is very low with respect to the lighter isotope (i.e., ^{14}N). In addition, ^{15}N-

enriched compounds have been used as tracers to measure the rates of in-stream specific biogeochemical N pathways. This approach has allowed measuring assimilatory uptake, nitrification or denitrification in laboratory mesocosm studies (Eppley, Rogers and McCarthy, 1969; Naldi and Wheeler, 2002; Bunch and Bernot, 2012) and specific rates at whole-reach scale (Mulholland et al. 2000; Peterson et al. 2001; Mulholland et al. 2008). Some studies have taken advantage of the distinct ^{15}N signature of WWTP effluents to trace biogeochemical pathways of NH_4^+ and NO_3^- along the streams based on the changes in ^{15}N fluxes in the receiving streams (Lofton, Hershey and Whalen, 2007). In the ^{15}N tracer experiments, addition of low quantities of $^{15}NO_3^-$ or $^{15}NH_4^+$ are required to produce strong isotopic enrichments of the DIN pool in the water column, while keeping the overall DIN concentration at ambient levels. This allows researchers to trace N across biogeochemical N pathways as well as to quantify its transference to in-stream biota at whole reach scale. Moreover, the low quantities involved in ^{15}N tracer additions result in a negligible increase of ambient NO_3^- or NH_4^+ concentrations, avoiding the confounding effects of in-stream N enrichment that typically occur when using the classical additions of regular nutrient salts (Fry, 2008;(Mulholland et al. 2000).

In this Thesis, we used a combination of the ^{15}N-related methodologies described above in field and mesocosm settings to measure the key biogeochemical DIN pathways in a range of nutrient concentrations (from ambient to experimental DIN enrichments).

1.6 Conceptual framework and objectives of the present Thesis

The inherent capacity of stream ecosystems to take up DIN depends on multiple factors operating both at catchment scale as well as within the stream channel. Since humans have modified large parts of the natural landscape,

there is a need to revisit the classical view of in-stream N dynamics within the context of humanized landscapes. A remarkable effort has been made in recent years to disentangle those factors that control in-stream DIN cycling in both pristine and human-impacted streams. Among these factors, DIN concentration has been shown to drive variability in N uptake among streams (O'Brien et al. 2007; Dodds et al. 2002; Newbold et al. 2006; Von Schiller et al. 2008b). However, only few studies have explicitly considered differences in in-stream N uptake between the two DIN species (i.e., NO_3^- and NH_4^+). This comparison is relevant within the current context of widespread in-stream DIN enrichment and the changes in the ratio between NO_3^- and NH_4^+ caused by land use change.

The general goal of the present Thesis is to understand how in-stream DIN uptake is affected by changes in DIN availability and speciation induced by human activities. To approach this objective, the Thesis has been developed within the context of a conceptual framework for DIN uptake in human-impacted streams, which considers catchment-scale controls, reach-scale processes and habitat-scale mechanisms (Fig. 1.3). Basically, we assume that human activities not only cause in-stream DIN enrichment, but also modify the NO_3^-:NH_4^+ ratio through changes in catchment land use (Fig. 1.3A). Therefore, in streams draining catchments with human activity, the total DIN concentration and the relative proportion between NO_3^- and NH_4^+ is influenced by both the degree of land transformation and the dominant type of human activity (urban, agricultural, industrial). DIN spiraling at whole-reach scale depends on the biogeochemical pathways of NO_3^- and NH_4^+ uptake in the stream (Fig. 1.3B). In-stream uptake (both assimilatory and dissimilatory) of DIN species is mainly mediated by microbial assemblages that develop as biofilms on benthic substrata (Fig. 1.3C). Within this conceptual framework, the working hypoThesis of the present Thesis is that DIN uptake in streams will be influenced by the relative availability of NO_3^- and NH_4^+ because biotic assimilatory uptake demand may differ between the two DIN species and dissimilatory uptake processes are distinct for each DIN species. Ultimately, this Thesis seeks to increase our understanding of DIN uptake in stream

ecosystems within the context of global change by particularly focusing on the potential distinct uptake of the two major DIN species (i.e., NO_3^- and NH_4^+), which can have implications for DIN downstream transport.

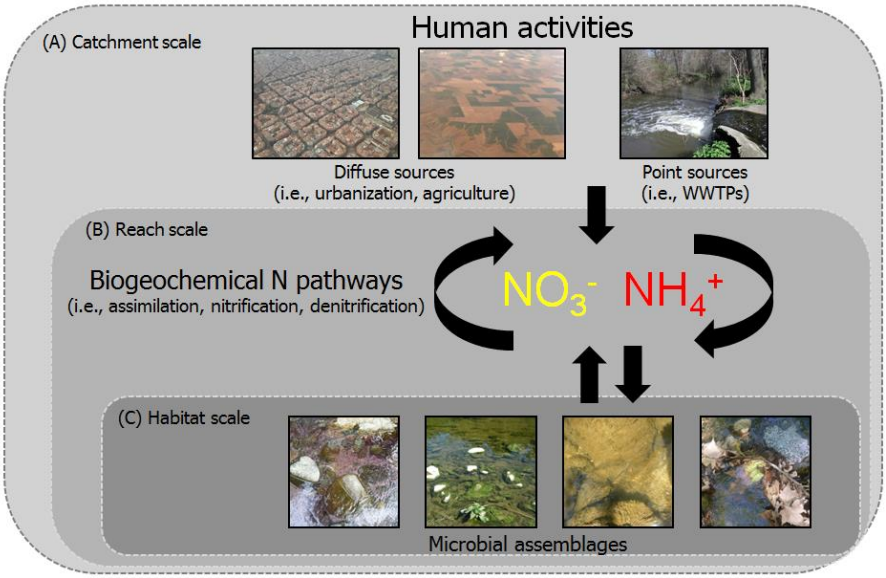

Figure 1.3. Conceptual framework of dissolved inorganic nitrogen (DIN as both NO_3^- and NH_4^+) spiraling in human-impacted streams used to approach the objectives of this Thesis. The framework considers land use changes in the catchment by human activity as sources of DIN species to streams, in-stream biogeochemical processes driving uptake of DIN species, and microbial assemblages developed on stream substrata as the major players of in-stream DIN uptake.

This Thesis is articulated in four chapters, each corresponding to independent specific questions, which are related to the general goal:

Chapter 2. This chapter examines biofilm DIN uptake kinetics and compares them between NO_3^- and NH_4^+. The study was conducted in two streams differing in ambient DIN concentration to better understand the responses of biofilms to increases in DIN availability. Results from this study contribute to understand the underlying mechanism responsible for differences in DIN uptake in streams with varying DIN concentrations.

Chapter 3: This chapter explores biofilm responses in terms of growth and DIN uptake to chronic increases in NO_3^- and NH_4^+ and compares them among streams differing in ambient DIN concentrations. Results from this study shed light on the effects of sustained DIN enrichments, typically induced by land use change, on the biofilm capacity to assimilate NO_3^- and NH_4^+ from the water column.

Chapter 4. This chapter investigates the capacity of a stream receiving N inputs from a WWTP effluent to process DIN by examining the main biogeochemical pathways involved and the potential role of benthic biofilms. Results contribute to understand DIN spiraling in a stream with high DIN concentration and characterized by a low NO_3^-:NH_4^+ ratio, as commonly observed in streams receiving inputs from urban WWTP effluents.

Chapter 5. This chapter compares uptake of NO_3^- and NH_4^+ at whole-reach scale by using two different approaches: (i) ^{15}N-tracer additions of either NO_3^- or NH_4^+ in a Mediterranean stream and, (ii) a literature survey of data of nutrient spiraling metrics for the two DIN species in streams worldwide. Results shed light on the pathways and biotic mechanisms associated with NO_3^- and NH_4^+ uptake as well as on the differences between the two DIN species, which are often considered interchangeable.

Chapter 2

Influence of nitrate and ammonium availability on uptake kinetics of stream biofilms

Original publication in Ribot M., von Schiller D., Peipoch M., Sabater F., Grimm N.B. & Martí E. (2013) Influence of nitrate and ammonium availability on uptake kinetics of stream biofilms. *Freshwater Science***,** 1155-1167

Cover: experimental channels filled by naturally colonized cobbles collected from the stream. Photograph by Miquel Ribot.

2.1. Abstract

Human activity has significantly increased dissolved inorganic N (DIN) availability and has modified the relative proportion of NO_3^- and NH_4^+ species in many streams. Understanding the relationship between DIN concentration and DIN uptake is crucial to predicting how streams will respond to increased DIN loading. Nonetheless, this relationship remains unclear because of the complex interactions governing DIN uptake. We aimed to evaluate how biofilms from 2 streams differing in background DIN concentration would respond to increases in availability and changes in speciation (NO_3^- or NH_4^+) of DIN. We measured DIN uptake by biofilms in artificial flumes in each stream, using separate ^{15}N-NO_3^- and ^{15}N-NH_4^+ additions in a graded series of increasing DIN concentrations. The ambient uptake rate (U) was higher for NO_3^- than for NH_4^+ in both streams, but only U for NH_4^+ differed between streams. Uptake efficiency ($U_{N\text{-}specific}$) in ambient conditions was higher in the low-N than in the high-N stream for both DIN species. A Michaelis–Menten model of uptake kinetics best fit the relationship between uptake and concentration in the case of NH_4^+ (for both streams) but not in the case of NO_3^- (neither stream). Moreover, saturation of NH_4^+ uptake occurred at lower rates (lower U_{max}) in the low-N than in the high-N stream, but affinity for NH_4^+ was higher (lower K_s) in the low-N stream. Together, these results indicate that the response capacity of biofilm communities to short-term increases of DIN concentration is determined primarily by the ambient DIN concentrations under which they develop. Our study also shows that DIN uptake by benthic biofilms varies with DIN availability and with DIN speciation, which often is modified by human activities.

2.2. Introduction

Human activities have significantly increased the concentration of dissolved inorganic N (DIN) in streams (Howarth et al. 1996, Carpenter et al. 1998). Understanding how stream DIN uptake (i.e., the process by which stream biota immobilize DIN from the water column) responds to human alteration of DIN availability has become a research focus for stream ecologists (Mulholland and Webster 2010). Some researchers have studied DIN uptake kinetics (i.e., changes in uptake rates [*U*] in response to changes in concentration) based on the relationship between whole-reach DIN uptake and DIN concentration by using measurements from different streams spanning a broad range of background DIN concentrations (Dodds et al. 2002, Bernot et al. 2006, Newbold et al. 2006, O'Brien et al. 2007). Other researchers have focused on DIN uptake kinetics within the same stream by following changes in whole-reach uptake in response to short-term DIN enrichment (Payn et al. 2005, Earl et al. 2006, Covino et al. 2010, O'Brien and Dodds 2010) or by investigating DIN uptake kinetics in mesocosms (Eppley et al. 1969, Kemp and Dodds 2002, O'Brien and Dodds 2008).

Three mathematical models describe the relationship between DIN uptake and concentration in streams. The first model corresponds to a 1st-order response in which uptake flux (μg N m^{-2} s^{-1}) is directly proportional to concentration of substrate (Dodds et al. 2002). The 2nd model, the efficiency–loss model, follows a power relationship in which *U* increases but efficiency declines with concentration (O'Brien et al. 2007). The 3rd model follows Michaelis–Menten kinetics and is characterized by saturation of uptake when availability exceeds biological demand (Earl et al. 2006). In general, results from interstream comparisons suggest that the linear and efficiency–loss models best fit the relationship between DIN uptake and concentration (Dodds et al. 2002, O'Brien et al. 2007). Conversely, results from enrichment experiments in the same stream or in mesocosms (i.e., with the same community) suggest that the Michaelis–Menten model best fits DIN uptake kinetics (Payn et al. 2005, Earl et al. 2006, Covino et al. 2010, O'Brien and

Dodds 2010).

Human activities also change the relative proportions of the 2 major DIN species: NO_3^- and NH_4^+ (Stanley and Maxted 2008, Lassaletta et al. 2009, Martí et al. 2010). *U* and kinetics are expected to differ between NO_3^- and NH_4^+ because energetic costs of assimilation associated with NO_3^- are generally higher than those associated with NH_4^+ (Dortch 1990, Naldi and Wheeler 2002). Furthermore, dissimilatory transformations, in which neither compound is incorporated into biomass, contribute to NH_4^+ and NO_3^- uptake. Nitrification (i.e., oxidization of NH_4^+ to NO_3^- by autotrophic or heterotrophic Bacteria and Archaea) will result in apparent NH_4^+ uptake, whereas apparent NO_3^- uptake may include denitrification (i.e., the respiratory process by which bacteria reduce NO_3^- to N_2). These transformations are carried out by different organisms and governed by different controlling factors (Bothe et al. 2007), and thus, may contribute to the expected differences between NO_3^- and NH_4^+ uptake kinetics. Most researchers have investigated NO_3^- or NH_4^+ uptake separately. Thus, we do not know how uptake kinetics differ between these 2 DIN species under similar environmental conditions. In addition, little is known about differences in uptake kinetics of NO_3^- or NH_4^+ of stream biofilms (i.e., the microbial communities that develop on stream substrata) associated with increases in DIN availability. Understanding DIN uptake kinetics of stream biofilms is especially important because biofilms are major contributors to nutrient dynamics in stream networks (Pusch et al. 1998, Battin et al. 2003) and, therefore, may help ameliorate anthropogenic DIN inputs.

We compared *U* and kinetics for NO_3^- and NH_4^+ between biofilms developed in 2 streams differing in background DIN concentrations. We measured biofilm *U* in experiments in which we separately added ^{15}N-labeled NO_3^- and NH_4^+ at increasing concentrations to artificial flumes in each stream. We predicted that ambient uptake flux would be higher for NO_3^- than for NH_4^+ and in the high-N than in the low-N stream because of higher availability of NO_3^- with respect to NH_4^+ and the overall higher DIN

availability in the high-N stream. In terms of uptake kinetics, we predicted that the Michaelis–Menten model would best fit the relationship between DIN uptake and concentration because DIN uptake is mediated by enzymatic processes. In particular, we expected lower maximum uptake (U_{max}) and ½-saturation constant (K_s) for NH_4^+ than for NO_3^- because of the lower energetic cost of assimilation of NH_4^+ than of NO_3^-. We further expected U_{max} and K_s to be lower in the low-N stream than in the high-N stream because of differences in N affinity between stream biofilms resulting from different histories of nutrient exposure.

2.3. Methods

Study sites

Font del Regàs (lat 2°27′00″E, long 41°49′32″N; 929 m asl) is a forested stream situated within the protected area of the Parc Natural del Montseny at the headwaters of the catchment of the river La Tordera. Santa Coloma (lat 2°37′52″E, long 41°52′18″N; 425 m asl) is an agricultural stream situated next to gardening plantations in a lower part of the same catchment. Discharge (mean ± SE) was 56 ± 12 L/s for Font del Regàs and 163 ± 35 L/s for Santa Coloma (biweekly samplings from September 2004–July 2007; MR, DvS, FS and EM, unpublished data). Concentrations of NO_3^- and NH_4^+ were 181 ± 11 µg N/L and 12 ± 1 µg N/L for Font del Regàs, and 780 ± 44 µg N/L and 19 ± 2 µg N/L for Santa Coloma (biweekly samplings from September 2004–July 2007; MR, DvS, FS and EM unpublished data). Hereafter, we refer to Font del Regàs as the low-N stream and to Santa Coloma as the high-N stream.

Channel experiments

We conducted experiments from 3 to 24 July 2007 in the low-N stream and from 23 October to 7 November 2007 in the high-N stream. We placed a set of 6 parallel polyvinyl chloride (PVC) channels (6 m long × 15 cm wide) on the stream bed in a metal structure that held them together and above the stream water (Fig. 2.1A). Water from an upstream tank fed all channels

continuously with a mean (± SE) flow rate of 1.8 ± 0.018 L/min (from measurements done daily throughout the experiments and in each channel). We filled the channels with stream cobbles of similar size and biofilm cover that were collected from the stream bed <50 m upstream from the channel setting. We exposed channels to 5 sequential 24-h fertilization cycles each with an increased concentration (1, 4, 8, 16, and 32× background concentration) of either NO_3^- or NH_4^+ (n = 3 channels each; Fig. 2.1A, B). We released solutions of NO_3^- (as $NaNO_3$) or NH_4^+ (as NH_4Cl) to the corresponding channels at a constant rate from a 3-output carboy (1/channel). We maintained a constant head in each carboy with a Masterflex (Vernon Hills, Illinois) L/S battery-powered peristaltic pump. We also added PO_4^{3-} (as $NaH_2PO_4 \cdot H_2O$) proportionally into the solution at each fertilization level to maintain the background stoichiometric ratio between DIN and soluble reactive P (SRP) throughout the fertilization cycles.

We conducted a tracer addition of either $^{15}NO_3^-$ (n = 3 channels) or $^{15}NH_4^+$ (n = 3 channels) over the last 6 h of each fertilization level to estimate U of biofilms. We added solutions amended with $^{15}NO_3^-$ (as 99% enriched $K^{15}NO_3$) or $^{15}NH_4^+$ (as 99% enriched $^{15}NH_4Cl$) and NaCl as a conservative tracer at a constant rate using a similar setup as described above. We calculated the amount of $K^{15}NO_3$ and $^{15}NH_4Cl$ needed to produce a target $\delta^{15}N$ enrichment of 3000‰ for both DIN species in the channels. To verify plateau conditions, we logged conductivity every 10 seconds at the end of each channel with a portable WTW conductivity meter (Weilheim, Germany).

Prior to fertilizations, we collected water at the downstream end of each channel for analysis of ambient nutrient concentrations (3 replicates/channel) and $^{15}NH_4^+$ and $^{15}NO_3^-$ signatures (1 replicate/channel). We also collected composite biofilm samples for the analysis of biomass, pigment content, and natural abundance of ^{15}N (1 replicate/channel) by scraping 3 randomly selected cobbles and filtering the biomass onto combusted, preweighed glass-fiber filters (GF/Fs; Whatman, Maidstone, UK). Before completing each fertilization period (when fertilization and ^{15}N addition were running

together), we collected another set of water and biofilm samples (3 replicates/channel) for analysis of nutrient concentration and $^{15}NH_4^+$ and $^{15}NO_3^-$ signatures. Then we stopped the additions, emptied the channels, cleaned them, and filled them again with cobbles from the stream to initiate the experiment with a higher fertilization level (Fig. 2.1B).

We filtered the water samples immediately through combusted GF/Fs into acid-washed, plastic containers and stored them on ice for transportation to the laboratory. We estimated the cobble surface area by covering it with Al foil and weighing the foil. We stored the filters with biofilm samples on ice in the field and froze (for chlorophyll *a* analysis) or oven-dried them (for ash-free dry mass [AFDM] and ^{15}N analysis) in the laboratory until further processing. We logged photosynthetically active radiation (PAR) every 10 min with a SKP215 quantum sensor (Skye, Powys, UK) connected to a Campbell Scientific data logger (Logan, Utah, USA). We measured temperature at plateau conditions with a WTW 340i portable conductivity meter (Weilheim, Germany).

Laboratory analyses

We analyzed water samples for concentrations of NO_3^-, NH_4^+, and SRP on a Bran+Luebbe (Norderstedt, Germany) TRAACS 2000 autoanalyzer with standard colorimetric methods (APHA 1995). We processed water samples for analysis of $^{15}NO_3^-$ and $^{15}NH_4^+$ with the NH_3-diffusion technique (Sigman et al. 1997 and Holmes et al. 1998, respectively). To measure $^{15}NO_3^-$, we amended a known volume of sample with 3 g of MgO and 5 g of NaCl and boiled it to remove the NH_4^+. We then added 0.5 mg MgO and 0.5 mg Devarda's alloy to reduce the NO_3^- to NH_4^+, and treated the remaining sample as for $^{15}NH_4^+$. For $^{15}NH_4^+$ determination, we amended a known volume of sample with 3 g/L of MgO and 50 g/L of NaCl and a Teflon filter packet containing a 1-cm-diameter combusted Whatman GF/D fiber glass filter acidified with 25 µL of 2.5 M $KHSO_4$ (to trap the volatilized NH_3), and incubated it on a shaker at 40°C for 4 wk. Once the incubation was

completed, we removed the filter packets and placed them in a desiccator for 4 d. We encapsulated filters in tins and stored them until ^{15}N analysis.

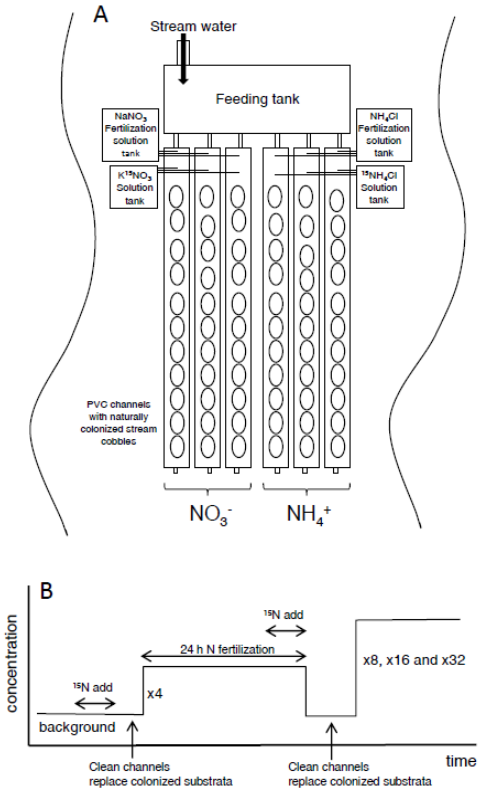

Figure 2.1. Scheme of the channel setting used to experimentally approach the objectives of our study. A.—In-situ channel structure. Upstream water supplied the feeding tank, which in turn, fed each polyvinyl chloride (PVC) channel independently. Fertilization and ^{15}N amended solutions for NO_3^- or NH_4^+ reached each single channel independently (3 channels for each dissolved inorganic N [DIN] species). B.—Detail of experimental design to conduct the different fertilization levels (24 h each) and the ^{15}N-tracer additions (add; during the last 6 h of each fertilization treatment) to measure biofilm N uptake for each DIN species (3 channels for each DIN species treatment). For each N fertilization cycle, we used a new set of colonized substrata collected upstream of the channel setting.

We oven-dried filters with biofilm samples at 60°C until they reached a constant mass. To estimate the biofilm AFDM (g/m^2), we weighed subsamples on a Sartorious (Göttingen, Germany) MC1 analytical balance and combusted them at 500°C for 5 h. We measured biofilm chlorophyll *a* content ($\mu g/cm^2$) following McIntire et al. (1996). We submerged frozen filters in a known volume of 90% volume/volume acetone and kept them in the dark at 4°C overnight. We sonicated the filters for 5 min and centrifuged them for 10 min at 4000 rpm. We measured the absorbance of the resultant supernatant at 664, 665, and 750 nm before and after acidification with a Shimadzu (Tokyo, Japan) ultraviolet (UV) spectrometer. To determine the ^{15}N signature of biofilms, we weighed 1-cm diameter subsamples to the nearest 0.001 mg on a Mettler-Toledo (Greifensee, Switzerland) MX5 microbalance and encapsulated them in tins. We sent the samples for analysis at the University of California Stable Isotope Facility (Davis, California). We measured the N content (as % dry mass) and the abundance of the heavier isotope, expressed as the ^{14}N:^{15}N ratio compared to that of a standard (N_2 from the atmosphere) using the notation of $\delta^{15}N$ 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).

Calculation of U and data analysis

We used independent *t*-tests to explore differences in ambient nutrient concentrations, biofilm AFDM, and biofilm chlorophyll *a* content between streams.

To calculate the uptake rates of NO_3^- and NH_4^+, we first calculated the amount of ^{15}N tracer contained in biofilm ($^{15}N_{biofim}$; µg N/m^2) with the equation:

$$^{15}N_{biofilm} = B_{biofilm}N(MF_i - MF_b)/100 \quad (1)$$

where $B_{biofilm}$ is the biofilm biomass as dry mass per unit area, N is the biofilm N content expressed as % dry mass, MF is the molar fraction of ^{15}N in biofilm at plateau conditions (MF_i) and at background conditions (MF_b).

We estimated the biofilm U (µg N m^{-2} s^{-1}) for NO_3^- or NH_4^+ with the equation (adapted from von Schiller et al. 2007):

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

where $^{15}N_{biofim}$ is the amount of ^{15}N tracer in biofilm biomass from eq. 1, $T_{addition}$ is the duration of the ^{15}N addition (6 h), $^{15}N_{flux}$ is the ^{15}N flux (as either NO_3^- or NH_4^+) at plateau conditions in the channel water and N_{flux} is the total N flux (as NO_3^- or NH_4^+) at each fertilization level in the channel water based on concentration and channel flow rate (µg N/s). We then calculated the biomass-specific U ($U_{N\text{-}specific}$; d^{-1}) for biofilm communities and DIN species as a surrogate of N uptake efficiency by dividing biofilm U (µg N m^{-2} s^{-1}) by the N content of dry mass (µg N/m^2).

To compare U and $U_{N\text{-}specific}$ for NO_3^- and NH_4^+ at ambient conditions within and between streams, we used a 2-way analysis of variance (ANOVA) with DIN species (NO_3^-, NH_4^+) and stream (low-N, high-N) as factors. We used post hoc Tukey Honestly Significant Difference tests after significant ANOVAs ($p < 0.05$) to further examine the effects of stream and DIN species on U and $U_{N\text{-}specific}$.

To explore the relationship between U and concentration of each DIN species at the different levels of fertilization, we determined the fit of our experimental data to the 3 mathematical models described in the introduction. The 1st-order response model followed the equation:

$$U = a + bC \quad (3)$$

where U is assumed to increase linearly with DIN concentration (C) and a and b are a constant and the slope, respectively. The Michaelis–Menten model

followed the equation:

$$U = \frac{U_{max}C}{K_s+C} \quad (4)$$

where C is the DIN concentration, U_{max} is the maximum U, and K_s is the concentration at which ½ U_{max} is reached. K_s is an indicator of the biofilm affinity for DIN. High values indicate lower affinity than low values. The efficiency–loss model followed the equation:

$$U = aC^b \quad (5)$$

where U is assumed to increase with DIN concentration (C) as a power law with exponent $b < 1$.

The parameters a and b from each mathematical model (for the Michaelis–Menten model, U_{max} corresponds to a and K_s corresponds to b), were calculated based on the Gauss–Newton algorithm, an iterative process that seeks the values of the parameters that minimize the sum of the squared differences between the observed and predicted values of the dependent variable. We estimated the confidence intervals (CIs; 95%) for each coefficient by the generic function *confint* powered by R software (version 2.14.0; R Development Core Team, Vienna, Austria). The default method assumes asymptotic normality, and requires that suitable *coef* and *vcov* methods to be available. The default method can be called directly for comparison with other methods. We used the Akaike Information Criterion (AIC) to estimate Akaike weights (w_i), which yield the relative likelihood of each model given a particular data set. Within the set of candidate models for the data, we selected the model with the highest w_i.

We conducted all statistical tests with R. When necessary, data were log(x)-transformed before analysis to meet assumptions of homogeneity of variance and normality (Zar 1996).

2.4. Results

Environmental conditions differed substantially between the 2 study streams during the experiments (Table 2.1). Mean water temperature and PAR were 1.4 and 7× higher, respectively, in the low-N stream than in the high-N stream. Consistent with the long-term trend (i.e, biweekly sampling), mean NO_3^- concentration was 2× higher in the high-N than in the low-N stream (*t*-test, $p < 0.001$; Table 2.1). In contrast to the long-term trend, mean NH_4^+ concentration was 2× higher in the low-N stream than in the high-N stream (*t*-test, $p < 0.001$; Table 2.1). Mean SRP concentration was 4× lower and mean DIN:SRP ratio was 8× higher in the high-N than in the low-N stream (*t*-test, $p < 0.001$). Mean biofilm AFDM and chlorophyll *a* content were higher (5 and 9×, respectively) in the high-N than in low-N stream (*t*-test, $p < 0.001$).

Table 2.1 Mean (± SE) water temperature, photosynthetically active radiation (PAR), background nutrient concentration for both dissolved inorganic N (DIN) species, soluble reactive P (SRP), and biofilm ash-free dry mass (AFDM) and chlorophyll *a* for both study streams during the experiments. Nutrient data from biweekly samplings from September 2004–July 2007 also provided (in brackets).

Variable	Low-N stream	High-N stream
Water temperature (ºC)	15.4 ± 0.1	11.0 ± 0.2
PAR (mol m^{-2} d^{-1})	9.5 ± 3.4	1.4 ± 0.3
NO_3^- (µg N/L)	222 ± 2 (181 ± 11)	400 ± 27 (780 ± 44)
NH_4^+ (µg N/L)	15 ± 1 (12 ± 1)	8 ± 1 (19 ± 2)
SRP (µg P/L)	11 ± 0.3 (4 ± 0.5)	3 ± 0.3 (15 ± 2.6)
DIN:SRP (molar)	48 ± 1 (192 ± 32)	394 ± 32 (429 ± 106)
AFDM (g/m^2)	0.9 ± 0.1	4.3 ± 0.3
Chlorophyll *a* ($\mu g/cm^2$)	0.3 ± 0.03	2.6 ± 0.2

DIN species, stream, and the DIN × stream interaction affected both *U* and $U_{N\text{-}specific}$ at ambient concentrations (ANOVA, all $p < 0.01$). U_{NO3-} (3.1 ± 0.6 µg N m^{-2} s^{-1} in the low-N stream, 4.1 ± 0.8 µg N m^{-2} s^{-1} in the high-N stream) was higher than U_{NH4+} (0.3 ± 0.02 µg N m^{-2} s^{-1} in the low-N stream, 0.06 ± 0.01 µg N m^{-2} s^{-1} in the high-N stream) in both streams (Fig. 2.2A).

U_{NH4+} differed between streams (Tukey HSD test, $p = 0.001$), whereas U_{NO3-} did not (Tukey HSD test, $p = 0.636$). $U_{N\text{-}specific}$ for NO_3^- (4.1 ± 0.8 d^{-1} in the low-N stream, 1.0 ± 0.2 d^{-1} in the high-N stream) was higher than $U_{N\text{-}specific}$ for NH_4^+ (0.4 ± 0.02 in the low-N stream, 0.01 ± 0.002 in the high-N stream) in both streams (Fig. 2.2B). In contrast to U, $U_{N\text{-}specific}$ for both NO_3^- and NH_4^+ differed between streams (Tukey HSD test, $p < 0.001$).

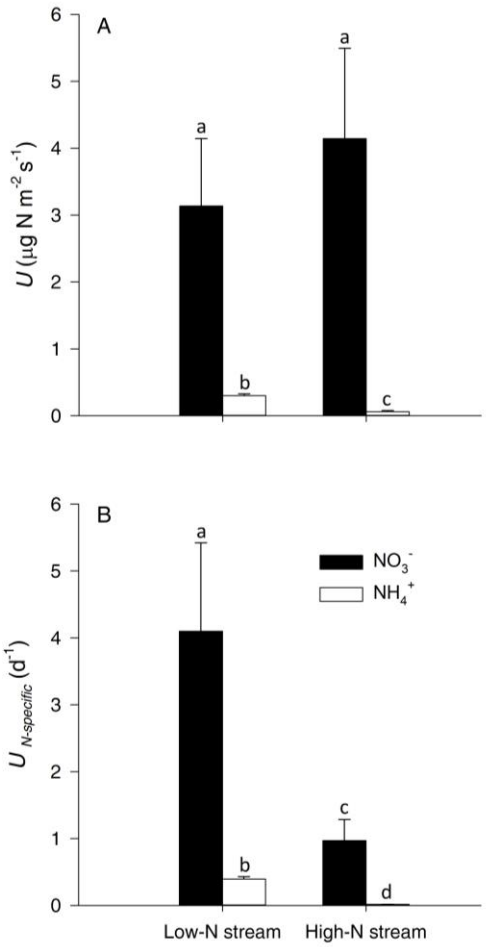

Figure 2.2 Mean (±1 SE; $n = 3$) uptake rate (U) (A) and biomass-specific N uptake rate ($U_{N\text{-}specific}$) (B) at ambient concentrations for the 2 dissolved inorganic N species (NO_3^- and NH_4^+) and study streams. Bars with the same letters are not significantly different ($p > 0.05$) based on post hoc Tukey Honestly Significant Difference test.

Uptake responses to increases in DIN concentration differed substantially between DIN species and streams (Fig. 2.3A–D). The relationship between *U* and NO_3^- concentration differed between streams, but uptake kinetics did not fit Michaelis–Menten model in neither stream (Fig. 2.3A, B). In the low-N stream, AIC analysis indicated that the relationship between *U* and NO_3^- concentration better fit a 1st-order model with a negative slope (Table 2.2). Conversely, in the high N-stream, 95% CIs for *b* in all 3 models contained 0, indicating no significant fit, and AIC analysis resulted in no clear model selection (Table 2.2).

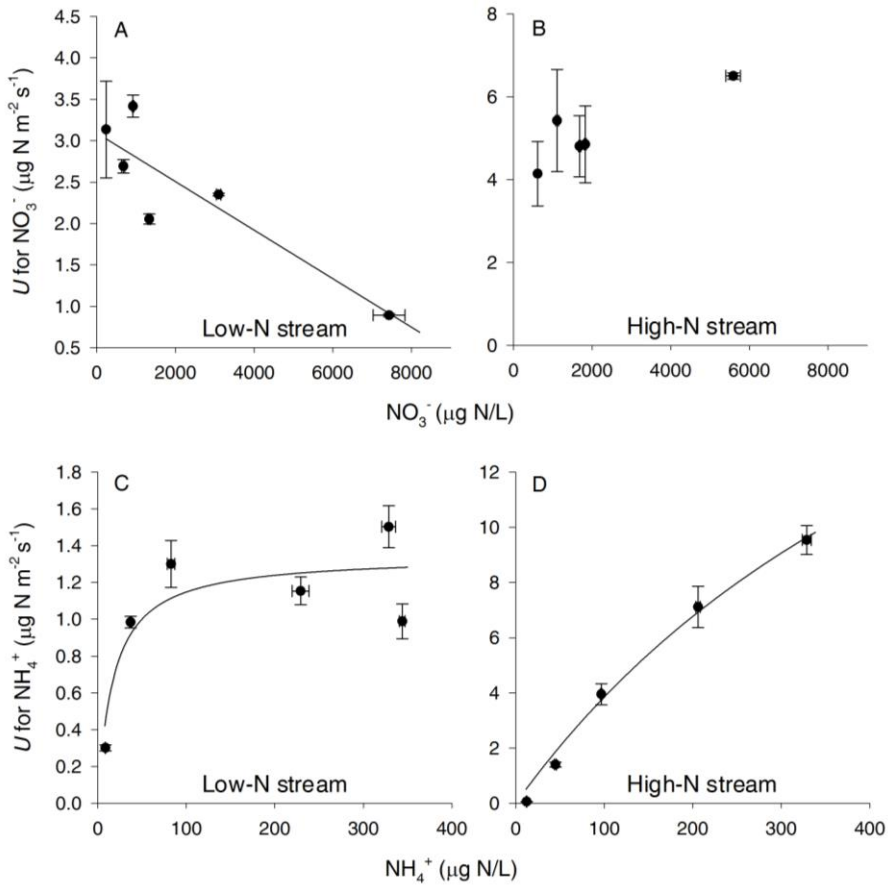

Figure 2.3 Mean (±1 SE; *n* = 3) uptake rates (*U*) for NO_3^- (U_{NO3-}) (A, B) and NH_4^+ (U_{NH4+}) (C, D) in the low-N (A, C) and high-N (B, D) streams. The first point in each panel corresponds to *U* measured at ambient concentration. Lines represent the selected regression model from Akaike Information Criterion analysis (see Table 2.2 for regression statistics).

U for NH_4^+ varied with increases in NH_4^+ concentrations (Fig. 2.3C, D). The AIC analysis indicated the Michaelis–Menten model as the best fit for the relationship between *U* and NH_4^+concentration in both streams (Table 2.2). However, uptake kinetic parameters differed between streams. U_{max} and K_s were lower in the low-N than in the high-N stream, and 95% CIs did not overlap (Table 2.2).

2.5. Discussion

We evaluated the response of biofilm *U* to changes in DIN concentration, and tested whether this response varied among DIN species. We used an experimental approach that combined nutrient fertilizations and ^{15}N-tracer additions in situ in artificial flumes. We predicted that *U* and uptake kinetics would depend on DIN species (NO_3^- vs NH_4^+) and ambient DIN concentration in the stream (low-N vs high-N). Our results supported these predictions only partially. *U* was higher for NO_3^- than for NH_4^+ in both streams, but only U_{NH4+} differed between streams, with lower values in the high-N stream. In addition, $U_{N\text{-}specific}$ at ambient conditions was higher in the low-N stream for both DIN species. In terms of uptake kinetics, the Michaelis–Menten model best fit the relationship between *U* and concentration in the case of NH_4^+ (for both streams), but not in the case of NO_3^- (neither stream). Moreover, saturation of NH_4^+ uptake occurred at lower U_{max} in the low-N stream than in the high-N stream, but affinity for NH_4^+ was higher (lower K_s) in the low-N stream.

Biofilm DIN uptake in streams of contrasting DIN availability and speciation

U of epilithic biofilm for both DIN species under ambient conditions in our study were similar to values reported from previous studies using whole-stream ^{15}N-tracer additions (Mulholland et al. 2000, Tank et al. 2000, Hamilton et al. 2001, Merriam et al. 2002, Ashkenas et al. 2004, von Schiller et al. 2009, Sobota et al. 2012). This result indicates that values of *U* in our channel experiments were representative of natural field conditions.

Table 2.2 Mean (95% CI) for statistical parameters of linear ($U = a + bC$), Michaelis–Menten ($U = a\,C/b + C$), and efficiency–loss ($U = aC^b$) models used to evaluate the model that best fit the relationship between uptake rate (U) and dissolved inorganic N (DIN) concentration (C) for both streams and DIN species (NO_3^- and NH_4^+). The Akaike Information Criterion (AIC) was used to estimate Akaike weights (w_i), which give the relative likelihood of each model. The highest relative likelihoods are marked in bold. For the Michaelis–Menten model, a corresponds to the maximum uptake rate (U_{max}; µg N m^{-2} s^{-1}) and b corresponds to the ½-saturation constant (K_s; µg N/L).

Model	Low-N stream				High-N stream			
	a	*b*	AIC	w_i	*a*	*b*	AIC	w_i
NO_3^-								
Linear	**3.1 (2.7–3.5)**	**–0.00029 (–0.0004 to –**	**33.4**	**0.97**	4.3 (3.1–5.5)	0.00040 (–0.000023–	55.1	0.36
Michaelis–Menten	2.1 (1.6–2.6)	–85.8 (–131.9 to –7.6)	48.0	0	6.5 (4.8–9.2)	384 (–36.5–1282)	55.6	0.28
Efficiency–loss	11.9 (5.3–	–0.2 (–0.4 to –0.1)	48.1	0.03	1.3 (0.3– .6)	0.2 (–0.010–0.4)	55.1	0.37
NH_4^+								
Linear	0.8 (0.5–1.0)	0.0016 (0.00029–0.0029)	17.3	0	0.3 (–0.5–1.1)	0.030 (0.025–0.034)	45.1	0.03
Michaelis–Menten	**1.3 (1.2–1.5)**	**17.1 (7.8–34.9)**	**2.6**	**0.98**	**28.0 (17.4–**	**628 (307–3449)**	**38.9**	**0.77**
Efficiency-loss	0.4 (0.2–0.7)	0.2 (0.093–0.3)	10.9	0.02	0.082 (0.030–	0.8 (0.7–1.0)	41.7	0.19

Ambient U_{NO3-} was 10× higher than U_{NH4+} in both streams, even though NH_4^+ is theoretically an energetically less costly DIN source and, thus, was expected to be preferentially assimilated over NO_3^- (Dortch 1990, Naldi and Wheeler 2002). Estimated values of the relative preference index (RPI) were ~1 in the 2 streams. This index was proposed by Dortch (1990) as a means to determine the preference for NH_4^+ over NO_3^- (values < 1) or for NO_3^- over NH_4^+ (values > 1). The RPI value of ~1 in our study suggests that biofilms in the 2 streams have no preference for either DIN species. Thus, the observed higher U_{NO3-} than U_{NH4+} was mostly attributable to the higher concentrations of NO_3^- than of NH_4^+.

Ambient U_{NO3-} did not differ between streams, but U_{NH4+} was 10× lower in the high-N than in the low-N stream. Higher NO_3^- availability relative to NH_4^+ availability in the high-N stream may have favored uptake of NO_3^- over NH_4^+ in the high-N stream, as suggested by other authors (Fellows et al. 2006, Newbold et al. 2006, Bunch and Bernot 2012). Furthermore, at low NH_4^+ concentration, the presence of NO_3^- can favor NO_3^- assimilation (Geisseler et al. 2010). Expression and biosynthesis of assimilatory nitrate reductase (the enzyme responsible for NO_3^- assimilation processes) is induced by NO_3^- and NO_2^- and suppressed by NH_4^+ (Gonzalez et al. 2006). Thus, the concurrence of high $NO_3^-$and low NH_4^+ concentration at ambient conditions in the high-N stream may have led to lower NH_4^+ assimilation rates than in the low-N stream.

Differences in nitrification, which can contribute to NH_4^+ uptake in biofilms, are another potential explanation for the differences in *U* between streams. If nitrification rate were constrained by the low substrate (NH_4^+) availability in the high-N stream, then we would expect the contribution of nitrification to total NH_4^+ uptake to be lower in that stream. In both streams, $\delta^{15}NO_3^-$ increased during plateau conditions in the channels where we did $^{15}NH_4^+$ additions, a result indicative of nitrification (2.6 ± 0.5‰ and 1.9 ± 0.9‰ in the low-N and the high-N streams, respectively). Based on these $\delta^{15}NO_3$ increases, we estimated the contribution of nitrification to total biofilm NH_4^+ uptake for each fertilization cycle. This contribution ranged from 0.2 to 7.6% in the low-N stream, whereas it was <0.2% in the high-N stream. These results contrast with findings from Bernhardt et al. (2002),

who found a higher contribution of nitrification to total NH_4^+ uptake in high-NO_3^- streams of Hubbard Brook (New Hampshire, USA). They hypothesized that when assimilatory processes switch to NO_3^- uptake (i.e., in high-NO_3^- streams), competition between nitrifiers and heterotrophs is ameliorated, resulting in higher nitrification rates. Our data do not support this mechanism because nitrification rate was probably lower in the high-N than in the low-N stream. Instead, we suggest that combination of lower NH_4^+ assimilation and lower nitrification by biofilms in the high-N stream explains the differences in U_{NH4+} between streams.

$U_{N\text{-}specific}$ values indicate that the biofilm from the high-N stream took up both NO_3^- and NH_4^+ from the water column less efficiently than the biofilm from the low-N stream. Lower uptake efficiencies often occur in streams with high DIN concentrations because of saturation of ssimilative processes (O'Brien et al. 2007). Thus, our results suggest functional differences in the way DIN is cycled within biofilm communities grown under low- and high-N conditions, which in turn, may lead to differences in the uptake kinetics for both DIN species between stream types.

Biofilm DIN uptake kinetics

Contrary to expectations from nutrient kinetic theory, increases in NO_3^- availability did not enhance biofilm U_{NO3-}. In the high-N stream, addition of NO_3^- had no effect on biofilm *U*, suggesting that uptake capacity of biofilm assemblages probably was saturated at the ambient NO_3^- concentration. Earl et al. (2006) suggested that when N is not limiting in streams, a 0-order mathematical model (i.e., constant rate with slope = 0) is more applicable than a higher-order model, a suggestion in concordance with our results in the high N-stream. Alternatively, the lack of biofilm uptake response to increases in NO_3^- concentration might be explained by tight coupling of NO_3^- uptake to availability of other nutrients (Fairchild et al. 1985, Sterner et al. 1992). Schanz and Juon (1983) suggested that P is a potentially limiting element at DIN:P >20 (others have suggested a transition from N to P limitation at DIN:P ≈ 16–17; Redfield 1958, Grimm and Fisher 1986). We added SRP in the fertilization solutions to maintain background DIN:P, but

ratios were well above the potential P-limitation thresholds, especially in the high-N stream (394 ± 32 µg P/L). In this sense, NO_3^- uptake in the high-N stream may have been constrained by P insufficiency. However, if P were the limiting nutrient, then increases in P availability should alleviate P limitation and, thus, enhance NO_3^- uptake. We think this alternative explanation is unlikely because previous nutrient-limitation bioassays in the high-N stream failed to show P limitation (von Schiller et al. 2007).

Increases in NO_3^- availability in the low-N stream produced a decrease in biofilm *U*, indicating a possible inhibitory effect of high NO_3^- concentrations on biofilm uptake in this stream. Inhibitory effects on the uptake of NH_4^+ or NO_2^- at high concentrations have been reported in the literature (usually associated with nitrification processes; Kim et al 2006, Vadivelu et al. 2007). However, as far as we know, no previous evidence exists for inhibition of NO_3^- uptake at high NO_3^- concentrations. However, inhibitory effects of long-term NO_3^- enrichment have been reported for periphyton growth in nutrient-diffusing substrate experiments (Bernhardt and Likens 2004), and a few investigators have shown potentially toxic effects of NO_3^- on freshwater animals and plants (Camargo and Alonso 2006, Lambert and Davy 2011). Our experiments do not allow us to identify the mechanisms underlying observed patterns but do provide evidence that a short-term, sharp increase in NO_3^- concentration may be inhibitory.

Michaelis–Menten kinetics described biofilm uptake responses to increases in NH_4^+ concentration in both streams. Values of K_s were higher than ambient concentrations of NH_4^+ in both streams, so we conclude that biofilm uptake for this DIN source was below saturation at ambient concentrations (Tilman 1982). Therefore, biofilms were able to respond positively to short-term increases in NH_4^+ concentration within a certain range in the 2 streams. Bunch and Bernot (2012) also compared uptake responses of microbial communities to NH_4^+ and NO_3^- enrichments. They observed that responses to NH_4^+ were immediate and pronounced, whereas responses to NO_3^- were delayed and more variable. They suggested that preference for NH_4^+ as a DIN source by microbial communities dictates stronger and more rapid uptake responses to changes in NH_4^+ than in NO_3^-

concentration.

Our results agree with those by Bunch and Bernot (2012) in showing rapid response to increases in NH_4^+. However, the values of RPI of ~1 in our study indicated no clear preference for NH_4^+ over NO_3^-, at least under ambient conditions. An alternative explanation for the difference in the kinetic responses between NO_3^- and NH_4^+ involves enzymatic responses to short-term changes in availability. Increased availability of NH_4^+ in NH_4^+-amended channels may have triggered repression of NO_3^- reductase and increased biofilm NH_4^+ uptake to meet N demand (Gonzalez et al. 2006). This mechanism could explain the positive biofilm NH_4^+ uptake response to increases in NH_4^+ concentration even though uptake responses for NO_3^- indicated that biofilm demand for this DIN species was saturated at ambient conditions. Previous investigators have found a Michaelis–Menten response of nitrification rates to increases in NH_4^+ concentration within a range of NH_4^+ concentrations similar to that used in our study (Koper et al. 2010). Nitrification probably was substrate-limited at the relatively low NH_4^+ concentrations in the 2 study streams, which would produce a positive response to increased NH_4^+ concentration that conforms to a Michaelis–Menten model. However, our a posteriori calculations of nitrification contribution to the whole-channel uptake suggest that nitrification is only a minor contributor to observed kinetics of NH_4^+ uptake. We suggest that a combination of several mechanisms best explains the different kinetic responses of NH_4^+ and NO_3^- in the study streams.

NH_4^+ uptake kinetics fit the Michaelis–Menten model in the 2 streams, but the kinetic parameters (K_s and U_{max}) clearly differed between streams, supporting our predictions. NH_4^+ U_{max} of the biofilm in the high-N stream was 21× higher than U_{max} of the biofilm in the low-N stream. The high-N stream had higher biofilm biomass and more photoautotrophic organisms (as indicated by chlorophyll *a* content) than the low-N stream, a result that could explain the higher U_{max} observed in the high-N stream. However, U_{max} weighted by N content of biofilm dry mass, a surrogate measure of uptake efficiency, was only 4× higher in the high-N stream. Therefore, biofilms were relatively more efficient in NH_4^+ uptake in the low-N than in the high-N stream, a result that is in agreement with uptake results measured at ambient

DIN conditions.

In contrast, biofilms showed a higher affinity (lower K_s) for NH_4^+ in the low-N stream than in the high N-stream. Higher affinities for substrate often are attributed to exposure of microorganisms to lower ambient concentrations (Collos et al. 2005, Martens-Habbena et al. 2009). This explanation may not apply to our study if we consider only ambient NH_4^+ concentration, which was similar and low in the 2 streams. However, when discussing nutrient limitation, it is more appropriate to consider total DIN concentration, which was 2× lower in the low-N than in the high-N stream, because biofilms can meet their N demand by uptake of either DIN species. Alternatively, differences in NH_4^+ affinity between streams could be caused by boundary-layer constraints arising from differences in biofilm structure (Dodds et al. 2002). In support of this idea, the higher AFDM content per unit area in the high N-stream implies thicker biofilms and limitation of diffusion of DIN to all cells in the biofilm (Stewart 2003, Teissier et al. 2007). Limitation by diffusion has been demonstrated for uptake of inorganic C and nitrification activity in model biofilms, with both processes restricted to the surface layer of the biofilm (Gieseke et al. 2005). As a result, the thickness of the biofilm in the high-N stream may contribute to an increase in the range of NH_4^+ concentrations within which U_{NH4+} responds positively. Constraints resulting from diffusion limitation in thicker biofilms operate for both N assimilation and nitrification, and thus, can amplify the range of NH_4^+ concentrations that can be reached before saturation occurs because the 2 processes may have different kinetics.

We cannot rule out differences in environmental conditions, such as light availability and temperature, between the 2 streams as potential causes of differences in biofilm uptake kinetics for NH_4^+. We tried to conduct experiments in streams with similar environmental conditions, but a large flood in the high-N stream forced us to postpone the experiment until the biofilm communities recovered fully. As a result, temperature and light availability were higher in the low-N than in the high-N stream during the experiments and could have enhanced biofilm activity and kinetic responses in the low-N stream. However, the effect of temperature on nutrient uptake kinetics is unclear, and Smith (2011) found no

evidence of sensitivity of Michaelis–Menten parameters to temperature. Light availability was higher in the low-N stream, but biofilm chlorophyll *a* content was 9× higher in the high-N than in the low-N stream. Thus, this factor could not have caused the observed kinetic differences, at least for the photoautotrophic component of the biofilms. Thus, observed differences in biofilm uptake kinetics between streams seem to be more influenced by differences in DIN concentrations and relative proportions of DIN species than by differences in other environmental factors.

Conclusions

Biofilm uptake responses to short-term changes in DIN concentration in the 3 Mediterranean streams investigated during the study period depended on ambient conditions, including DIN concentrations, where biofilm developed, and the DIN species considered. Under short pulses of increased DIN concentration, the stream biofilms in our study were more reactive to changes in NH_4^+ than to changes in NO_3^- concentration, but ambient U_{NO3-} far exceeded ambient U_{NH4+}, largely because NO_3^- was present at much higher concentration. The greater kinetic response to NH_4^+ may be attributable to repression of enzymes associated with NO_3^- uptake or the contribution of a different process (nitrification) to total uptake. Lack of response to NO_3^- suggests this species was present in saturating concentrations. Our results contrast with findings from laboratory-scale experiments, in which NO_3^- kinetics conformed to the Michaelis–Menten model (Eppley et al. 1969, Kemp and Dodds 2002, Maguer et al. 2011). In our study, stream biofilm communities were able to respond to increases in NH_4^+ concentration, which is an energetically cheaper N source than NO_3^- and is the substrate for nitrification. However, we found clear differences between streams in biofilm responses to NH_4^+ that probably arose from differences in biofilm characteristics, interactions with other N species, such as NO_3^-, or adaptive changes in affinity.

Human activities associated with different land uses may enrich adjacent streams with DIN and alter the proportion of DIN species in the streams. Thus, streams draining catchments dominated by agricultural practices tend to be NO_3^-

enriched, whereas streams draining urbanized catchments are often NH_4^+ enriched (Stanley and Maxted 2008, Lasaletta et al. 2009, Martí et al. 2010). Given widespread changes in land use, our results have implications for understanding and managing N losses to downstream ecosystems. The N species that reach stream ecosystems potentially could be retained by in-stream biofilm communities (NH_4^+) or exported downstream with the subsequent enrichment of receiving waters (NO_3^-).

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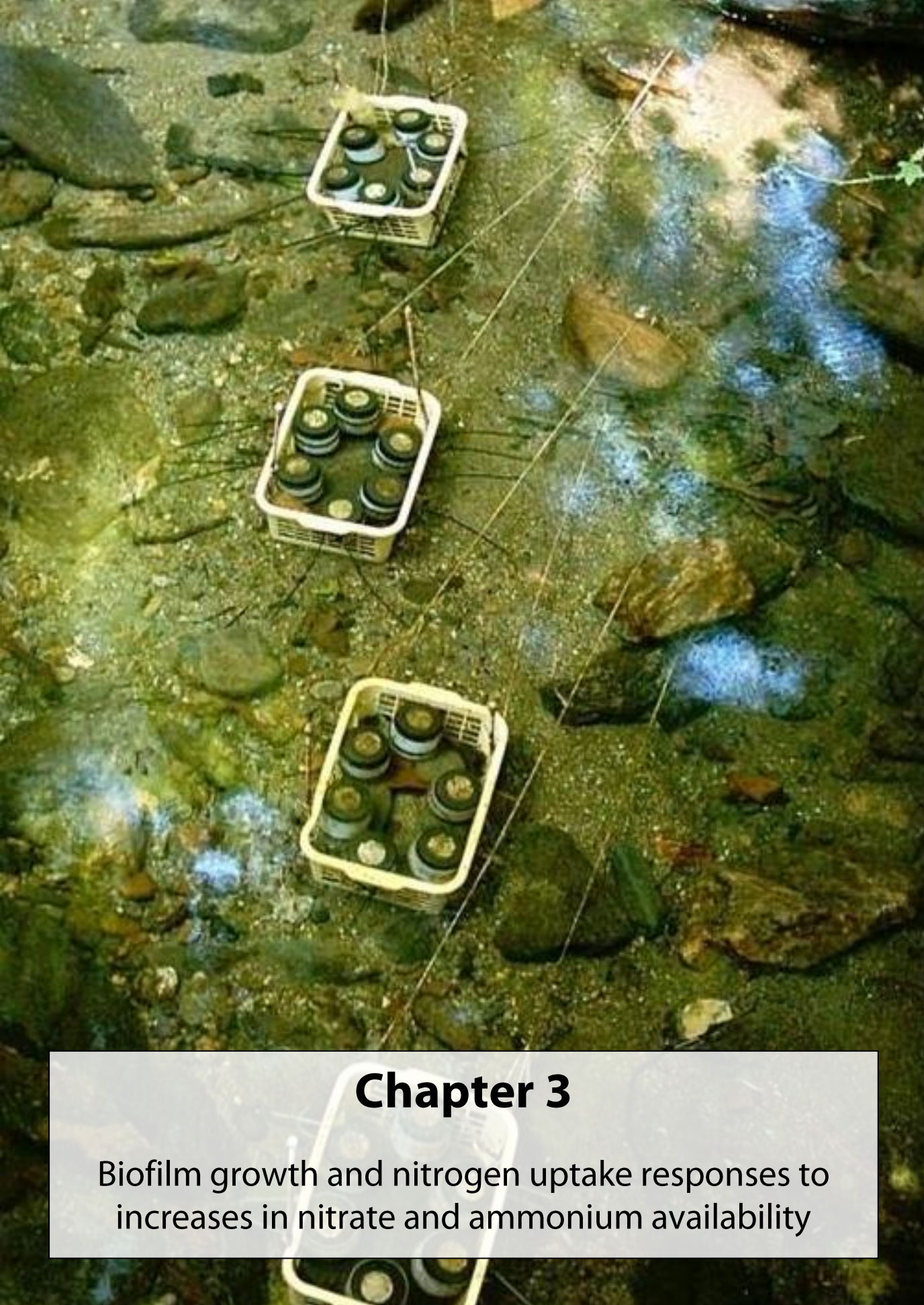

Chapter 3

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

Ribot M., von Schiller D., Sabater F & Martí E. Biofilm growth and nitrogen uptake responses to increases in nitrate and ammonium availability. Aquatic Sciences (In review).

Cover: nutrient diffusing substrata bioassays during incubation in Santa Coloma stream. Photograph by Daniel von Schiller.

3.1. Abstract

Nitrate (NO_3^-) and ammonium (NH_4^+) are the two major dissolved inorganic nitrogen (DIN) species available in streams. Human activities not only cause in-stream DIN enrichment, but also modify the NO_3^-:NH_4^+ ratio. We examined biofilm responses in terms of growth and DIN uptake to variation in ambient concentrations and enrichments in either NO_3^- or NH_4^+. We incubated nutrient diffusing substrata (NDS) bioassays with 3 treatments (DIN-free, +NO_3^- and +NH_4^+) in 5 streams. Biofilm specific uptake rates (U_{spec}) of NO_3^- and NH_4^+ were then measured under similar environmental conditions using *in-situ* additions of ^{15}N-labeled NO_3^- and NH_4^+. Biomass, algal accrual rates, and U_{spec}-NO_3^- of biofilms in DIN-free treatments varied among the streams in which the NDS had been incubated. Higher ambient DIN concentrations tended to enhance biofilm growth rates and DIN uptake efficiency. U_{spec}-NO_3^- was one order of magnitude higher and more variable than U_{spec}-NH_4^+, but biofilms did not show a clear relative preference for either DIN species. Biofilm growth and DIN uptake in DIN-amended NDS (i.e., +NO_3^- and +NH_4^+) were consistently lower than in DIN-free NDS. Negative biofilm responses were consistently more pronounced for algal accrual rates and U_{spec}-NO_3^- and for the +NH_4^+ than for the +NO_3^- treatments. The most relevant response was the reduction of biofilm U_{spec}-NO_3^- in NH_4^+ enrichments. Overall, our findings indicate that DIN uptake by biofilms can be reduced mostly by increases in NH_4^+ concentration, which may result in higher DIN downstream export.

3.2. Introduction

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

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

NO_3^- and NH_4^+ can also undergo a variety of energy-yielding dissimilatory pathways associated with microbial activity, which may be altered by increases in availability of these two DIN species. The most common processes in streams are nitrification, the aerobic oxidization of NH_4^+ to NO_3^- carried out by autotrophic or heterotrophic bacteria and archaea (Lin et al. 2009, Daims and Wagner 2010) and denitrification, the anaeorbic

respiratory process by which bacteria reduce NO_3^- to N-gas using the former as an electron acceptor (Seitzinger 1988; Lin et al. 2009). Furthermore, other dissimilatory processes such us dissimilatory nitrate reduction to ammonium (DNRA) or anaerobic ammonium oxidation (Anammox) may also consume and/or produce NO_3^- and NH_4^+, yet these processes are poorly understood in lotic systems (Burgin and Hamilton 2007). Nevertheless, this study is basically focused on understanding the effects of increases of these two DIN species on N assimilation as a starting step on overall effects.

Understanding how in-stream biofilms respond to increases of either NO_3^- or NH_4^+ availability is particularly relevant since several studies have indicated that human activity not only increases DIN availability in streams, but also modifies the relative abundance of the two DIN species (Stanley and Maxted 2008; Von Schiller et al. 2008b; Lassaletta et al. 2009; Martí et al. 2010). From those studies we learned that streams draining catchments dominated by agricultural practices tend to have higher NO_3^-:NH_4^+ ratios than streams dominated by urban activity. Urban streams tend to be NH_4^+ enriched because effluent inputs from wastewater treatment plants (WWTP) are subjected to the partial nitrification capacity of the WWTP systems. Studies addressing the effect of increases in DIN availability on the growth of stream biofilms with explicit consideration of the two DIN species (i.e., NO_3^- and NH_4^+) are scarce (but see von Schiller et al. 2007 and Hoellein et al. 2010). In addition, available results are contradictory, showing either preference for NH_4^+ as an N source for DIN assimilatory uptake (von Schiller et al. 2007) or no differential effect between the two DIN species on biofilm growth (Hoellein et al. 2010). In addition, studies designed to compare biofilm uptake responses to increases in NO_3^- and NH_4^+ concentration have mostly been from laboratory tests (Kemp and Dodds 2002; O'Brien and Dodds 2008; Domingues et al. 2011; Bunch and Bernot 2012) with little research knowledge from field experiments (but see Bernot et al. 2006 and Ribot et al. 2013). NH_4^+ is usually considered the preferred DIN source for DIN uptake (Dortch 1990; Naldi and Wheeler 2002); however, instances when NO_3^- is the

main N source for microorganisms are common due to the generally higher NO_3^- availability (Domingues et al. 2011; Bunch and Bernot 2012; Ribot et al. 2013). These studies also contend that biofilms may respond to changes in DIN availability both through functional and structural modifications such us enhancing DIN uptake or by shifts in the species composition.

The goal of this study was to examine biofilm responses in terms of growth and DIN uptake to variation in ambient concentrations and enrichments of either NO_3^- or NH_4^+. In particular, we test how induced changes in DIN concentration and relative dominance of the two DIN species derived from land use activity can influence the relative contribution of biofilm N assimilation to in-stream downstream transport. To approach this goal, we conducted nutrient diffusing substrata (NDS) bioassays with 3 treatments (DIN-free, $+NO_3^-$ and $+NH_4^+$) in 5 streams spanning a range in ambient DIN availability. The NDS allowed measuring biomass and algal growth under the different treatments in the different streams. In addition, at the end of NDS incubations, we exposed the different biofilms developed on the NDS to ^{15}N additions of either NO_3^- or NH_4^+ in a single location to measure their capacity for DIN assimilation of the two species as well as their relative preference for the uptake of the two DIN species. Comparison of assimilation rates between biofilms under control and DIN amended conditions allow estimating the effect of DIN species enrichments on N assimilation rates of biofilms.

Biofilms rely on DIN from the water column and may show some biochemical preference on DIN species for assimilatory uptake; therefore, we expected that biofilms that developed in streams with higher ambient DIN concentration would have higher growth rates and higher N demand (i.e., higher DIN uptake rates) if they are not limited by other environmental factors. We further expected that responses of biofilms exposed to NH_4^+ enrichments would be higher than those of biofilms exposed to NO_3^- enrichments because the latter DIN species has a higher energetic cost for N assimilation than the former (Dortch 1990).

3.3. Methods

Study sites

La Tordera catchment (Catalonia, NE Spain) has an area of 868.5 km^2 dominated by siliceous geology, and covers a 1700-m altitudinal gradient from the headwaters to the sea level within a 35 km distance. Climate in this region is typically Mediterranean, with warm, dry summers, and mild, humid winters. Although most of the catchment is forested, agricultural, urban and industrial areas tend to concentrate in the river valley, resulting in a heterogeneous land use template along the lowlands of the river network, which affects stream N concentrations (von Schiller et al. 2008b). Within this catchment, we selected 5 streams draining sub-catchments with different land uses (Table 3.1). Selection of the streams was based on DIN concentration data from biweekly samplings conducted from September 2004–July 2007 on a continuous synoptic survey across the La Tordera stream network. Santa Fe del Montseny (MON; mean ± SE from the survey = 120 ± 11 µgN L^{-1}), Font del Regàs (FR; 190 ± 11 µgN L^{-1}) and Castanyet (CAS; 290 ± 35 µgN L^{-1}) are headwater-forested streams. Gualba (GUA; 281 ± 18 µgN L^{-1}) and Santa Coloma (COL; 802 ± 43 µgN L^{-1}) are situated in the river valley and influenced by human activity. GUA is surrounded by an urban development, whereas COL is situated next to a gardening plantation (Table 3.1).

Experimental approach

Our experiment consisted of two separated sets of nutrient diffusing substrata (NDS) bioassays with enrichments in either NO_3^- or NH_4^+ (see description below) in each of the 5 study streams to follow the responses in biofilm development. These incubation experiments were followed by two separated ^{15}N tracer additions (one with $^{15}NO_3^-$ and the other with $^{15}NH_4^+$) in a single stream (i.e., COL stream). This allowed quantifying N assimilatory uptake rates and comparing them among biofilms grown under the two DIN species enrichments in the 5 streams. We selected a single stream to conduct the ^{15}N additions to better isolate the effect of different NO_3^- and NH_4^+

enrichments on N assimilation rates by the different biofilms. In this sense, we selected the stream with the highest DIN concentration (COL) to maximize biofilm N assimilation and allow a better estimation of the differences among the biofilm treatments. The first set of NDS bioassays started on June 21st 2006 and lasted for 16 days. After the incubation, we replaced the agar solution of all treatments by fresh DIN-free agar solution to ensure biofilm DIN uptake from the water column. These DIN-free NDS with grown biofilms were transferred to COL stream in containers filled with stream water. NDS were left in the stream during 5 days prior to the $^{15}NO_3^-$ addition (see description below) to estimate rates of NO_3^- assimilation by all the biofilms. We repeated the procedure for the second set of NDS bioassays, which started on July 7th and lasted for 21 days, with an acclimation period of 4 days before conducting the $^{15}NH_4^+$ addition (see description below) to estimate rates of NH_4^+ assimilation by all the biofilms. We acknowledge that the acclimation period (4-5 days) of all biofilms in the COL stream may have caused some changes in biofilm composition; and thus, in their uptake responses. However, we considered that it was better to allow biofilms to acclimatize to new conditions before the measurement of N uptake rates. In addition the acclimatization time was much shorter than the time biofilms were exposed to all the DIN treatments in the different streams; and thus, this treatment conditions should dictate biofilm responses.

NDS bioassays

We constructed NDS following the method outlined in Tank and Dodds (2003). The NDS consisted of 60 mL plastic containers filled with a 2% (by weight) agar solution, which was not amended (i.e., DIN-free treatments) or was amended either with nitrate (0.5 M KNO_3; hereafter referred as $+NO_3^-$) or ammonium (0.5 M NH_4Cl; hereafter referred as $+NH_4^+$). We placed Whatman GF/F glass fiber filters on the top of the plastic containers to cover the agar completely and to serve as the substrata for biofilm colonization. In each stream, we placed 6 replicates for each treatment (DIN-free, $+NO_3^-$ and

$+NH_4^+$). NDS were glued in plastic baskets that were fixed on the streambed to allow their colonization. We placed the NDS in pools of similar water depth and velocity. The stream substratum of all the selected stream reaches was basically composed of cobbles and pebbles with sand patches. During the study period, a well-developed riparian canopy cover shaded all the selected reaches.

Table 3.1 Physical and chemical characteristics of the streams in which the nutrient diffusing substrata (NDS) were incubated. Data reported are the mean ± SE of samples collected on three different dates during each of the two NDS incubation periods (n = 6). Note that streams are listed in order of increasing DIN availability (sum of NH_4^+ and NO_3^- concentrations).

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

During the two NDS incubation periods, we collected water samples in each stream on 3 evenly spaced dates for ambient nutrient concentration analyses. We collected water samples with plastic syringes and filtered them

immediately through ashed Whatman (Maidstone, UK) GF/F fiber glass filters into acid-washed plastic containers and stored them on ice for transportation to the laboratory until analysis. On the same dates, we measured water conductivity and water temperature with a portable WTW conductivity meter (Weilheim, Germany). In addition, we determined discharge on a single cross-sectional transect by measuring mean wetted width, mean depth and mean water velocity (Gordon et al. 1992).

^{15}N constant rate additions

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

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

Laboratory analyses

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

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

We analyzed water samples for the concentrations of NO_3^-, NH_4^+, and soluble reactive phosphorus (SRP) on a Bran+Luebbe (Norderstedt, Germany) TRAACS 2000 autoanalyzer following standard colorimetric methods (APHA, 1995). We processed water samples for the analysis of $^{15}NH_4^+$ and $^{15}NO_3^-$ as described in Holmes et al. (1998) and Sigman et al. (1997), respectively. Briefly, for $^{15}NH_4^+$ determination, we amended a known volume of sample with 3 g L^{-1} of MgO and 50 g L^{-1} of NaCl and a Teflon filter packet containing an acidified 1-cm-diameter ashed Whatman GF/D fiber glass filter to trap the volatilized NH_3, and incubated it on a shaker at 40ºC for 4 weeks. For $^{15}NO_3^-$ determination, we amended a known volume of the sample with 3 g of MgO and 5 g of NaCl and boiled it to remove the NH_4^+. We then added 0.5 mg of MgO and 0.5 mg Devarda's alloy to reduce the NO_3^- to NH_4^+, and treated the remaining sample as for $^{15}NH_4^+$. We also diffused a set of standards of known volume for volume-related fractionation corrections. Once the incubation was completed, we removed the filter packets and placed them in a desiccator for 4 days. We then encapsulated the filters in tins and stored them until ^{15}N analysis.

Samples for the determination of the ^{15}N signature were analyzed at the University of California Stable Isotope Facility (Davis, California, USA). The C and N content (as a percentage of dry mass) and the abundance of the heavier isotope, expressed as the ^{15}N:^{14}N ratio compared to that of a standard (i.e., N_2 from the atmosphere) using the notation of $\delta^{15}N$ in units of ‰, were measured by continuous-flow isotope-ratio mass spectrometry (20–20 mass spectrometer; PDZ Europa, Northwich, UK) after sample combustion in an on-line elemental analyzer (PDZ Europa ANCA-GSL).

Parameter calculations

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

To calculate biofilm DIN uptake rates of NO_3^- and NH_4^+ from the $^{15}NO_3$ and $^{15}NH_4$ additions, respectively, we first calculated the amount of ^{15}N tracer contained in biofilm biomass ($^{15}N_{biofilm}$; in µg N/m^2) at the end of the addition using the following equation:

$$^{15}N_{biofilm} = B_{biofilm} \times N/100 \times (MF_i - MF_b) \qquad (1)$$

where $B_{biofilm}$ is the biofilm as dry mass per unit of area (µg m^{-2}), N is the biofilm N content expressed as percentage of dry mass, MF is the molar fraction of ^{15}N in biofilm at plateau conditions (MF_i) and at background conditions (MF_b).

We then estimated the DIN uptake rate (U; in µg N m^{-2} s^{-1}) for either NO_3^- or NH_4^+ using the following equation:

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

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

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

$$RPI_{NO_3} = \frac{U_{NO_3}/\Sigma U_{DIN}}{NO_3/DIN} \quad (3)$$

where U_{NO_3} is the biofilm NO_3^- uptake rate (U for NO_3^- from eq. 2; in µg N m^{-2} s^{-1}) in a given NDS filter, ΣU_{DIN} is the sum of the mean biofilm uptake rate of NO_3^- and NH_4^+ (U for NH_4^+ from eq. 2; in µg N m^{-2} s^{-1}) within a NDS treatment, NO_3 is the mean nitrate concentration in COL during the two ^{15}N additions and DIN is the sum of the mean concentrations of NH_4^+ and NO_3^- in COL during the two ^{15}N additions. RPI is an indicator of the relevance of NO_3^- uptake relative to total DIN uptake weighed by the relative importance of NO_3^- concentration to total DIN concentration. For example if NO_3^- uptake

is 50% of DIN uptake, but NO_3^- is only 25% of DIN, the RPI value is 0.5/0.25 = 2, indicating preference for NO_3^- given the available DIN species. An RPI value <1 indicates a preference for NH_4^+.

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

Statistical analyses

We pooled the data from control treatments (DIN-free) from the two NDS incubations to explore differences in biofilm growth at ambient concentrations among streams in which the NDS were incubated. We compared biomass and algal accrual rates and C:N molar ratios using a linear mixed-effects model with stream as fixed factor (n=5) and incubation date as a random factor (n=2). We included the random effect 'incubation date' in the model to account for the potential temporal variation in biofilm responses between the two sets of NDS bioassays, despite initial analysis indicated that this effect was negligible. However, the inclusion of a non-significant random effect factor does not influence the inference on fixed effects factors (Zuur et al., 2009). On the other hand, since U_{spec}-NO_3^- and U_{spec}-NH_4^+ for control treatments were calculated separately from the first and the second NDS incubations respectively, we compared U_{spec}-NO_3^-, U_{spec}-NH_4^+ and RPI using one-way ANOVA with stream as a fixed factor (n=5) to explore differences in these variables at ambient concentrations among streams in which the NDS were incubated.

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

We ran Pearson correlations to explore if biofilm growth and DIN uptake were related to the ambient concentrations of NO_3^- and NH_4^+ of the study streams in which the NDS were incubated as well as to explore the relationships between biofilm growth and DIN uptake. Correlations were only explored if the fixed factor 'stream' was significant in the linear mixed-effects or ANOVA models.

We ran all statistical tests with R 2.15.0 (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/.). Linear mixed-effects models were done with the R package 'nlme'. Post-hoc multiple comparisons for nmle models followed significant fixed factor ($p<0.05$) using the R package 'multcomp'. Post-hoc Tukey HSD tests followed significant ANOVA ($p < 0.05$). When necessary, data were log-transformed before analysis to meet assumptions of homogeneity of variance and normality (Zar 1996).

3.4. Results

Physical and chemical characteristics of the study streams

During the study period, mean discharge was relatively low at all streams and averaged 9.6 L s^{-1} (Table 3.1). Stream water temperature and conductivity ranged from 14.2 to 21.4 ºC and 61 to 310 µS cm^{-1}, respectively, across streams. Concentration of NH_4^+ was low and relatively similar among streams, ranging from 14 to 22 µgN L^{-1}. In contrast, NO_3^- concentration ranged from 140 to 600 µgN L^{-1}, and SRP concentrations ranged from 4 to 46

µgP L^{-1} (Table 3.1). The lowest NO_3^- and SRP concentrations were observed in two of the forested streams (CAS and FR), whereas the highest concentrations were observed in COL, the stream with the highest percentage of agricultural land use. As a result of the high variability in nutrient concentrations, we observed a wide range in the NO_3^-:NH_4^+ ratio (from 8 to 27) and in the DIN:SRP molar ratio (23 to 95; Table 3.1).

Biofilm responses to ambient DIN variability

Mean biomass accrual rates of biofilms in DIN-free treatments ranged from 43 to 126 µg C cm^{-2} d^{-1}, and differed significantly among the streams in which the NDS were incubated (Fig. 3.1a, Table 3.2). However we only observed significant differences between GUA and FR (Tukey HSD tests, $p < 0.020$; Fig. 3.1a). The biomass accrual rates of biofilms in DIN-free treatments were positively correlated with ambient NO_3^- concentration ($r = 0.30$, $p = 0.029$; Fig. 3.2a) and NH_4^+ concentration ($r = 0.41$, $p = 0.002$; Fig. 3.2b) of the streams in which NDS were incubated. Algal accrual rates of biofilms in DIN-free treatments were similar among streams, except in CAS where rates were 5 times higher (Tukey HSD tests, $p < 0.001$; Fig. 3.1b, Table 3.2). Algal accrual rates of biofilms in DIN-free treatments were positively correlated with ambient NH_4^+ concentration of the streams in which NDS were incubated ($r = 0.31$, $p = 0.023$; Fig. 3.2d). Furthermore, algal accrual rates of biofilms in DIN-free treatments were positively correlated with biomass accrual rates in the same treatments ($r = 0.38$, $p = 0.005$). The C:N molar ratios of biofilms in DIN-free treatments (mean = 8.9) did not differ significantly among the streams in which the NDS were incubated (Fig. 3.1c; Table 3.2).

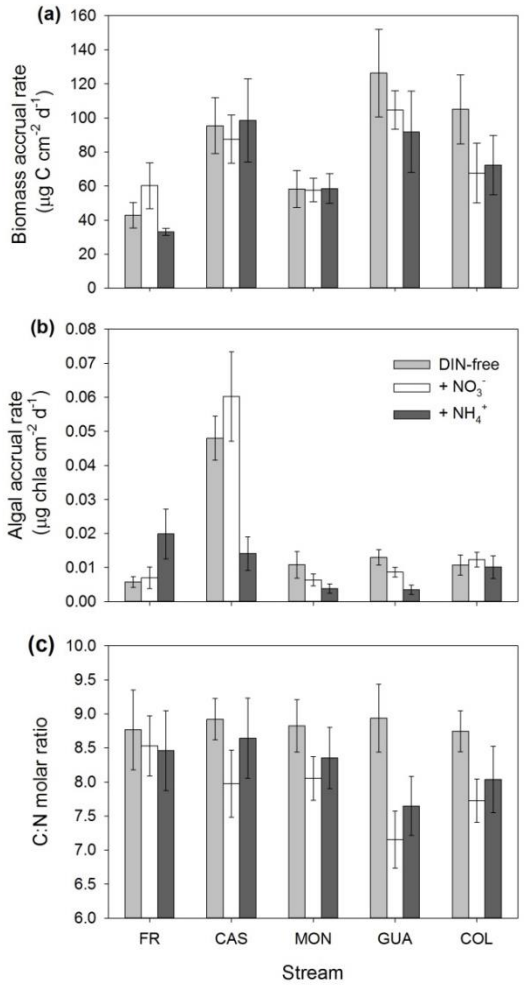

Figure 3.1. Biomass accrual rate (a), algal accrual rate (b) and C:N molar ratio (c) of biofilms developed on nutrient diffusing substrata (NDS) for the different streams and nutrient treatments in which the NDS were incubated. Data reported are the mean ± SE.

U_{spec}-NO_3^- of biofilms in DIN-free treatments was one order of magnitude higher (mean = 0.04 h^{-1} vs. mean = 0.005 h^{-1}) and more variable (CV = 71% vs CV = 26%) than U_{spec}-NH_4^+ (Fig. 3.3a and b). U_{spec}-NO_3^- of biofilms in DIN-free treatments varied significantly depending on the stream in which the NDS were incubated (one-way ANOVA, $F = 7.40$, $p < 0.001$). U_{spec}-NO_3^- was highest in biofilms developed in MON, and FR (Tukey HSD tests, $p < 0.012$). Conversely, U_{spec}-NH_4^+ of biofilms in DIN-free treatments

did not differ significantly among the streams in which the NDS were incubated (one-way ANOVA, F = 1.66, p = 0.224). U_{spec}-NO_3^- of biofilms in DIN-free treatments was negatively correlated with the ambient NH_4^+ concentration of the streams in which NDS were incubated (r = -0.37 and p = 0.045; Fig. 3.2f). Furthermore, U_{spec}-NO_3^- of biofilms in DIN-free treatments was negatively correlated with algal accrual rates in the same NDS treatments (r = -0.37 and p = 0.046).

Mean RPI values of biofilms in DIN-free treatments were similar among biofilms developed in the different streams (one-way ANOVA, F = 0.54, p = 0.712) and close to 1, indicating no clear preference for any of the two DIN species (Fig. 3.3c).

Table 3.2. Results from the linear mixed-effects model with stream as fixed factor and incubation as random factor on the biomass accrual rate, algal accrual rate and C:N molar ratio of biofilms in DIN-free treatments. Significance of the random factor incubation was obtained with the Likelihood Ratio Test. Values highlighted in bold indicate significant effects ($p < 0.05$).

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

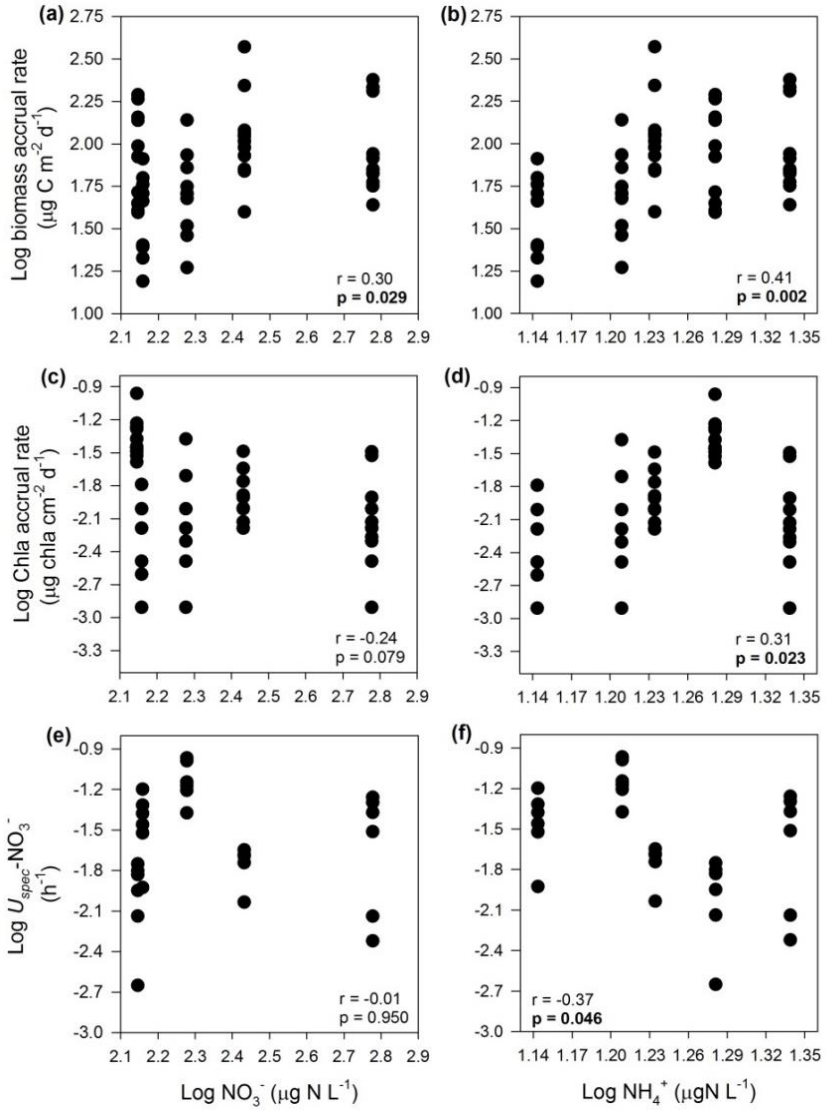

Figure 3.2. Relationships between biofilm variables and ambient concentrations of NO_3^- and NH_4^+ in the streams in which the NDS were incubated. Biomass accrual rates and NO_3^- (a) or NH_4^+ (b), algal accrual rates and NO_3^- (c) or NH_4^+ (d), and biomass-specific uptake for NO_3^- (U_{spec}-NO_3^-) and NO_3^- (e) or NH_4^+ (f). Results are for Pearson correlations. Values highlighted in bold indicate significant correlations ($p< 0.05$).

Biofilm responses to NO_3^- and NH_4^+ enrichments

In general, the comparison between DIN-free and DIN-enriched NDS treatments (i.e., the response ratio, RR) showed that both biofilm growth (for

the 3 variables measured) and DIN uptake had a negative response to NO_3^- and NH_4^+ enrichments (Fig. 3.4 and 3.5). The RRs of biomass accrual rates differed significantly among the streams in which the NDS were incubated (Fig. 3.4a; Table 3.3), but they did not differ significantly between $+NO_3^-$ and $+NH_4^+$ treatments (mean = -0.09 and -0.11 for $+NO_3^-$ and $+NH_4^+$ treatments, respectively; Fig. 3.4a). Biomass accrual response to DIN enrichments was null in those streams with lower DIN ambient availability and most negative in biofilms developed in COL, the stream with the highest ambient DIN availability (Tukey HSD tests, $p < 0.036$). In addition, the RRs of biomass accrual rates in $+NO_3^-$ treatments were negatively correlated with ambient NO_3^- concentration ($r = -0.39$, $p = 0.004$) and NH_4^+ concentration ($r = -0.38$, $p = 0.004$) of the streams in which the NDS were incubated. The RRs of biofilm accrual rates in $+NH_4^+$ treatments were also negatively correlated with the ambient NO_3^- concentration of the streams in which the NDS were incubated ($r = -0.34$ and $p = 0.022$). These correlations suggest that inhibition of biomass accrual increased with rising DIN concentration among streams.

The RRs of algal accrual rates in biofilms differed significantly among the streams and between $+NO_3^-$ and $+NH_4^+$ treatments (Fig. 3.4b; Table 3.3). The RRs for the two DIN enrichment treatments were negative in the biofilms developed in the 3 streams with intermediate ambient DIN concentrations (Tukey HSD tests, $p < 0.030$; Fig. 3.4b) and null in the 2 streams located in the extremes of the DIN gradient (Tukey HSD tests, $p < 0.005$; Fig. 3.4b). On average, the RRs of algal accrual rates were more negative in $+NH_4^+$ than in $+NO_3^-$ treatments (mean = -0.42 and -0.09, respectively; Fig. 3.5b; Table 3.3). The RRs of algal accrual rates for both $+NO_3^-$ and $+NH_4^+$ treatments was not correlated with either ambient NO_3^- or NH_4^+ concentration of the streams where the NDS were incubated.

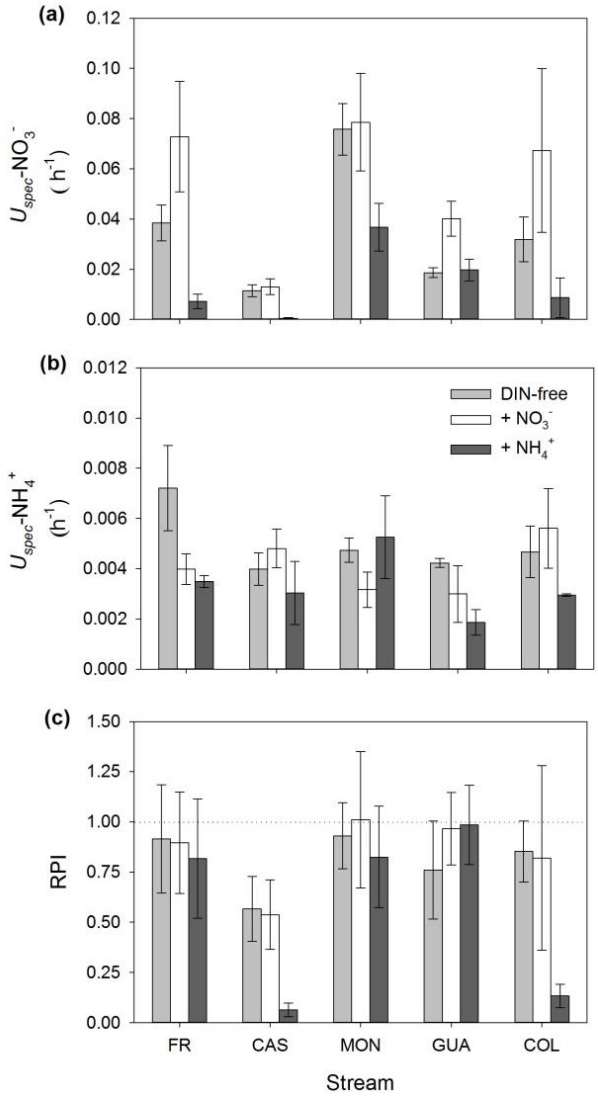

Figure 3.3. Biomass-specific uptake for NO_3^- (U_{spec}-NO_3^-; a), for NH_4^+ (U_{spec}-NH_4^+; b) and relative preference index (RPI; c) of biofilms developed on nutrient diffusing substrata (NDS) in the different streams and nutrient treatments. Note that the y-axis from panel b is one order of magnitude lower than that from panel a. In panel c, the horizontal dashed line at 1 denotes the shift from NH_4^+ to NO_3^- preference. Values <1 indicate preference for NH_4^+, whereas values >1 indicate preference for NO_3^-. Data reported are the mean ± SE.

The RRs of the biofilm C:N molar ratio were consistently negative across the streams and for both $+NH_4^+$ and $+NO_3^-$ treatments. Thus, biofilms exposed to DIN enrichments increased their N content relative to their C content.

Differences in RRs of C:N were significant among streams, but not between $+NO_3^-$ and $+NH_4^+$ treatments (Fig. 3.4c; Table 3.3). The responses to DIN enrichments were more negative in biofilms developed in GUA (Tukey HSD tests, $p < 0.005$).

Table 3.3. Results from the linear mixed-effects model with stream and NDS treatment as fixed factors and incubation as random factor on biofilm growth responses to DIN enrichments in the form of NO_3^- and NH_4^+ among streams in terms of biomass accrual rate, algal accrual rate and C:N molar ratio. Significance of the random factor incubation was obtained with the Likelihood Ratio Test. Values highlighted in bold indicate significant effects ($p< 0.05$).

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

The RRs of U_{spec}-NO_3^- for biofilms and DIN species enrichments, but measured in COL stream, differed significantly depending on the stream in which the biofilms had developed (two-way ANOVA, $F = 9.57$, $p < 0.001$) and between $+NO_3^-$ and $+NH_4^+$ treatments (two-way ANOVA, $F = 58.13$, $p<0.001$; Fig. 3.5a). The interaction between the two factors was also significant (two-way ANOVA, $F = 6.12$, $p < 0.001$). The RRs of U_{spec}-NO_3^-

tended to be null in biofilms grown in $+NO_3^-$ treatments and particularly negative for biofilms grown in $+NH_4^+$ treatments (Fig. 3.5a). This indicates that efficiency for NO_3^- uptake decreased especially in biofilms exposed to $+NH_4^+$ enrichment.

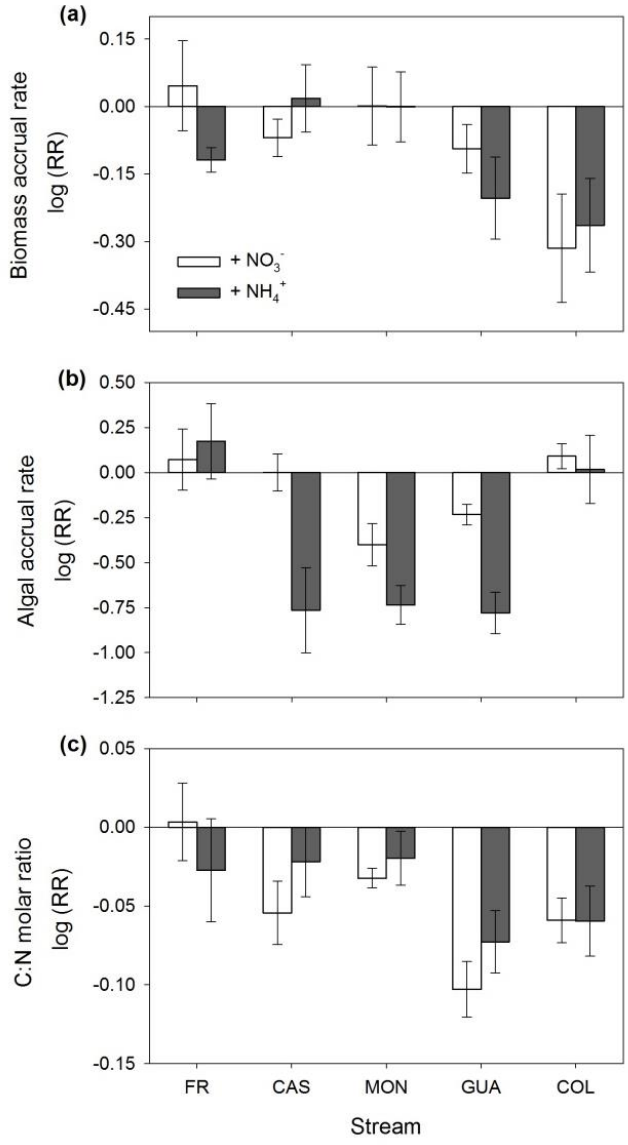

Figure 3.4. Biofilm growth response ratio (RR) to enrichments of NO_3^- and NH_4^+ in terms of biomass accrual rate (a), algal accrual rate (b) and C:N molar ratio (c) for the different streams in which the nutrient diffusing substrata (NDS) were incubated. Data reported are the mean ± SE.

The RRs of U_{spec}-NH_4^+ for biofilms developed in different streams and DIN species enrichments, but measured in COL stream, were similar regardless of the stream considered and the NDS treatment at which they developed (two-way ANOVA, F = 1.99, p = 0.118 and F = 1.06, p = 0.311 for stream and treatment respectively; Fig. 3.5b). In general, the RRs of U_{spec}-NH_4^+ were negative, but lower than the RRs of U_{spec}-NO_3^-, indicating a lower effect of DIN enrichments on U_{spec}- NH_4^+ than on U_{spec}-NO_3^-. The RRs of biofilm RPI differed significantly depending on the stream in which the NDS were incubated (two-way ANOVA, F = 5.38, p = 0.001) and between +NO_3^- and +NH_4^+ treatments (two-way ANOVA, F = 4.81, p = 0.034), with no significant interaction between factors (two-way ANOVA, F = 2.30, p = 0.075). However, despite these differences, the RRs of RPI were not different from 0 in 7 out of 10 cases (Fig. 3.5c), indicating no overall preference for any of the two DIN species.

3.5. Discussion

Biofilm responses to ambient DIN variability

We expected that differences in ambient NO_3^- and NH_4^+ concentrations among the streams in which the NDS were incubated would affect biofilm development and its N demand from the water column. Specifically, we expected that biofilm growth and DIN uptake would be higher in those biofilms that had developed in streams with higher ambient DIN availability if ambient concentrations of the two investigated DIN species were below saturation levels and if biofilms were not limited by other environmental factors (Dodds et al. 2002; O'Brien et al. 2007; von Schiller et al. 2007; O'Brien and Dodds 2008). We observed that streams with higher ambient NO_3^- and NH_4^+ concentrations showed higher biofilm biomass and algal accrual rates, supporting our expectations and suggesting that biofilms development and its contribution to stream water DIN uptake is enhanced under higher availability of DIN. On the other hand, lack of significant

variation in the biofilm C:N ratios at ambient levels suggests that the range of ambient DIN concentration was not broad enough to cause significant stoichiometric differences in the biofilms among the studied streams (Dodds et al. 2004).

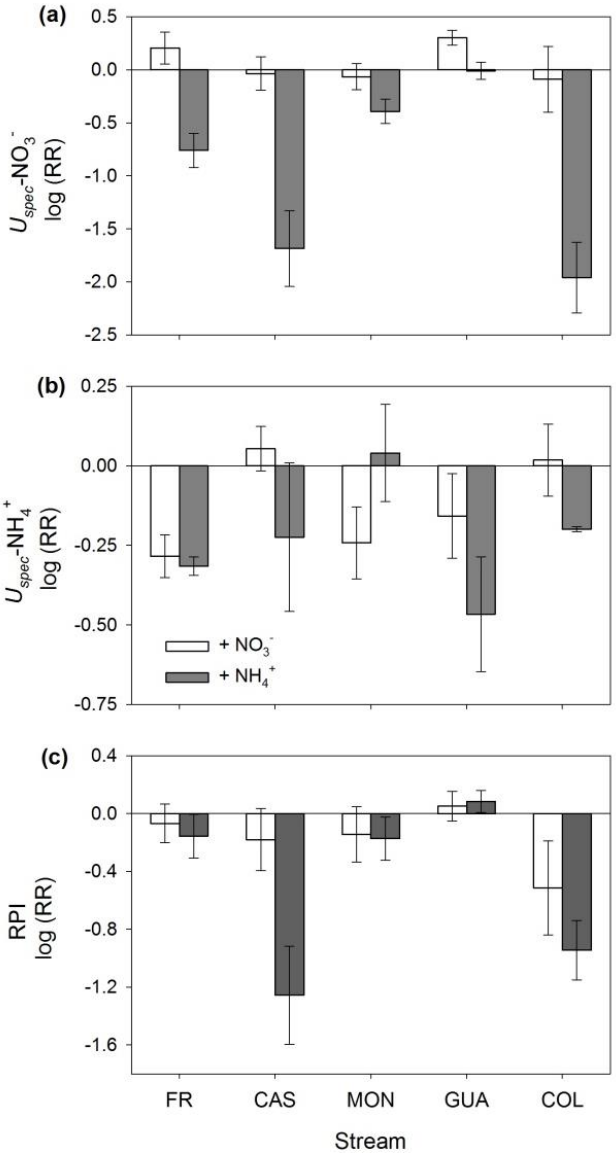

Figure 3.5. Biofilm DIN uptake response ratio (RR) to enrichments of NO_3^- and NH_4^+ in terms of biomass-specific uptake for NO_3^- (U_{spec}-NO_3^-; a) and for NH_4^+ (U_{spec}-NH_4^+; b), and relative preference index (RPI; c) for the different streams in which the nutrient diffusing substrata (NDS) were incubated. Data reported are the mean ± SE.

U_{spec}-NO_3^- of biofilms developed in DIN-free treatments in the different streams differed significantly, despite uptake measures were done in a single stream. Furthermore, biofilm U_{spec}-NO_3^- was consistently higher than U_{spec}-NH_4^+ regardless of the differences in the concentrations of the two DIN species among the study streams, suggesting that biofilms have a consistently higher reliance on NO_3^- than on NH_4^+ from the water column to meet their N requirements. Our results are in line with previous studies showing that the generally higher NO_3^- availability as a DIN source ultimately drives the use of this DIN species by biofilms to meet their N demand (Fellows et al. 2006; Newbold et al. 2006; Bunch and Bernot 2012). RPI values close to 1, indicating no preference for either DIN species, support this explanation. These results contrast the general idea that microbial assemblages in biofilms have a preference for DIN assimilation in the form of NH_4^+ due to the lower energetic cost associated with the assimilation of this DIN species (Dortch 1990; Naldi and Wheeler 2002). However, the results are in line with empirical data from a previous study which showed an unclear pattern of biofilm preference for NH_4^+ relative to NO_3^- availability (Hoellein et al. 2010).

According to previous studies (O'Brien et al. 2007; von Schiller et al. 2007), we expected that variability in U_{spec} of the two DIN species among biofilms would be positively related to differences in ambient DIN concentration of the streams in which the NDS were previously incubated. However, the results did not support our expectations. Higher U_{spec}-NO_3^- was observed in biofilms that developed in 2 of the 3 streams with the lowest NO_3^- concentrations, and no differences among streams in biofilm U_{spec}-NH_4^+ were found. In fact, we observed lower biofilm U_{spec}-NO_3^- in streams with higher NH_4^+ concentration, which supports previous studies indicating that NH_4^+ availability may regulate the uptake of DIN in the form of NO_3^- (Gonzalez et al. 2006; Dugdale et al. 2007; Domingues et al. 2011). The low range of variation in NH_4^+ concentration among streams where biofilms developed (from 14 to 22 µg N/L) may have precluded observing differences U_{spec}-NH_4^+, despite previous studies have shown that the concentration of NH_4^+ can

control NH_4^+ uptake rates at whole-reach scale (Dodds et al. 2002; O'Brien and Dodds 2008). Alternatively, lack of U_{spec}-NH_4^+ variation among biofilms developed in the different streams also suggests that biofilms assimilated NH_4^+ equally efficiently among streams, regardless of the differences in biomass accrual and algal growth observed, probably due to the lower range of NH_4^+ concentration among streams.

Variation in biomass accrual rates among streams was positively related to algal accrual rates, indicating that algae had a similar response to that of the bulk biofilm. In this context, the negative correlation between algal accrual rates and U_{spec}-NO_3^-, contrasts with other studies indicating that algae in biofilms rely mostly on NO_3^- (Bernhardt et al. 2002; Bechtold et al. 2012). It is worth noting that the streams where the NDS were incubated were heavily shaded by riparian vegetation, which may have limited N demand, especially by algae in biofilms (Hill et al. 1995; Sabater et al. 2000; von Schiller et al. 2007). Therefore, it is possible that light-limitation may have masked the effects of other factors such as variation in DIN concentration or relative availability between DIN and SRP among streams, on algal uptake (von Schiller et al. 2007).

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

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

Furthermore, algal accrual, U_{spec}-NO_3^- and RPI response ratios were consistently more negative in those biofilms that developed under NH_4^+ enriched conditions compared to NO_3^- enriched conditions, suggesting a differential effect of the two DIN species on biofilm development and biogeochemical activity.

The negative response to DIN enrichments was more pronounced for algal accrual than for bulk biomass accrual. This may be explained by the low light availability (i.e, closed canopy reaches) during the experiments, which had a higher constrain on algal development in biofilms than on whole-bulk biofilm biomass. Interestingly, we also observed that the negative responses of algal growth were more pronounced in $+NH_4^+$ than in $+NO_3^-$ treatments. Instances of lower biofilm and algal growth in DIN-enriched substrates with respect to control treatments are relatively common in the literature (Francoeur 2001; Tank and Dodds 2003; Bernhardt and Likens 2004; von Schiller et al. 2007), despite these studies have mainly focussed on NO_3^- enrichments. Several mechanisms have been proposed to explain this response: (i) preference of grazing invertebrates for biofilms developed on nutrient-rich substrates, (ii) nutrient enrichment up to toxic levels, or (iii) changes in the species composition of biofilms (Bernhardt and Likens 2004; Hoellein et al. 2010; Domingues et al. 2011). Field observations during both NDS incubations confirmed low presence of grazers on NDS filters, which excludes the first explanation. Alternatively, we cannot exclude the fact that $+NH_4^+$ treatments lead to toxic effects (Camargo and Alonso 2006) or that either NO_3^- or NH_4^+ enrichments lead to changes in biofilm assemblage composition because the experiment was not aimed to provide these mechanistic results. However, we need to consider that our results suggest a shift in DIN uptake efficiency of biofilms, with those exposed to NH_4^+ enrichment potentially favoring the development of nitrifier assemblages, enhancing the process of aerobic oxidation of NH_4^+ to NO_3^-.

The most relevant biofilm responses to enrichment of the two DIN species were observed for N uptake. In absolute terms, the negative response

observed was greater for U_{spec}-NO_3^- than for U_{spec}-NH_4^+ and mostly associated with NH_4^+ enrichments. NO_3^- enrichment caused only minor changes in either U_{spec}-NO_3^- or U_{spec}-NH_4^+ when compared with NH_4^+enrichment. Overall, results suggest that biofilm exposures to NH_4^+ enrichment may induce some functional and/or structural changes in the biofilms resulting in a lower demand for NO_3^-. In addition, NH_4^+ enrichments might have enhanced internal N cycling within the biofilms; thereby decreasing the biofilm NO_3^- dependence from the water column (von Schiller et al. 2007). An alternative explanation is that the enrichment of NH_4^+ can favor the development of nitrifiers, which is supported by results from previous studies (Bernhardt and Likens 2004; Merbt et al. 2014). Nitrifying microorganisms have lower growth efficiencies compared to other microbial components of the biofilms (Risgaard-Petersen et al. 2004) and they also have a preferential demand for NH_4^+, which is used as reduction power in anabolic activity. This potential shift in the microbial composition of biofilms could at least partially explain the more negative effects on U_{spec}-NO_3^- in NH_4^+ enrichments consistently observed for biofilms developed in all streams studied.

Conclusions

NDS bioassays have been commonly used to assess nutrient limitation of P and N in a large variety of freshwater environments (Francoeur 2001; King et al. 2014). However, NDS have been rarely employed to address other ecologically relevant questions, such as to contrast biofilm responses to different DIN species (but see von Schiller et al. 2007 and Hoellein et al. 2010). In addition, studies using NDS have mostly focused on the biofilm response in terms of biomass accrual, and less attention has been paid on how the nutrient enrichments affect biofilm function, such as the demand of nutrients from the water column. In this regard, we found that the most relevant biofilm responses to enrichment of the two DIN species were observed for N uptake, and more specifically, that NH_4^+ enrichments caused a clear decrease in U_{spec}-NO_3^-. Knowledge on these responses provides a better

understanding of the effects of increases in the availability of DIN species on biofilm development and contribution to in-stream N uptake. Our results suggest that biofilms developing in streams with high NO_3^- concentration, such as those draining agricultural catchments (Stanley and Maxted 2008; Lassaletta et al. 2009) may have a limited capacity to retain excess NO_3^-. On the other hand, streams with low NO_3^-:NH_4^+ ratios due to inputs of NH_4^+-rich sources, such as streams receiving WWTP effluents (Marti et al. 2004; Martí et al. 2010), may cause significant decreases in the capacity of biofilms for NO_3^- uptake. All together these biofilm responses to increases in the concentration of the DIN species, which can be driven by land use changes, may have significant implications for the export of DIN to downstream ecosystems.

Acknowledgments

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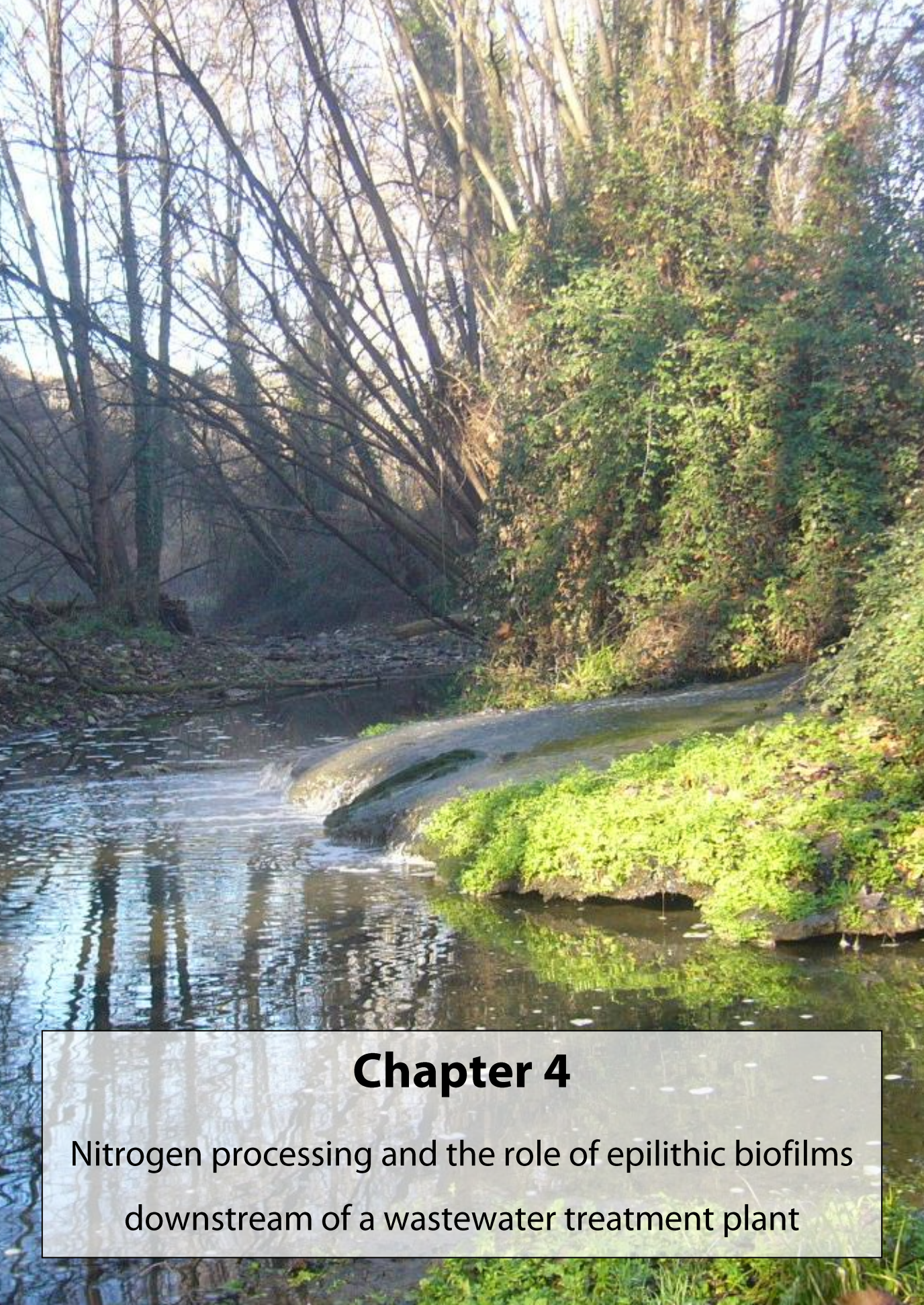

Chapter 4

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

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Cover: effluent of the waste water treatment plant of Santa Maria de Palautordera. Photograph by Miquel Ribot.

4.1. 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.

4.2. Introduction

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 (Kendal 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 (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 type 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.

4.3. 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 et al. 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 this site because the stream was dry upstream of the WWTP input.

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; 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 ($S_{W\text{-}net}$) (Martí et al. 2004), in which the net variation of nutrient concentration along the reach can be described as:

$$N_x = N_1(C_x/C_1)e^{-K_c x} \quad (1)$$

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.

4.4. 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. 4.1A, 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. 4.1A). 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. 4.1A). 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. 4.1A). In summer, the NH_4^+ concentration decreased sharply along the reach to yield a relatively short $S_{W\text{-}net}$ (157 m; Fig. 4.1B). In contrast, NO_3^- concentration showed a hump-shaped longitudinal pattern (Fig. 4.1B). 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. 4.1B).

Table 4.1. 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
EC (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

The magnitude and longitudinal patterns of the $\delta^{15}N$ values also differed between seasons (Fig. 4.1C, D). In winter, $\delta^{15}NH_4^+$ values increased along the study reach (linear regression, $p < 0.001$; Fig. 4.1C), whereas $\delta^{15}NO_3^-$ values

decreased (linear regression, $p = 0.001$; Fig. 4.1C). 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. 4.1D). $\delta^{15}NO_3^-$ 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.

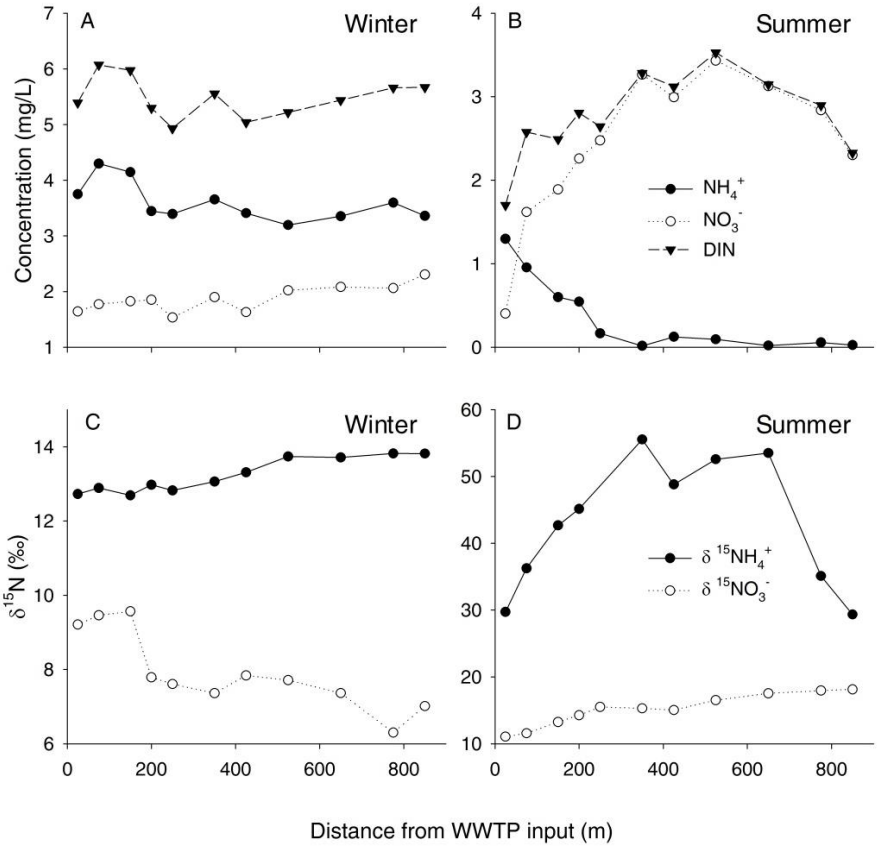

Figure 4.1. Variation of ambient concentrations (A, B) and $\delta^{15}N$ signatures (C, D) of dissolved N species along the study reach in winter (A, C) and summer (B, D). WWTP= wastewater treatment plant.

The relationships between the concentrations of DIN species and their $\delta^{15}N$ signatures differed between seasons (Fig. 4.2A–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. 4.2A), whereas NO_3^- concentrations and

$\delta^{15}NO_3^-$ were significantly correlated (Spearman rank correlation, r = –0.67, p = 0.03; Fig. 4.2B). 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. 4.2C, D).

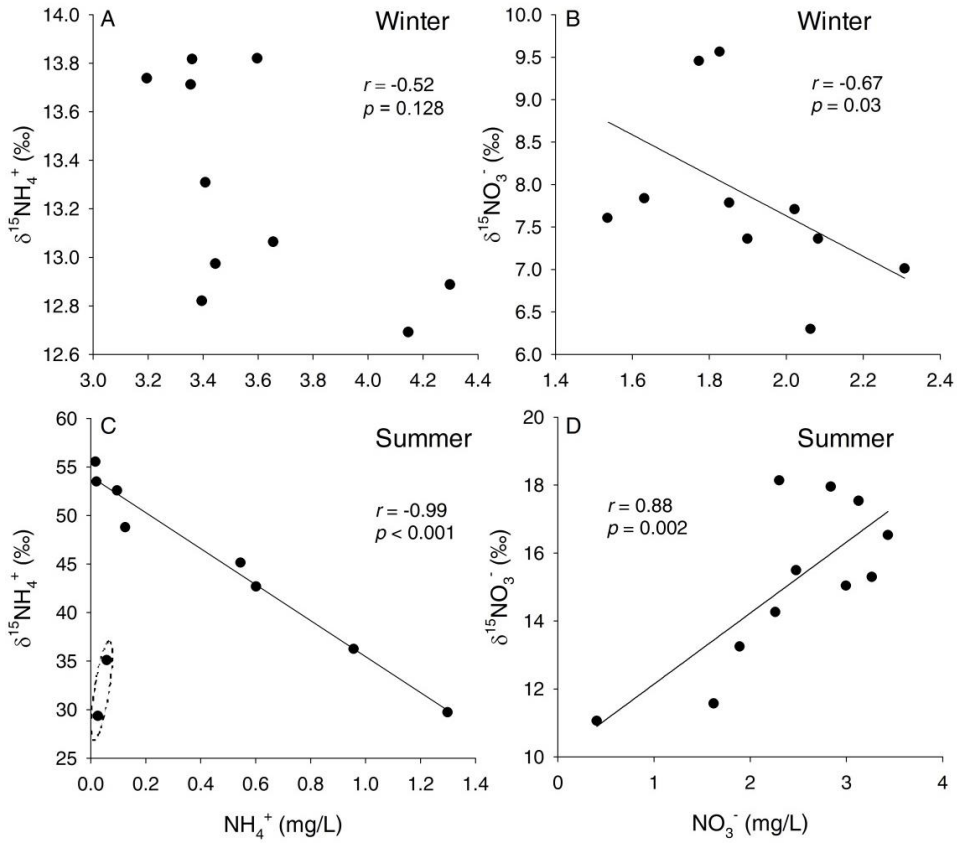

Figure 4.2. Relationships between the concentrations of NH_4^+ (A, C) and NO_3^- (B, D) and their respective $\delta^{15}N$ signatures in winter (A, B) and summer (C, D). The dashed ellipse in C indicates 2 outliers of the correlation corresponding with the last 2 sampling sites. Results are for Spearman rank correlations.

$\delta^{15}N$ 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. 4.3A). 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. 4.3A). In summer, $\delta^{15}N$ values did not differ between biofilm types (Wilcoxon matched pair test, $p = 0.213$; Fig. 4.3B), and $\delta^{15}N$ values of both biofilm types increased along the reach downstream of the WWTP (linear regression, $p < 0.001$; Fig. 4.3B).

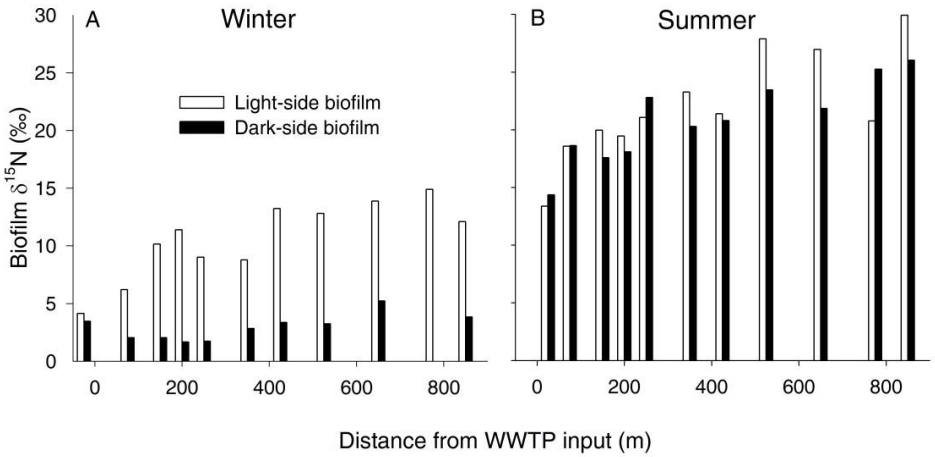

Figure 4.3. Variation along the study reach in $\delta^{15}N$ values of biofilm types from the light and dark sides of cobbles measured in winter (A) and summer (B). Negative values for distance indicate the site upstream of the wastewater treatment plant (WWTP) input (0 m).

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. 4.4A), 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. 4.4B).

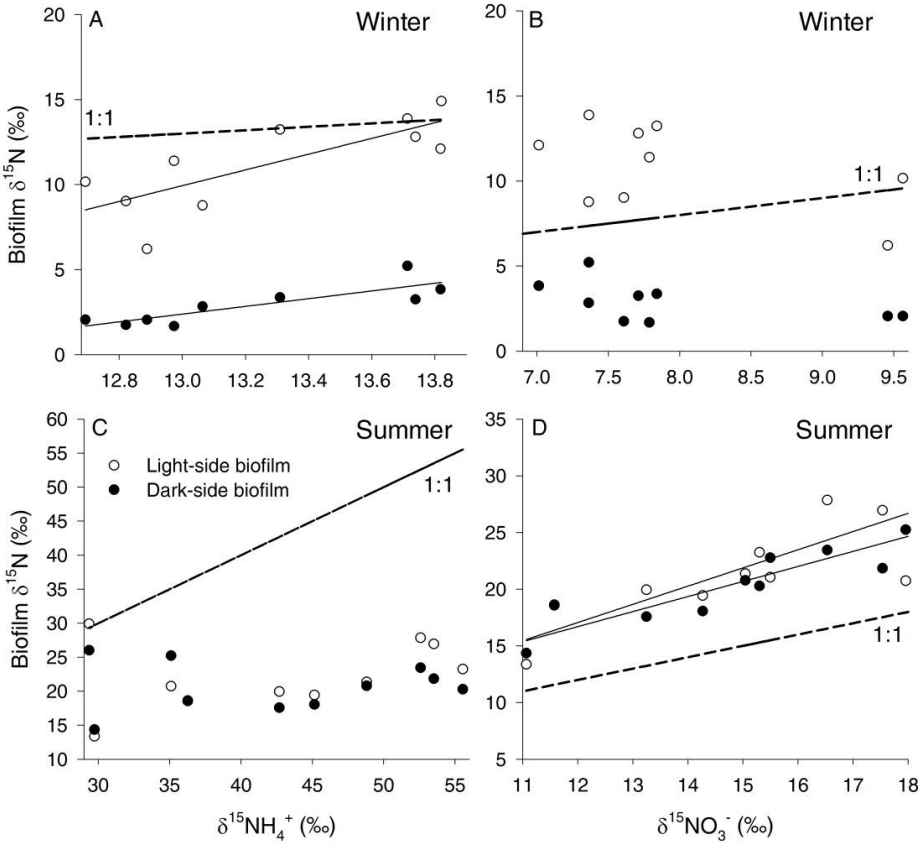

Figure 4.4. Relationships between $\delta^{15}N$ signature of NH_4^+ (A, C) and NO_3^- (B, D) and $\delta^{15}N$ signature of the biofilm from the light and dark sides of cobbles in winter (A, B) and summer (C, D). Significant Spearman rank correlations ($p < 0.05$) are indicated by lines. Dashed lines denote 1:1 relationships.

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. 4.4C). 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. 4.4D).

4.5. 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 differential ^{15}N fractionation involved in each process (Kendal 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. 2010, 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 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. Chérnier et al. (2006) showed

close coupling between photoautorophic 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 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 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 further have 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 et al. 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 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 communities and provides 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 2004). NH_4^+-oxidizing bacteria grow slower and have lower N uptake rates than photoautotrophs (Risgaard-Petersen 2003, 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 which 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 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 et al. (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 $\delta^{15}N$ 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 $\delta^{15}N$ 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, $\delta^{15}N$ 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 biolfim in summer than in winter.

Overall, our study revealed that the longitudinal patterns of stream DIN concentrations and $\delta^{15}N$ 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 $\delta^{15}N$ 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, 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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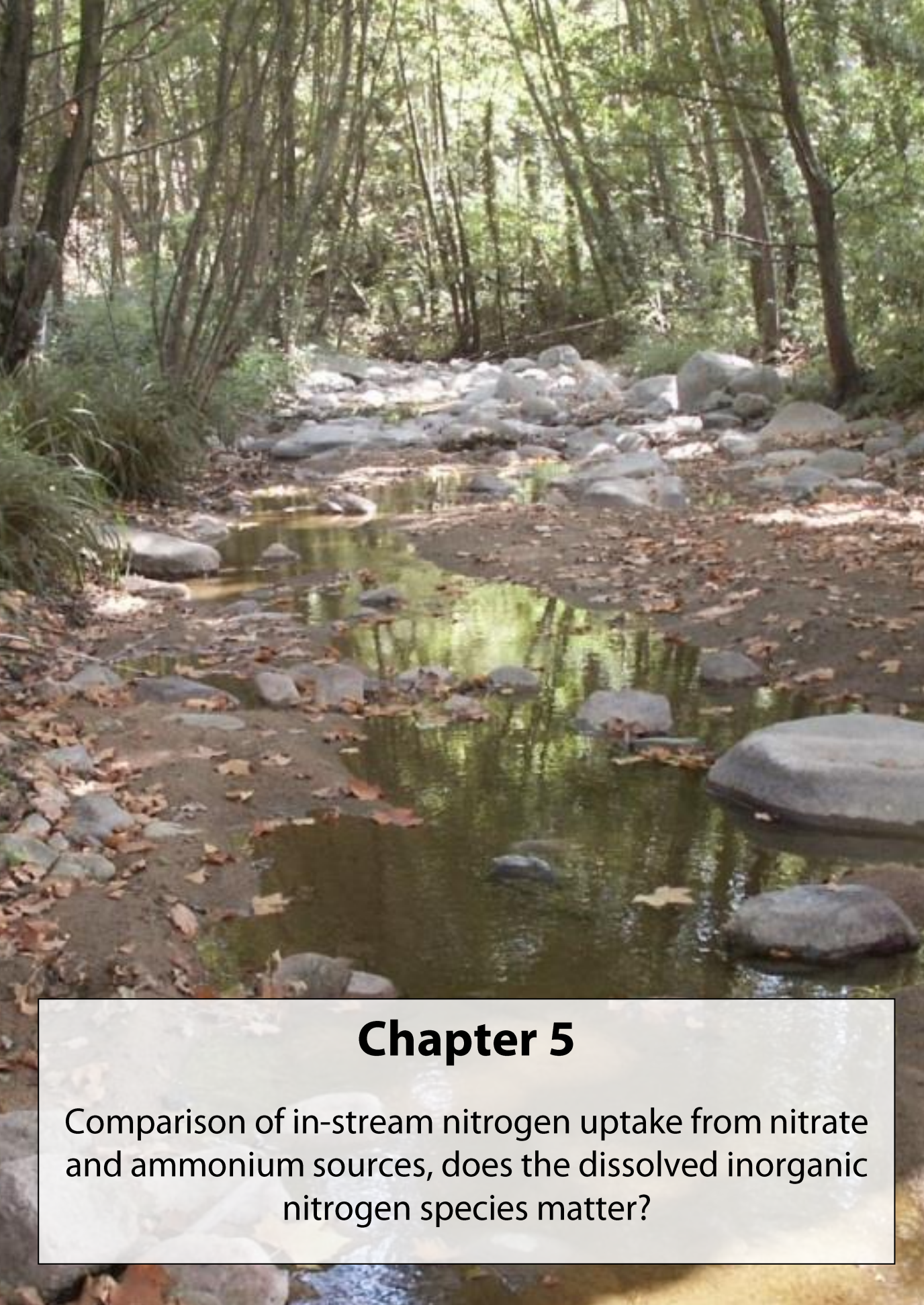

Chapter 5

Comparison of in-stream nitrogen uptake from nitrate and ammonium sources, does the dissolved inorganic nitrogen species matter?

With permissions from Daniel von Schiller and Eugènia Martí, who are co-authors of this study.

Cover: general view of the Santa Coloma stream. Photograph by Daniel von Schiller.

5.1. Abstract

Dissolved inorganic nitrogen (DIN) in streams is mostly available as two different species, nitrate (NO_3^-) and ammonium (NH_4^+). These two DIN species undergo different biogeochemical uptake pathways, mostly driven by benthic microbial assemblages (i.e., biofilms), which ultimately dictate the fate of in-stream DIN. We characterized the key in-stream N uptake pathways and primary uptake compartments (PUCs) responsible for NO_3^- and NH_4^+ uptake in a Mediterranean stream using field ^{15}N tracer additions of the two DIN species. Furthermore, we examined how experimentally-observed patterns from a single stream fit within existing results by analyzing trends from the literature. Our results indicated remarkable differences between the two DIN species. Reach-scale uptake efficiency and demand of in-stream biota was higher for NH_4^+, but total NO_3^- uptake was higher than that of NH_4^+ due to the higher availability of the former. Denitrification and DNRA had a low incidence on the total NO_3^- uptake (< 1%). Conversely, nitrification accounted for 43% of the total NH_4^+ uptake. Assimilatory uptake by PUCs accounted for only 17 % of the total NO_3^- uptake, whereas it accounted for 76% of total NH_4^+ uptake. In absolute terms, assimilatory uptake was higher for NH_4^+ than for NO_3^- and for the two DIN species was mostly driven by biofilms from fine benthic organic matter. Results from the literature survey support the results from our single stream, pointing to the fact that the two DIN species are not exchangeable and in-stream uptake metrics are DIN-species specific.

5.2. Introduction

Over the past 30 years, much effort has been placed to quantify how streams contribute to regulate nitrogen (N) inputs from terrestrial ecosystems (Alexander et al. 2000; Peterson et al. 2001) and to disentangle the drivers of in-stream N uptake (Mulholland and Webster 2010). N is a key element for organisms, therefore its availability can control in-stream primary production (Grimm and Fisher 1986; Francoeur 2001). During the last decades, in-stream dissolved inorganic N (DIN) concentrations have increased due to human activities developed on the draining catchments (Seitzinger and Kroeze 1998; Boyer et al. 2002; Galloway et al. 2004). Furthermore, recent studies have highlighted that land use changes driven by humans have modified the relative proportion of the two main DIN species: nitrate (NO_3^-) and ammonium (NH_4^+). Agriculture causes N enrichment of the draining streams, mainly in the form of NO_3^- (Stanley and Maxted 2008; Lassaletta et al. 2009; Ballantine and Davies-Colley 2014), whereas urban storm water runoff and WWTP effluents cause N enrichments in receiving streams mainly in the form of NH_4^+ (Marti et al. 2004; Merseburger et al. 2005; von Schiller et al. 2008b; Martí et al. 2010).

In-stream assimilatory DIN uptake is mostly driven by microbial assemblages (i.e. biofilms), which develop on submersed substrata (Lock et al. 1984; Pusch et al. 1998; Battin et al. 2003). Biofilms mainly develop on mineral sediments such as sand and cobbles, commonly referred to as epilithon, and on organic detritus such as leaves, small wood and fine benthic organic matter (FBOM). Other primary uptake compartments (PUCs) developing on the stream channel such as macrophytes or riparian vegetation growing of the stream edges may also contribute substantially to in-stream DIN uptake (Schade et al. 2001; von Schiller et al. 2009; Riis et al. 2012). PUCs can directly assimilate the two DIN species from the water column to meet their N demand (Kemp and Dodds 2002; Pastor et al. 2013). The uptake fluxes of NO_3^- and NH_4^+ not only rely on the ambient concentrations of each DIN species (Dodds et al. 2002; Ribot et al. 2013) but also on the relative

proportion between them (Geisseler et al. 2010). Despite NH_4^+ is typically less available than NO_3^- in streams, the NH_4^+ uptake flux may be similar or even higher than that of NO_3^- (Peterson et al. 2001; Ensign and Doyle 2006), likely because NH_4^+ is preferred over NO_3^- due to the lower assimilatory cost of the former DIN species (Naldi and Wheeler 2002; Hildebrand 2005).

Besides assimilatory uptake, NO_3^- and NH_4^+ can also undergo specific energy-yielding dissimilatory pathways. Nitrate serves as the substrate for two microbially-mediated pathways that occur under sub-oxic to anoxic conditions: denitrification and dissimilatory nitrate reduction to ammonium (DNRA). In denitrification, NO_3^- is sequentially reduced to N_2O and N_2 gas in presence of organic matter, thereby decreasing the NO_3^- load from the ecosystem (Seitzinger et al. 2006; Lin et al. 2009). In DNRA, NO_3^- is reduced to bioavailable NH_4^+, thus preserving N within the ecosystem (Tiedge JM, 1988; Silver et al. 2001). While denitrification may account for a significant fraction of the total NO_3^- uptake in streams (Mulholland et al. 2008), the importance of DNRA is relatively unknown (Burgin and Hamilton 2007). On the other hand, NH_4^+ serves as the substrate for two other microbially-mediated dissimilatory pathways: nitrification and anaerobic ammonium oxidation (anammox). Nitrification refers to the oxidation of NH_4^+ to NO_3^- by which some chemoautotrophic bacteria and archaea meet their energy demand (Prosser 1989; Lin et al. 2009). Anammox refers to the anaerobic oxidation of NH_4^+ to N_2 using NO_2^- as the electron acceptor (Op Den Camp et al. 2006). Previous studies have highlighted that nitrification may account for a large portion of total NH_4^+ uptake in streams (e.g. Peterson et al. 2001), while anammox has been less studied and its importance is still poorly understood (Burgin and Hamilton 2007).

In summary, in-stream NO_3^- uptake mainly relies on the specific uptake fluxes associated to assimilatory uptake, denitrification and DNRA as well as to the relative dominance among them. On the other hand, in-stream NH_4^+ uptake mainly relies on the specific uptake fluxes associated to assimilatory uptake and nitrification and the relative dominance between them. Therefore,

general conclusions on the characterization of in-stream N uptake may be subjected to the DIN species under consideration. However, when exploring N spiraling in streams, only few studies, have focused on explicitly contrasting the fate of the two DIN species at whole-reach scale under the same environmental conditions (but see Bernot et al. 2006; Tank et al. 2008). Together, changes in land uses driven by human and the different pathways undergone by the two DIN species in the stream may have consequences for the downstream export of DIN. Therefore consideration of the two major DIN species within a single study would allow us to better understand in-stream DIN processing and its downstream fate.

The goal of this study was to compare the uptake rates of NO_3^- and NH_4^+ at whole-reach scale and among PUCs. To approach our objective we used two field ^{15}N tracer additions to measure the key biogeochemical uptake pathways responsible of in-stream NO_3^- and NH_4^+ uptake in a reach of a Mediterranean stream. To put our results in a wider context, we surveyed the literature for data from streams worldwide with uptake metrics of the two DIN species. Using this data set, we explored the relationships between NO_3^- and NH_4^+ uptake to assess if the two DIN species behave similarly, and thus are interchangeable, or, as we expected, if in-stream DIN uptake metrics are DIN-species specific. On the other hand, data from the two ^{15}N additions within the same stream reach allowed us to compare the assimilatory uptake fluxes of NO_3^- and NH_4^+ among the different PUCs and assess the relative contribution of each PUC to the total assimilatory uptake fluxes.

5.3. Methods

Study site

The study was conducted in the Riera de Santa Coloma, a third-order stream located in Catalonia, NE Spain. The dominant climate in this region is typically Mediterranean, with warm-dry summers and cool-wet winters. Mean annual temperature and precipitation are 14ºC and 800 mm, respectively. At the study site (2º39´32´´E, 41º51´48´´N; 240 m a.s.l.), the stream drains a 45-

km^2 catchment dominated by siliceous geology. Most of the catchment is forested (93%), but it also includes some small towns (4%) and agricultural fields (3%), which are concentrated along the stream valley. For the experiments, we selected a 250-m long reach that runs along an area of ornamental plantations. The reach has a well-developed riparian vegetation that consists mainly of alder (*Alnus glutinosa*) and sycamore (*Platanus hispanica*), with a dense herbaceous understory. Channel morphology in the study reach is well preserved, with sand (40%), boulders (29%) and cobbles (19%) being the most abundant substrate types.

Field methods

We conducted the study during summer 2006, a period characterized by low stream flow and dense riparian tree coverage at the study reach. We defined 6 sampling sites along the reach and a sampling site upstream of it to serve as a reference of background conditions. We conducted two consecutive additions of ^{15}N using $^{15}NO_3$ (12 July 2006) and $^{15}NH_4^+$ (1 August 2006) as different DIN sources to measure whole-reach N uptake and transformation fluxes, and N assimilation fluxes for specific PUCs. The two additions were performed under similar environmental conditions so uptake fluxes for the two DIN species could be compared (Table. 5.1).

For each ^{15}N addition we prepared a solution amended with either $^{15}NO_3^-$ (as 99% enriched $K^{15}NO_3$) or $^{15}NH_4^+$ (as 99% enriched $^{15}NH_4Cl$) in conjunction with NaCl as a conservative tracer. The amount of $K^{15}NO_3$ and $^{15}NH_4Cl$ and the pump flow rate were set to achieve a target $\delta^{15}N$ enrichment of 10,000‰ for each DIN species in the water column. We released the ^{15}N solutions at the top of the reach at constant rate using a Masterflex (Vernon Hills, Illinois, USA) L/S battery-powered peristaltic pump. The two ^{15}N additions started at midnight (00:00) and lasted for 12 hours. To verify plateau conditions during each ^{15}N addition, we automatically recorded conductivity every 10 s at the end of the reach using a portable WTW conductivity meter connected to a Campbell Scientific (Logan, Utah, USA) CR510 data logger.

We measured conductivity and collected samples of water and different PUCs at each sampling site before each addition started for background characterization (i.e., pre-sampling). We repeated the water sampling at each site at 06:00 and at 12:00, during plateau conditions, to determine whole-reach N uptake metrics at night- and day-time, respectively. One day after the addition was stopped, we conducted another sampling (i.e. post-24 h sampling) of water, to determine whole-reach N regeneration rates, and of each PUC, to determine dry mass (DM) standing stocks, N content and ^{15}N labeling.

All water samples were immediately filtered through ashed Albet (Barcelona, Spain) FVF glass fiber filters (0.7 µm pore size). We stored filtered samples for nutrient chemistry (40 mL, two replicates per station) and $^{15}NO_3^-$ (0.5 L, two replicates per station) on ice in the field, and then refrigerated them at 4 ºC or keep them frozen in the laboratory until further processing and analysis. Processing of $^{15}NH_4^+$ samples (3 L, one sample per station) started immediately in the field and followed the procedures explained in the laboratory methods section.

At the pre- and plateau samplings of the $^{15}NO_3^-$ addition, we also collected water samples to analyze dissolved $^{15}N_2$ and $^{15}N_2O$ (40 mL, two replicates per station) as outlined in Hamilton and Ostrom (2007). Briefly, we collected water samples in 60-mL plastic syringes fitted with stopcocks, avoiding the inclusion of air bubbles in the samples. Then, we added 20 mL of high purity He to each water sample. We shook the syringes for 10 min to allow equilibration of the N-gas in the water into the He space. Afterwards, a gas sample from the He space was collected in evacuated 12-mL Labco (High Wycombe, UK) Type 3 exetainers. We stored the exetainers in water-filled centrifuge tubes until analysis.

Sampling of the different PUCs present in the reach (accounting for a reach coverage >10%) at each sampling site followed standard procedures used in previous ^{15}N experiments (Mulholland et al. 2000; von Schiller et al. 2009). For dry mass standing stocks, we sampled organic detrital

compartments (i.e., leaves and small wood sticks) and water-submerged alder roots by collecting all material found within a 0.04 m^2 metal frame. We also sampled FBOM from the surface (1 cm depth; FBOMsurf) and from the sub-surface (3 to 5 cm depth; FBOMsub) of the streambed sediments. We collected an aliquot of the suspended FBOMsurf and FBOMsub material contained within a plastic core of 0.05 m^2 by manual agitation; and filtered it onto ashed pre-weighted FVF glass fiber filters. Samples from epilithic biofilms were collected by scraping a cobble surface and filtering the sludge onto ashed pre-weighted FVF glass fiber filters. We estimated the scraped cobble surface area by covering it with aluminum foil and applying a weight to area relationship. Additionally, composite samples from each PUC type (3 replicates per station) were collected from each sampling station on the pre- and post 24h-samplings using the same methods as described above to analyze ^{15}N content of each PUC.

We estimated whole-reach metabolism on the days of the ^{15}N additions using the open-system, single station approach (Bott, 2006). We recorded dissolved oxygen (DO) concentration and water temperature at the bottom of the study reach at 10-min intervals during a 24 h period with a WTW (Weilheim, Germany) 340i portable oxygen meter. During the same period, we also recorded photosynthetically active radiation (PAR) every 10 min with a Skye (Powys, UK) SKP215 quantum sensor connected to a Campbell Scientific (Logan, Utah, USA) CR510 data logger.

Laboratory methods

We analyzed stream water samples for NO_3^-, NH_4^+ and soluble reactive phosphorus (SRP) concentrations with standard colorimetric methods (APHA, 1995) on a Bran+Luebbe (Nordersted, Germany) TRAACS 2000 Autoanalyzer. We also determined the concentration of dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) on a Shimadzu (Tokyo, Japan) TOC-VCSH analyzer.

We processed water samples for the analysis of $^{15}NO_3^-$ and $^{15}NH_4^+$ as described in Sigman et al. (1997) and Holmes et al. (1998), respectively. Briefly, for $^{15}NO_3^-$ determination, we amended a known volume of the sample with 3 g of MgO and 5 g of NaCl and boiled it to remove the NH_4^+. We then added 0.5 mg of MgO and 0.5 mg Devarda's alloy to reduce the NO_3^- to NH_4^+, and treated the remaining sample as for $^{15}NH_4^+$. For $^{15}NH_4^+$ determination, we amended a known volume of sample with 3 g L^{-1} of MgO and 50 g L^{-1} of NaCl and a Teflon filter packet containing an acidified 1-cm-diameter ashed Whatman GF/D fiber glass filter to trap the volatilized NH_3, and incubated it on a shaker at 40ºC for 4 weeks. We also diffused a set of standards of known volume for volume-related fractionation corrections. Once the incubation was completed, we removed the filter packets and placed them in a desiccator for 4 days. We then encapsulated the filters in tins and stored them until ^{15}N analysis.

For ^{15}N analysis of leaves, wood and alder roots, we grounded oven-dried subsamples to fine powder, weighted them to the nearest 0.001 mg on a Mettler-Toledo (Greifensee, Switzerland) MX5 microbalance, encapsulated them in tins, and stored until analysis. For ^{15}N analysis of FBOMsurf, FBOMsub, and epilithon, we cut out a disc of a known surface area (1 cm diameter) from the oven-dried filters and processed these subsamples as described above.

Encapsulated samples of $^{15}NO_3^-$, $^{15}NH_4^+$, and PUCs were analyzed at the University of California Stable Isotope Facility (Davis, California, USA). The content (as a percent of dry mass) and the stable isotope ratios of C and N 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). One set of N-gas samples was analyzed for $^{15}N_2O$ on a Finnigan (Sacramento, California, USA) MAT 251 Isotope Ratio Mass Spectrometer at the same stable isotope laboratory. The other set of N-gas samples was analyzed for $^{15}N_2$ on a multi-collector CV Instruments (Manchester, UK) Isoprime Mass Spectrometer at

the Michigan State University Stable Isotope Laboratory (Lansing, Michigan, USA).

To estimate the DM standing stock (mg m^{-2}) for each PUC in the reach, we oven-dried area-specific samples at 60 ºC and weighted them to the nearest 0.1 mg on a Sartorius (Goettingen, Germany) MC1 analytical balance. The area-specific DM estimates were multiplied by the percent coverage of each PUC along the reach to calculate reach-weighed DM standing stocks. Reach-weighed N standing stocks (mgN m^{-2}) for each PUC were calculated by multiplying the reach-weighed DM standing stock (mg m^{-2}) by the percentage of N.

Parameter calculations

Whole-reach metabolism. We calculated daily rates of gross primary production (GPP) and ecosystem respiration (ER) by integrating the DO measurements at the bottom of the reach during the 24 h period following Bott (2006). For each measurement time of DO concentration, we estimated the DO concentration at saturation using DO and water temperature together with a standard altitude-air pressure algorithm to correct for site altitude. Comparison between observed values and estimated values at saturation were used to calculate the DO deficit. Reaeration coefficients of DO along the reach were estimated from the night-time reaeration method (Young and Huryn 1998) using the relationship between the DO deficits and the net changes in DO for measurements done at night time. We estimated the average instantaneous net DO change rates, corrected for reaeration fluxes, at night and extrapolated it to 24 h to compute daily rates of ER. We computed the daily rate of GPP by integrating the difference between the instantaneous net DO change rates (corrected by the reaeration flux) measured during day time and the extrapolated average instantaneous respiration rate. We multiplied GPP and ER by the mean reach depth to obtain daily rate estimates per unit of surface reach area.

Whole-reach N-NO_3^- and N-NH_4^+ uptake. To measure total uptake rate at reach scale for each DIN species we followed the procedures described in Mulholland et al. (2000 and 2004). We first calculated the flux of ^{15}N at each station by multiplying the background-corrected concentration at plateau of either $^{15}NO_3^-$ or $^{15}NH_4^+$ by the station-specific discharge. We used data from the two plateau samplings (i.e., night and day) separately. Discharge at each station was determined based on the dilution data along the reach obtained from the conservative tracer addition. We estimated the fractional uptake rate per unit distance (k_w, m^{-1}) for either $^{15}NO_3^-$ or $^{15}NH_4^+$ from the regression of the ln-transformed tracer ^{15}N fluxes versus site distance from the top of the reach. The inverse of k_W is the uptake length (S_w; m), which was converted to the uptake velocity (V_f; mm min^{-1}) by dividing the stream specific discharge (Q/w) by S_w (Stream Solute Workshop 1990). The N uptake fluxes (U; µgN m^2 s^{-1}) for each DIN species were calculated by multiplying V_f for either NO_3^- or NH_4^+ by the mean ambient NO_3^- or NH_4^+ concentration.

The fractional rates per unit distance for denitrification (k_{DEN}), DNRA (k_{DNRA}) and nitrification (k_{NIT}) were estimated by fitting ln-transformed tracer ^{15}N fluxes versus distance to two-box models as proposed by (Mulholland et al. 2004; Mulholland et al. 2008) using the Solver option in Microsoft Excel (Redmond, Washington, USA). In particular, we calculated k_{DEN} using the fluxes of $^{15}N_2O$ along the reach because we did not detect labeled $^{15}N_2$. We only used data from stations with a significant label of N_2O (defined as $\delta^{15}N$ values greater than the upper 97.5% confidence interval of background values (Mulholland et al. 2008). We corrected the $^{15}N_2O$ fluxes by the air-water exchange of N_2O using a molecular transformation of the reaeration DO fluxes calculated for metabolism. We calculated k_{DNRA} using $^{15}NH_4$ fluxes along the reach from the post-addition sampling of the $^{15}NO_3^-$ addition. We calculated k_{NIT} using the fluxes of $^{15}NO_3^-$ along the reach from the plateau samplings of the $^{15}NH_4$ addition. The denitrification flux (U_{DEN}; µg N m^{-2} s^{-1}), the DNRA flux (U_{DNRA}; µg N m^{-2} s^{-1}), and the nitrification flux (U_{NIT}; µg N m^-

^{2} s^{-1}) were calculated from $k_{DEN,}$ k_{DNRA}, *and* $k_{NIT,}$ *respectively*, as previously for total N uptake flux (*U*).

Whole-reach DIN regeneration. We used the data collected at the post-24 h sampling for each ^{15}N addition to calculate whole-reach rates of DIN regeneration from N assimilated by PUCs. For each ^{15}N addition, we used the longitudinal tracer fluxes of $^{15}NH_4^+$ to estimate the fractional rate for ammonification per unit time from biomass to water column (k_{AM}; s^{-1}) and we used the longitudinal tracer fluxes of $^{15}NO_3^-$ to estimate the subsequent transformation of $^{15}NH_4^+$ to $^{15}NO_3^-$ via nitrification (K_{NIT}; s^{-1}). We multiplied the rates of N uptake for the two DIN species (i.e., total uptake, denitrification, DNRA, and direct nitrification, in m^{-1}) by the mean stream water velocity (m s^{-1}) to compare them with rates of N regeneration (i.e., ammonification and indirect nitrification, in s^{-1}).

Assimilatory N-NH_4^+ and N-NO_3^- uptake by PUCs. We calculated the compartment-specific assimilatory N uptake flux (U_{BIO}; µg N m^{-2} s^{-1}) of each PUC for the NO_3^- and NH_4^+ additions following (Mulholland et al. 2000). We divided the reach-weighted mass of background-corrected ^{15}N tracer per m^2 measured in each PUC by the time of the addition (12 h) and the fraction of ^{15}N in the stream water flux of either NO_3^- or NH_4^+. For this calculation, we only considered data from the two uppermost sampling sites (i.e., 25m and 50m downstream of the ^{15}N addition point) to avoid confounding effects of potential N regeneration within the reach (Mulholland et al. 2000). The total U_{BIO} (either as NO_3^- or NH_4^+) at whole-reach scale was calculated as the sum of the mean compartment-specific U_{BIO} of each PUC. Then, for each PUC, we calculated the biomass-specific N uptake rate for the two DIN species (U_{spec}; d^{-1}) by dividing U_{BIO} by the N content in PUC biomass. U_{spec} has been used as an indicator of N turnover time within a biotic compartment (Dodds et al. 2004), but it can also be interpreted as an uptake efficiency as it expresses the N demand from the water column per unit of N biomass and time for a given PUC. Therefore U_{spec} allowed us to compare the N uptake of NO_3^- and NH_4^+ of the same PUC regardless of their biomass during each ^{15}N additions. We

used total U_{BIO} and compartment-specific U_{BIO} to estimate a) the contribution of N assimilation by PUCs to total uptake of the two DIN species and b) to evaluate the relative contribution of each PUC to the assimilation of NH_4^+ and NO_3^-.

Furthermore, the contribution PUCs to DIN uptake was also estimated by calculating the percentage of ^{15}N retained within the reach by PUCs with respect to the total ^{15}N added in each addition. The mass of ^{15}N assimilated by each PUC along the reach was estimated by integrating values obtained at each station using the best-fit exponential decay model (Mulholland et al. 2000). Total ^{15}N retained in the reach was calculated as the sum of integrated ^{15}N mass values for each PUC along the reach. For each ^{15}N addition, this ^{15}N mass was divided by the total ^{15}N mass added as inorganic dissolved form during the addition to compute the percentage of ^{15}N retained by the PUCs in the reach.

Literature survey of in-stream N-NH_4^+ and N-NO_3^- uptake

To place the empirical results from this study into a wider context, we conducted a literature search for data of uptake metrics (i.e., S_w, V_f and U) based on solute addition techniques. We identified a total of 69 streams where values for the two DIN species were available. The majority of the data was based on short-term nutrient enrichment studies (63 out of 69 streams), and only data from 6 streams was based on ^{15}N additions for both NO_3^- and NH_4^+. About half of the studies had more than one value for the same DIN species uptake metric. In those cases, we used the mean value for a given DIN species to be compared to the value for the other DIN species. The data set was complemented with environmental variables for each stream such as discharge, width, depth and water velocity and NO_3^- and NH_4^+ ambient concentrations. In instances where data were provided only graphically, we obtained values by digitalization of the graph with Adobe Illustrator CS5 15.0.0 (Adobe Systems Software Ireland Ltd). When studies did not provide the 3 uptake metrics (i.e., S_w, V_f and U), we derived the remaining metrics

based on the equations provided in the Stream Solute Workshop (1990) using the data from the environmental variables mentioned above.

Statistical analyses

For each DIN species, we tested if the different fractional rates per unit distance (i.e., k_i) differed between night and day samplings using a *t*-test for the comparison of slopes (Fowler and Cohen, 1990) and applying a Bonferroni correction.

We compared compartment-specific U_{BIO} and U_{spec} using a two-way ANOVA with DIN species (n=2) and PUC type (n=6) as fixed factors. Post-hoc Tukey HSD tests followed significant differences ($p < 0.05$) among cases both within factors and between factors.

Using data from the literature survey we evaluated differences between NO_3^- and NH_4^+ for ambient concentrations and for each uptake metric (i.e., S_w, V_f and U) across streams using a Student t-test. To explore if values for NO_3^- and NH_4^+ from the 4 variables mentioned above were related to each other across streams we ran Pearson correlation analysis. Finally, we explored if variability among streams for the NO_3^-: NH_4^+ ratios from the 4 variables was related to stream size (in terms of discharge) or DIN concentration by using linear regression analysis.

We ran all statistical tests with R 2.15.0 (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/.). When necessary, data were log-transformed before analysis to meet assumptions of homogeneity of variance and normality (Zar 1996).

5.5. Results

Characteristics of the study reach during the ^{15}N additions

Physical and chemical characteristics of the reach as well as daily rates of in-stream metabolism were similar during the two ^{15}N additions (Table 5.1). Characteristics of the stream reach were typical of the study period, with low discharge (~4 L s^{-1}) and warm water temperature (~22 ºC). Water conductivity

was moderate (mean= 328 µS cm^{-1}) and water was relatively well oxygenated (mean DO = 6.8 mg L^{-1}). Dissolved N concentration was mainly available as inorganic forms (~80 %) and mostly as NO_3^- (~98 % of DIN). Concentration of NO_3^- was relatively high, while concentration of NH_4^+ was low (Table 5.1). Concentration of SRP averaged 16 µg L^{-1} and dissolved organic carbon (DOC) ~ 2 mg L^{-1}. Daily rates of GPP were low and more than one order of magnitude lower than daily rates of ER (Table 5.1). Therefore, the metabolism of the study reach was clearly dominated by heterotrophic activity (GPP:ER ~ 0.04).

Table 5.1. Physico-chemical characteristics and daily rates of whole-reach metabolism for the study reach during the two ^{15}N additions. Data for physicochemical parameters are the mean values from samples collected at all sampling sites along the study reach before the start of each ^{15}N addition. Daily rates of whole-reach gross primary production (GPP) and ecosystem respiration (ER) were estimated on the day of each addition.

		$^{15}NO_3^-$ addition	$^{15}NH_4^+$ addition
Physical			
	Discharge (L s^{-1})	4.3	4.3
	Velocity (cm s^{-1})	1.7	1.6
	Width (m)	4.6	4.5
	Depth (cm)	5.4	6.1
	Temperature (ºC)	21.3	22.5
Chemical			
	Conductivity (µS cm^{-1})	326	330
	Dissolved oxygen (mg L^{-1})	6.9	6.6
	NO_3^- (µg N L^{-1})	666	772
	NH_4^+ (µg N L^{-1})	13	13
	SRP (µg P L^{-1})	8	23
	DON (µg N L-1)	53	79
	DOC (mg L^{-1})	2.0	1.9
Metabolism			
	GPP (g O_2 m^{-2} d^{-1})	0.06	0.42
	ER (g O_2 m^{-2} d^{-1})	6.84	7.31
	GPP:ER	0.01	0.06

The total N standing stock in PUCs in the reach was 2-fold higher during the $^{15}NH_4^+$ addition than during the $^{15}NO_3^-$ addition (Table 5.2). Nonetheless, each PUC accounted for a similar proportion of the total N standing stock during the two ^{15}N-tracer additions. In both cases, detrital compartments (the two types of FBOM and leaves) accounted for more than 80% of the total N standing stock, whereas the contribution of the rest of PUCs was <10% (Table 5.2). The %N and C:N ratios of the PUCs were similar between the two ^{15}N additions, but differed among PUCs (Table 5.2). The %N was highest in roots and leaves, and C:N was highest in wood and leaves (Table 5.2).

Whole-reach N-NO_3^- and N-NH_4^+ uptake and DIN regeneration

During the $^{15}NO_3^-$ addition, there was no significant decay of the $^{15}NO_3^-$ flux along the reach at the nighttime plateau; thus, NO_3^- uptake metrics could only be estimated for the daytime plateau (Fig. 5.1). During the $^{15}NH_4^+$ addition, there were significant decays of the $^{15}NH_4^+$ flux, which were similar (*t*-test for the comparison of slopes, $p \geq 0.05$; Fig. 5.1) for the two plateau samplings. The value of k_w-NO_3^- was 2 orders of magnitude lower than k_w-NH_4^+ (Fig. 5.1); and thus, S_w-NO_3^- was at the km range whereas S_w-NH_4^+ was <100 m (Table 5.3). Accordingly, V_f-NO_3^- was 2 orders of magnitude lower than V_f-NH_4^+; however, U-NO_3^- was only 1.7-fold higher than U-NH_4^+ (Table 5.3).

The contribution of dissimilatory uptake pathways to total uptake differed between NO_3^- and NH_4^+. U_{DEN} was low and accounted for <1% of the total NO_3^- uptake at the whole-reach scale (Fig. 5.1C; Table 5.3). Increases in the $^{15}NH_4^+$ flux along the reach during the two plateaus of the $^{15}NO_3^-$ addition suggest DNRA activity (Fig. 5.1E). However, the estimated U_{DNRA} was low and accounted for <1% to the total NO_3^- uptake at the whole-reach scale (Table 5.3). On the contrary, U_{NIT} was relatively high and accounted for 43% of the total NH_4^+ uptake at the whole-reach scale (Table 5.3).

Table 5.2. Reach-weighted N standing stocks (mg N m^{-2}), contribution to the total N standing stock (in %) in the reach, percentage of N in dry mass (%N), and carbon to nitrogen ratio (C:N by mass) for the different primary uptake compartments (PUCs). Data are mean values and SE (in brackets) from samples collected at all sampling sites for the two ^{15}N additions separately.

	N standing stock (mgN m^{-2})				% N		C:N	
PUC	$^{15}NO_3^-$ addition	(%)	$^{15}NH_4^+$ addition	(%)	$^{15}NO_3^-$ addition	$^{15}NH_4^+$ addition	$^{15}NO_3^-$ addition	$^{15}NH_4^+$ addition
Epilithon	50 (29)	4.3	52 (1)	2.3	1.6 (0.2)	1.6 (0.1)	5.9 (0.1)	6.2 (0.05)
FBOM								
surface	381 (71)	33.4	843 (165)	37.9	0.9 (0.05)	0.9 (0.04)	9.1(0.1)	8.9 (0.2)
sub-surface	423 (90)	37.2	735 (72)	33.0	0.8 (0.1)	0.8 (0.04)	9.1 (0.1)	9.0 (0.2)
Leaves	220 (5)	19.3	450 (7)	20.2	2.3 (0.3)	2.9 (0.2)	21.0 (4.7)	15.5 (0.7)
Wood	35 (0.2)	3.1	101 (7)	4.5	1.0 (0.1)	1.1 (0.1)	46.9 (6.1)	43.2 (2.5)
Roots	30 81)	2.6	45 (1)	2.0	2.9 (0.3)	2.8 (0.2)	15.3 (1.2)	16.9 (1.0)
TOTAL	1139		2225					

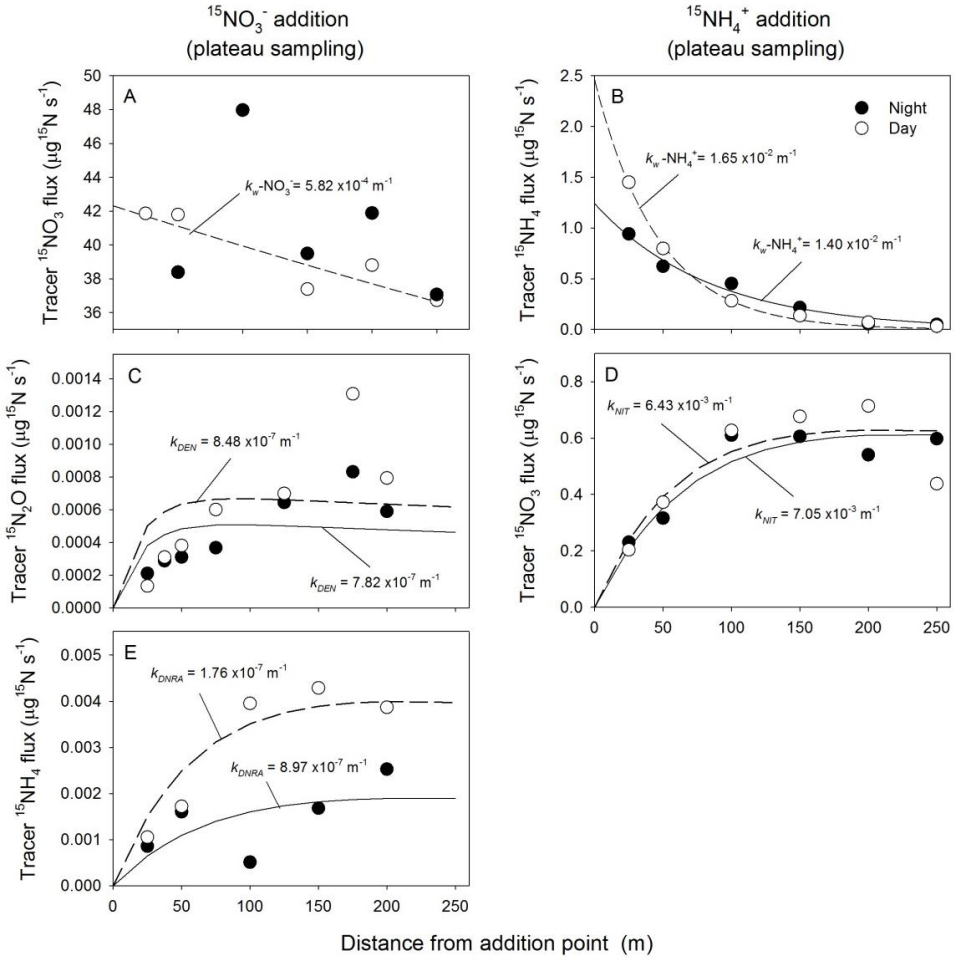

Figure 5.1. Patterns of ^{15}N fluxes along the study reach during plateau samplings at daytime (white circles) and nighttime (black circles) for calculations of total NO_3^- (A) and NH_4^+ (B) uptake rates, denitrification (C) and DNRA (E) during the $^{15}NO_3^-$ addition, and nitrification during the $^{15}NH_4^+$ addition (D). Fractional N uptake rates per unit distance (k; m^{-1}) are shown. Solid and dashed lines show the best fit for data during night and day plateaus, respectively. Lack of solid line in A denotes non-significant decay in $^{15}NO_3^-$ flux during the night plateau sampling.

Overall, N regeneration pathways estimated after each ^{15}N addition differed between additions (Fig. 5.2). k_{AM} estimated from the $^{15}NO_3^-$ addition was roughly 2-fold higher than that estimated from the $^{15}NH_4^-$ addition. On the other hand, k_{NIT} estimated from $^{15}NO_3^-$ addition was 2 orders of magnitude higher than that estimated from the $^{15}NH_4^-$ addition (Fig. 5.2). When we

compared the rates of N release (i.e, N regeneration) with those of N uptake, we observed remarkable differences between the two DIN species. After the $^{15}NO_3^-$ addition, k_{AM} was one order of magnitude higher than that of k_w whereas after $^{15}NH_4^+$ addition, k_{AM} and *kw* were relatively balanced to each other (Table 5.3).

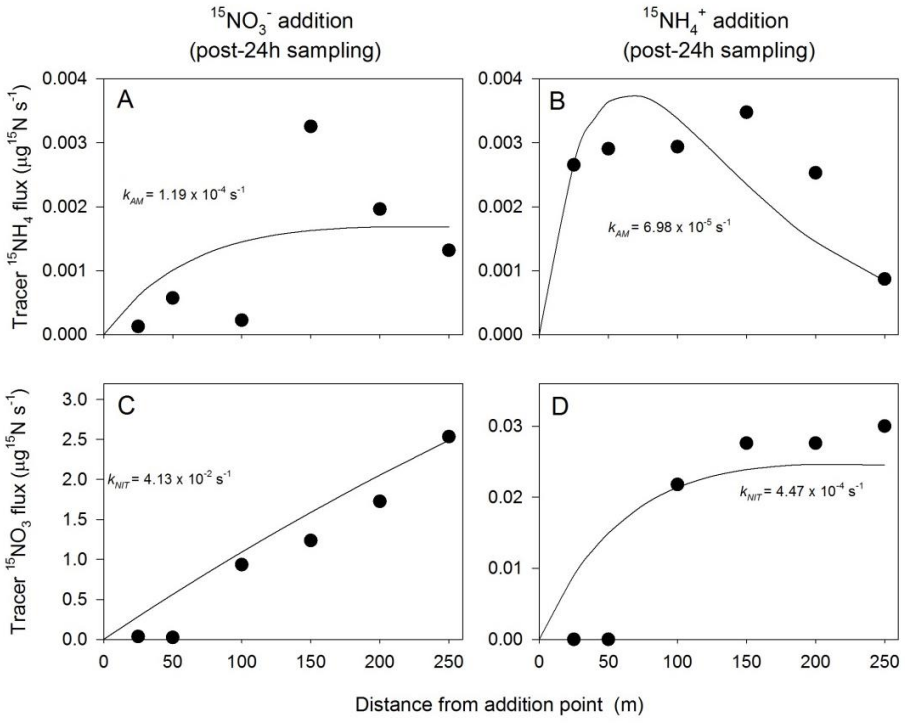

Figure 5.2. ^{15}N fluxes in the study reach during post-24h samplings used for calculations of N regeneration rates. Fractional rates of ammonification per unit time (k_{AM}; s^{-1}) and nitrification (k_{NIT}; s^{-1}) after the $^{15}NO_3^-$ addition (A and C) and after the $^{15}NH_4^+$ addition (B and D).

Table 5.3. Summary of biogeochemical NO_3^- and NH_4^+ spiraling metrics calculated from the $^{15}NO_3^-$ or the $^{15}NH_4^+$ addition. See Fig. 5.1 and Fig. 5.4 for ^{15}N fluxes used in calculations during night and day plateaus and post-24h sampling, respectively. The fractional uptake rate per unit distance showed in Fig. 1 for each N uptake pathways (k_w, m^{-1}) were transformed to s^{-1} by multiplying k_w with the mean water velocity of each ^{15}N addition ($m\ s^{-1}$) to better compare with the rates of N regeneration pathways.

		$^{15}NO_3^-$ addition	$^{15}NH_4^+$ addition
Total N uptake[1]			
	k_w (s^{-1})	1.02×10^{-5}*	2.37×10^{-4}
	S_w (m)	1719*	66
	V_f ($mm\ min^{-1}$)	0.035*	0.86
	U ($\mu gN\ m^{-2}\ s^{-1}$)	0.36*	0.21
Denitrification[1]			
	k_{DEN} (s^{-1})	1.43×10^{-8}	–
	U_{DEN} ($\mu gN\ m^{-2}\ s^{-1}$)	6.15×10^{-4}	–
	% of the total NO_3^- uptake	0.17	–
DNRA[1]			
	k_{DNRA} (s^{-1})	2.32×10^{-8}	–
	U_{DNRA} ($\mu gN\ m^{-2}\ s^{-1}$)	9.50×10^{-4}	–
	% of the total NO_3^- uptake	0.26	–
Nitrification[1]			
	k_{NIT} (s^{-1})	–	1.05×10^{-4}
	U_{NIT} ($\mu gN\ m^{-2}\ s^{-1}$)	–	0.09
	% of the total NH_4^+ uptake	–	43.2
Assimilatory uptake[1]			
	U_{BIO} ($\mu gN\ m^{-2}\ s^{-1}$)	0.06	0.16
	% of the total N uptake	16.7	75.6
	% ^{15}N retained of total added	2.0	39.1
N regeneration[2]			
	k_{AM} (s^{-1})	1.19×10^{-4}	6.98×10^{-5}
	k_{NIT} (s^{-1})	–	4.47×10^{-4}

Parameters calculated from the mean values of night and day plateaus, except for total N uptake for NO_3^- in which we only used data from the day plateau (*).

[2] Parameters calculated using data from the sampling 24h after the end of the ^{15}N additions.

Assimilatory N-NO_3^- and N-NH_4^+ uptake by PUCs

The total U_{BIO}-NH_4^+ was roughly 3-fold higher than the total U_{BIO}-NO_3^- (Table 5.3, Fig. 5.3A). The contribution of total U_{BIO} to U varied between the two DIN species. U_{BIO}-NO_3^- only accounted for ~17% of U-NO_3^- whereas U_{BIO}-NH_4^+ accounted for a major part of U-NH_4^+ (~76%). The percentage of ^{15}N tracer retained assimilated by PUCs with respect to the total ^{15}N tracer added during the 12h additions also varied between the two DIN species (Table 5.3). Only 2% of the total $^{15}NO_3^-$ added to the stream reach was retained by PUCs, whereas in the case of NH_4^+, PUCs retained 39% of the total $^{15}NH_4^+$ added (Table 5.3).

Regarding to the uptake fluxes by which the different PUCs assimilated DIN from the water column, the two-way ANOVA indicated that U_{BIO} differed between DIN species (Table 5.4). All PUCs with the exception of alder roots, showed higher U_{BIO}-NH_4^+ than U_{BIO}-NO_3^- (Fig. 5.3A). We also found significant differences in U_{BIO} among PUC types (Table 5.4, Fig. 5.3A). Post-hoc tests indicated that U_{BIO} of wood was the lowest (Tukey HSD test, $p \leq 0.013$) followed by the rest of PUCs with no significant differences among them (Tukey HSD test, $p > 0.05$). We did not find a significant interaction between the two fixed factors (Table 5.4). Regarding U_{spec}, the two-way ANOVA also showed significant differences between the two DIN species and among PUCs (Table 5.4; Fig. 5.3B). All PUC with the exception of alder roots showed U_{spec}-NH_4^+ was higher than U_{spec}-NO_3^- (Fig. 5.3B). Regarding to differences among PUC type, post-hoc tests indicated that U_{spec} of roots was the highest (Tukey HSD test, $p \leq 0.014$) followed by the rest of PUC with no significant differences among them (Tukey HSD test, $p > 0.05$). The interaction between the two fixed factors was not significant (Table 5.4).

Table 5.4. Results from the two-way ANOVA with DIN species (n = 2) and primary uptake compartment (PUC) type (n= 6) on N assimilatory uptake fluxes (U_{BIO}) and biomass-specific N uptake (U_{spec}). Values highlighted in bold indicate significant effects ($p < 0.05$). Df = degrees of freedom.

Variable	*df*	*F*	*P*
U_{BIO}			
DIN species	1	21.57	**<0.001**
PUC type	5	8.48	**<0.001**
DIN species x PUC type	5	2.51	0.089
U_{spec}			
DIN species	1	5.08	**0.044**
PUC type	5	11.22	**<0.001**
DIN species x PUC type	5	1.83	0.182

The relative contribution of each PUC to the total U_{BIO} also varied between the two DIN species (Fig. 5.4A and B). The two types of FBOM accounted for a similar proportion of the total U_{BIO}-NO_3^- (41%), followed by roots (34%), and leaves (18%). Epilithon and wood only accounted for a small proportion of total U_{BIO}-NO_3^- (6 and 1% respectively). Regarding NH_4^+, the contribution of FBOMsurf to total U_{BIO}-NH_4^+ doubled that of FBOMsub summing 42% of the total U_{BIO}-NH_4^+. Leaves accounted for 35%, followed by roots and wood with a similar contribution (8% each) and epilithon (7%).

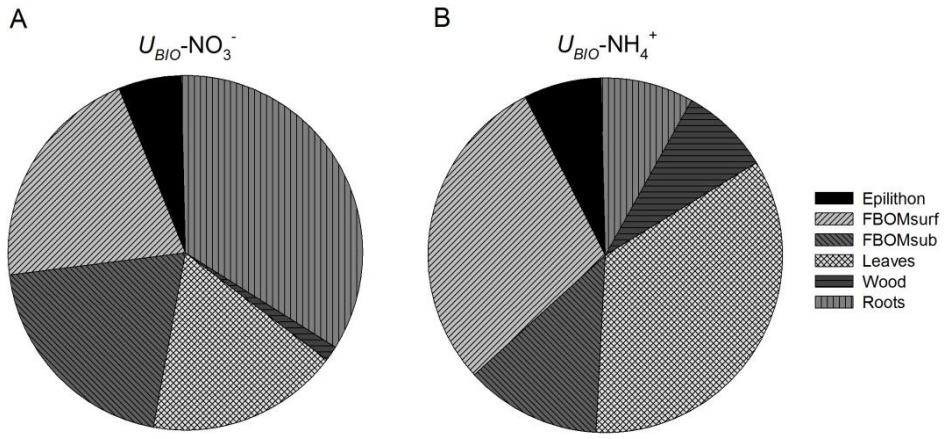

Figure 5.4. Percentage contribution of each primary uptake compartment (PUC) to the total N assimilatory uptake (U_{BIO}) for NO_3^- (A) and NH_4^+ (B).

Comparison of whole-reach N-NO_3^- and N-NH_4^+ uptake from the literature survey

The streams from the literature survey showed a wide range of NO_3^- and NH_4^+ concentrations, but NO_3^- concentration was on average one order of magnitude higher than NH_4^+ concentration (Student t-test, $p < 0.001$; Fig. 5.5A). On average, NO_3^- concentration accounted for 81% of the total DIN concentration. Despite remarkable variation among streams, uptake metrics also differed significantly between the two DIN species. Mean S_w-NO_3^- was significantly longer than S_w-NH_4^+ (mean ± SE; 2735 ± 1023 m and 604 ± 163 m, respectively; Student t-test, $p < 0.001$; Fig. 5.5B), and V_f-NH_4^+ was significantly higher than V_f-NO_3^- (mean ± SE; 3.4 ± 0.7mm min^{-1} and 6.8 ± 1.1 mm min^{-1} , respectively; Student t-test, $p < 0.001$; Fig. 5.5C). Despite mean U-NO_3^- was roughly 8-fold higher than U-NH_4^+ (mean ± SE; 9.8 ± 3.2 µN m^{-2} s^{-1} and 1.3 ± 0.3 µN m^{-2} s^{-1},respectively) this difference was only marginally significant due to a large variability among streams, especially for U-NO_3^- (Student t-test, $p = 0.07$; Fig. 5.5D). Relationships between NH_4^+ vs NO_3^- for the log-transformed variables of background concentrations and the 3 uptake metrics (S_w, V_f and U) showed significant positive correlations (Pearson correlations; $r \geq 0.41$ and $p < 0.001$ for all cases, Fig. 5.6).

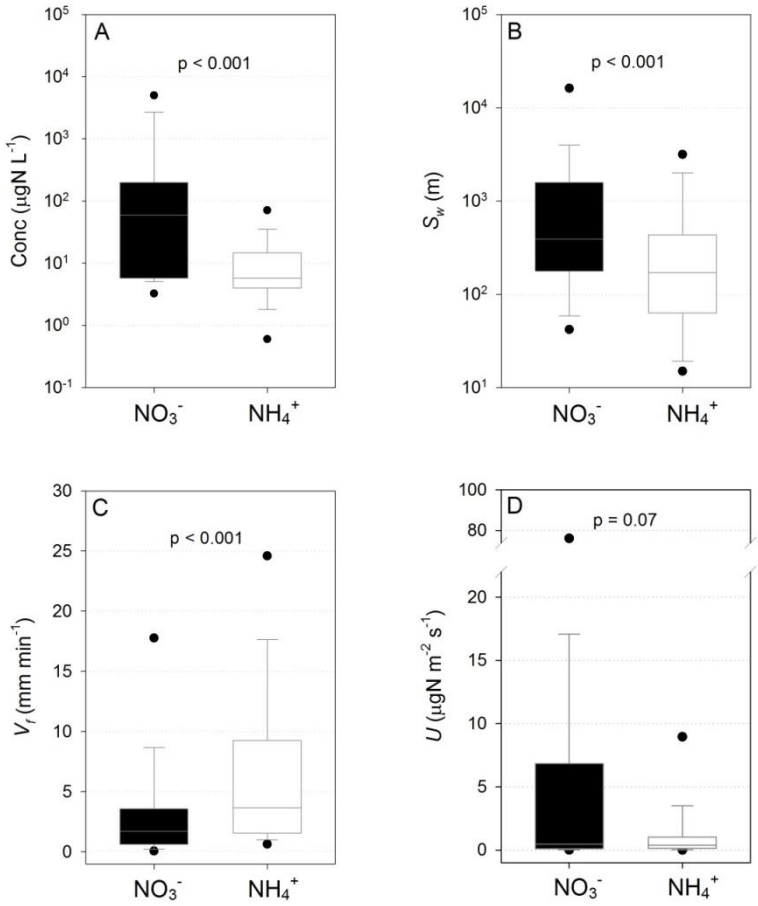

Figure 5.5. Data from the literature survey encompassing studies conducted in streams in which the spiraling of both NO_3^- and NH_4^+ were investigated. Boxplots display the median, 10th, 25th, 75th and 90th percentiles of background concentrations (A), uptake lengths (S_w; B), uptake velocities (V_f; C) and uptake fluxes (U; D) of NO_3^- and NH_4^+. Dots denote individual data points outside the 5th and 95th percentiles. Mean values for each metric were compared using a Student t-test with log-transformed data. The *p*-values are shown

5.5. Discussion

Whole-reach spiraling of NH_4^+ and NO_3^-

Our study stream was moderately efficient in taking up NO_3^- (i.e., S_w-NO_3^- in the km-range), whereas it was highly efficient in taking up NH_4^+ (mean S_w-NH_4^+ = 66 m). The S_w values for the two DIN species were on the same order of magnitude as those from other whole-reach ^{15}N-tracer addition studies in the same watershed (range = 802-2620 and 56-60 m for NO_3^- and

NH_4^+, respectively; von Schiller et al. 2009; M. Peipoch unpubl.). In a wider context, our S_w values were well bracketed by those from a $^{15}NO_3^-$ tracer study conducted in 72 streams across the United States of America (U.S.A) and Puerto Rico (range = 1-10km; Mulholland et al. 2008) and from another $^{15}NH_4^+$ tracer study conducted in 12 streams across the same area (range = 10-1000m; Peterson et al. 2001). Overall, results indicated a consistent pattern of higher reach-scale uptake efficiency for NH_4^+ with respect to that of NO_3^- across streams in different biomes (Ensign and Doyle 2006). V_f-NH_4^+ in our stream was an order of magnitude higher than V_f-NO_3^- (0.86 and 0.035 mm min^{-1}, respectively). Thus, our results indicated a greater in-stream demand for NH_4^+ with respect to NO_3^- (Webster and Valett 2006). This pattern was consistent with values from the nearby streams (range = 2.1-3.4 and 0.04-0.16 mm min^{-1} for NH_4^+ and NO_3^- and respectively (von Schiller et al. 2009; M. Peipoch unpubl.) and from streams across USA and Puerto Rico (2.0 - 41.2 and 0.004 - 17.8 mm min^{-1} for NH_4^+ and NO_3^- respectively (Peterson et al. 2001; Mulholland et al. 2008). Previous studies showed that in-stream metabolism may control N uptake in streams (Hall and Tank 2003), thus lower V_f for the two DIN species with respect to those estimated elsewhere, may be explained by the low GPP rates measured in our study streams (Hall and Tank 2003). Higher in-stream S_w and V_f for NH_4^+ with respect to NO_3^- indicated that NH_4^+ was preferred over NO_3^- as an N source (Naldi and Wheeler 2002; Hildebrand 2005). However total U-NO_3^- was similar or even higher than U-NH_4^+, indicating that despite a preference for NH_4^+, NO_3^- is the main source of N, likely due to the generally higher availability of this DIN species (Fellows et al. 2006; Newbold et al. 2006; Ribot et al. 2013). U values from the streams within the same catchment (0.2-1.1 and 0.7-0.8 µg N m^{-2} s^{-1} for NO_3^- and NH_4^+, respectively) and from streams across U.S.A and Puerto Rico (0-347 and 0.3-3.8 µgN m^{-2} s^{-1} for NO_3^- and NH_4^+, respectively) suggest that our stream had a limited capacity to take up DIN.

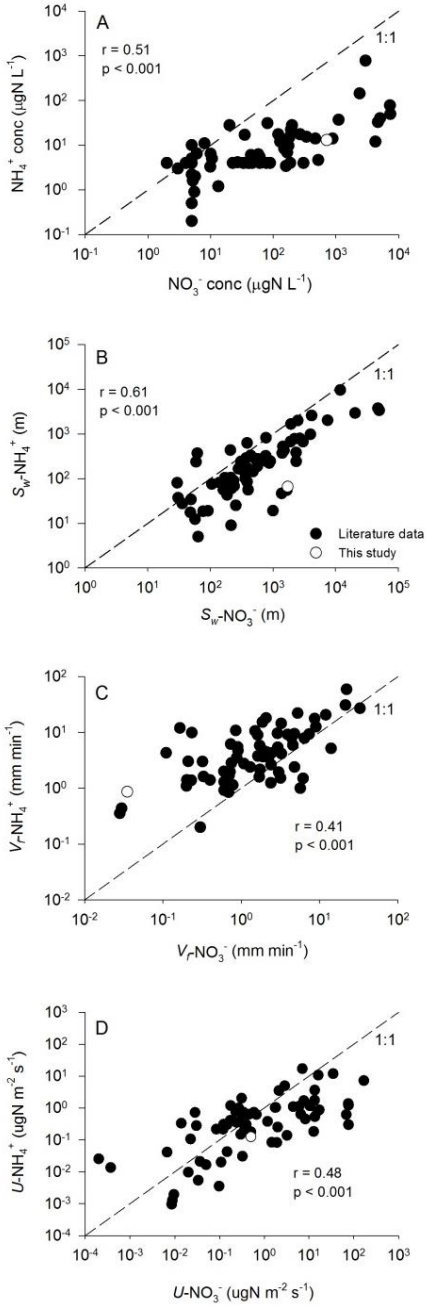

Figure 5.6. Relationship between NO_3^- and NH_4^+ in terms of background concentrations (A), uptake lengths (S_w; B), uptake velocities (V_f; C) and uptake rates (U; D) at whole-reach scale. The r and p-values are from Pearson correlations of log-transformed data. Dashed lines denote 1:1 lines. Data are from a literature survey of streams with nutrient spiraling metrics for the two DIN species.

N regeneration from N assimilated by PUCs within the stream reach was a remarkable source of in-stream DIN. Ammonification was a relevant source of in-stream NH_4^+ which in turn was immediately oxidized to NO_3^- via the coupled ammonification-nitrification pathway (i.e., indirect nitrification; (Mulholland et al. 2000). Rates of ammonification estimated after the two ^{15}N additions were in the same order of magnitude as the respective k_w of either NO_3^- or NH_4^+. This result suggests that the rates of gross N uptake and release processes were balanced. However, due to methodological constraints, we could not estimate the fluxes of DIN regeneration as those of DIN uptake (in units of $\mu N\ m^{-2}\ s^{-1}$). Therefore we could not assert that our stream reach was also at short-term biogeochemical equilibrium as indicated in previous studies which were assessed by estimating the net N uptake and release fluxes within a reach (Bernal et al. 2012; von Schiller et al. 2015).

Contribution of dissimilatory N uptake pathways to NO_3^- and NH_4^+ uptake

Dissimilatory pathways associated NO_3^- only accounted for a small fraction of *U*-NO_3^- (i.e., 0.2 and 0.3% for denitrification and DNRA, respectively). These results indicate that NO_3^- dissimilatory pathways in our stream had a low incidence on removing in-stream NO_3^- from the system which may result in an enrichment of the downstream water bodies. Nevertheless, we acknowledge that our estimation of DNRA uptake rates should be viewed with great caution. Rapid ammonification also may have rendered $^{15}NH_4^+$ to the water column, so we could not assert which proportion of $^{15}NH_4^+$ from the water column pool came from either DNRA or ammonification during the $^{15}NO_3^-$ addition. Denitrification contribution to *U*-NO_3^- from nearby streams ranged from 0 to 67% (von Schiller et al. 2009) and 0 up to 100% in streams across USA (Mulholland et al. 2008). Results indicated that denitrification was very variable among streams. However on average denitrification contribution to *U*-NO_3^- in the two studies mentioned above were 25% and 18% respectively, which suggests that other uptake processes play a major role on NO_3^- uptake. On the contrary, dissimilatory

pathways associated with in-stream NH_4^+ uptake accounted for almost one half of *U*-NH_4^+, indicating that nitrification was a remarkable sink for in-stream NH_4^+ in our stream, but at the same time was a source to NO_3^- to the water column (Peterson et al. 2001). This result in turn may explain to some extent, the observed higher concentrations of NO_3^- relative to those of NH_4^+. The nitrification values were clearly higher than those reported from a $^{15}NH_4^+$ addition study conducted in a nearby stream, in which nitrification rates accounted for less than 5% of *U*-NH_4^+ (M. Peipoch unpubl.). Yet, our values were in the upper range of values reported from streams across USA and Puerto Rico, in which nitrification ranged from less than 3% up to 60% of *U*-NH_4^+ (Peterson et al. 2001).

Contribution of N assimilation by PUCs to NO_3^- and NH_4^+ uptake

N assimilation by PUCs contributed to a remarkable fraction of the total N uptake but varied regarding the DIN species considered. U_{BIO}-NO_3^- represented the 17% of *U*-NO_3^-whereas U_{BIO}-NH_4^+ represented the 76 % of *U*-NH_4^+. Our results agreed with those from (Sobota et al. 2012) where the relative contribution of U_{BIO} to *U* was higher for NH_4^+ with respect to NO_3^- (32 % and 15%, respectively). Overall, those results indicated that PUCs had a preference to assimilate N as NH_4^+ to meet their N demand, likely due to the lower energetic cost associated to its incorporation into cells with respect to NO_3^- (Mccarty 1995). Higher percentage of ^{15}N tracer retention within our study reach for NH_4^+ with respect to NO_3^- agreed with the results reported in (Sobota et al. 2012) which further corroborated the in-stream biota preference for this N source (Dortch 1990).

We acknowledge that differences in total U_{BIO}-NH_4^+ with respect to those of U_{BIO}-NO_3^- within our study should be viewed with caution because the total biomass of PUCs during the $^{15}NH_4^+$ addition was 2-fold higher with respect to that during the $^{15}NO_3^-$ additions. Higher assimilatory biomass during the $^{15}NH_4^+$ addition with respect to that of $^{15}NO_3^-$ may be due to the fact that we ran the $^{15}NH_4^+$ addition one month later than that of $^{15}NO_3^-$ and discharge was

very low (~4 L s^{-1}) which may have favored the accumulation of detrital material within the stream channel (Acuna et al. 2005). To overcome this limitation we estimated U_{spec}. U_{spec}-NH_4^+ was higher than U_{spec}-NO_3^- which further corroborated that in-stream PUCs had preference for the former DIN species (Dortch 1990). On the other hand, U_{BIO} values reported here were in the same range of those reported in the nearby streams (range = 0.14 - 0.15 and 0.06 - 0.16 µgN m^{-2} s^{-1} for NO_3^- and NH_4^+ respectively (von Schiller et al. 2009; M. Peipoch unpubl.) Available data of U_{BIO}-NH_4^+ from the literature mostly emerged from studies within the Lotic Intersite Nitrogen eXperiment, phase I (LINX I, but see Riis et al. 2012). Mean U_{BIO} -NH_4^+ from the LINX I project was 5-fold higher than that reported in our study stream (Peterson et al. 2001) indicating that in-stream PUCs from our stream had a relatively low uptake capacity for NH_4^+. U_{BIO}-NO_3^- from a study conducted in the same streams within the LINX I study was in the range of values of streams within our study area (range = 0.07-0.3 µgN m^{-2} s^{-1}; Sobota et al. 2012). Altogether, these results point out that the assimilatory fluxes for the two DIN species may be at a similar range despite often the ambient NO_3^- concentration exceeded by far that of NH_4^+ which suggest PUC preference for the later DIN species (Pastor et al. 2013).

Microbial communities associated with detrital compartments of the stream benthos (i.e., two types of FBOM, leaves and wood) showed the highest contribution to in-stream assimilatory DIN uptake for both DIN species, which agreed with other studies (Mulholland et al. 2000; Dodds et al. 2000; von Schiller et al. 2009). Dominance of FBOM in the assimilatory DIN uptake has been previously reported using either $^{15}NO_3^-$ or $^{15}NH_4^+$ tracer studies (Dodds et al. 2000; Ashkenas et al. 2004; Riis et al. 2012). Furthermore our results indicated that FBOMsub and FBOMsurf had a similar contribution to the total U_{BIO}-NO_3^-, whereas FBOMsurf had a higher contribution than FBOMsub to the total U_{BIO}-NH_4^+.We do not know the underlying mechanism responsible for this pattern, but our data suggest that NH_4^+ was mostly taken up at the surface part of the stream bed. Differences in

the redox conditions (i.e., O_2 availability) between the surface and deeper layers of sediments may explain this pattern (Kemp and Dodds 2002). Overall our results indicated that DIN assimilation in our stream reach was mostly mediated by biofilms associated to detrital compartments. However, in the case of NO_3^-, up to 34 % of the U_{BIO} was attributed to submerged alder roots which may contribute to reduce the in-stream NO_3^- pool. These results reinforce the role that riparian vegetation may play in taking up in-stream DIN both via assimilation by microbial assemblages developing on decomposing leaf litter (Webster et al. 2009) or via direct uptake by submerged roots (Schade et al. 2001; von Schiller et al. 2009).

Comparison of whole-reach N-NO_3^- and N-NH_4^+ uptake from literature survey

In the relationship between NH_4^+ vs NO_3^- concentration, the majority of data falls above the 1:1 line, indicating that in those streams where uptake metrics were estimated, NO_3^- was the dominant DIN species. This data agreed with other studies which indicated that NO_3^- was the most conservative DIN species and therefore was exported in a major proportion with respect to NH_4^+ to downstream water bodies (Merriam et al. 2002; von Schiller et al. 2009). Furthermore, other factors operating at catchment scale such as changes in land use may explain this pattern (Stanley and Maxted 2008; von Schiller et al. 2008b; Ballantine and Davies-Colley 2014). Thus, dominance of agriculture in the stream catchments with respect to urbanization or undeveloped land may explain the widespread low in-stream ambient NH_4^+:NO_3^- concentration ratios.

Positive significant correlations between the two DIN species for the 3 uptake metrics suggested that NO_3^- and NH_4^+ respond in a similar manner to factors driving DIN uptake in streams. However, the majority of data points in the S_w-NH_4^+ vs S_w-NO_3^- relationship fall above the 1:1 line, indicating consistent lower reach-scale uptake efficiency for NO_3^- with respect to NH_4^+ across streams. Higher S_w is often attributed to higher discharge (Peterson et

al. 2001; Hall et al. 2013) but it has also been related to higher in-stream N concentrations (Gucker et al. 2006). Therefore, consistent higher in stream NO_3^- concentrations as indicated above may explain consistent longer S_w-NO_3^- with respect to S_w-NH_4^+. On the other hand, since nitrification may account for a large fraction of U-NH_4^+ (Peterson et al. 2001), nitrified NO_3^- may further contribute to lengthen the S_w-NO_3^- (Tank et al. 2008).

In terms of in-stream N demand, we also observed that generally V_f-NH_4^+ was higher than V_f-NO_3^- for a given stream, which agreed with previous studies (Simon et al. 2005; Ensign and Doyle 2006). Interestingly the V_f-NH_4^+:V_f-NO_3^- ratio tends to be close to the 1:1 line in the upper range of the two DIN species (i.e., at higher in-stream N demand). High N demand has been commonly associated to low N availability and increases in either NO_3^- or NH_4^+ availability often decrease their respective V_f (Dodds et al. 2002; Newbold et al. 2006; Mulholland et al. 2008). Therefore we expected that in-stream biota indistinctly rely on to the two DIN species (i.e., V_f-NH_4^+:V_f-NO_3^- ratio approaches 1) in those streams with limiting ambient DIN concentration. However relationships between log-transformed data of background concentration vs. their respective V_f for neither DIN species were significantly correlated (linear regression, r^2 = -0.01 and 0.02 and $p \geq 0.05$ for NO_3^- and NH_4^+, respectively). The relationship between log transformed data of V_f-NH_4^+:V_f-NO_3^- vs total DIN was also weak and not significant (linear regression, $r^2 = 0.04$, $p = 0.07$).

Despite a consistent higher in-stream N demand for NH_4^+, ambient availability of NO_3^- and NH_4^+ likely drive the uptake fluxes associated to each DIN species. Therefore, since NO_3^- was the predominant DIN species (~81% of the total DIN), in-stream U were relatively balanced (i.e., U-NH_4^+:U-NO_3^- ratio tend to be close to the 1:1 line). However, similar U for the two DIN species also suggested that NH_4^+ is been taken up preferably over NO_3^- because the mean ambient NH_4^+ concentration was on average one order of magnitude lower than that of NO_3^-.

Conclusions

Results from the study stream together with those from the literature survey indicate that streams are in general more efficient taking up NH_4^+ and have a higher N demand for this DIN species than for NO_3^-. Nonetheless, the generally low NH_4^+:NO_3^- concentration ratios found in streams results in a similar or higher in-stream *U* for NO_3^- than for NH_4^+. *U* for each DIN species was highly variable among streams, and mostly relies on the fact that the two DIN species undergo different N uptake pathways. In this regard, our empirical results indicated that dissimilatory uptake pathways associated to NO_3^- (denitrification and DNRA) had a low incidence on *U*-NO_3^- whereas that of NH_4^+ (i.e., nitrification) had a higher incidence on *U*-NH_4^+. Results from literature agreed with the overall relevance of dissimilatory uptake pathways to the *U* of each DIN specie. In-stream assimilatory uptake by biota had also a relevant contribution to *U* which in turn varied between the two DIN species. Furthermore, all these biogeochemical DIN uptake pathways are sensitive to the concentration of the two DIN species. Therefore changes in the NO_3^-:NH_4^+ concentration ratio driven by changes in human land uses may have a relevant effect on the uptake of the two DIN species as well as on their downstream export.

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Chapter 6

General discussion

6.1. Introduction

Human activities have caused general increases in dissolved inorganic nitrogen (DIN) availability in streams and rivers (Seitzinger and Kroeze 1998; Boyer et al. 2002; Galloway et al. 2004; Stanley and Maxted 2008; Lassaletta et al. 2009). Moreover, these activities have also modified the relative proportion of DIN as NO_3^- or NH_4^+ (von Schiller et al. 2008b; Martí et al. 2010). Nitrogen (N) is an essential element for in-stream biota; and thus, in-stream increases in DIN availability derived from human activity can alter biological N demand as well as dominant biogeochemical uptake pathways, which may ultimately have implications not only for DIN cycling within stream reaches, but also for DIN downstream transport. Within this context, several studies have already indicated a decrease in the in-stream demand for DIN under high DIN concentration (Marti et al. 2004; Newbold et al. 2006; O'Brien et al. 2007; Mulholland et al. 2008). However, few studies have examined in detail the in-stream responses specifically associated to the availability of the two DIN species. In this context, research conducted in the present Thesis aimed to increase our understanding of DIN uptake in stream ecosystems within the context of global change by particularly focusing on comparing processing rates associated with the uptake of the two major DIN species (NO_3^- and NH_4^+). We hypothesized that DIN uptake in streams would be influenced by the relative availability of NO_3^- and NH_4^+ because biotic assimilatory uptake demand may differ between the two DIN species and dissimilatory uptake processes are distinct for each DIN species.

We addressed the general objective of the PhD by conducting 4 experimental studies, introduced as different chapters in the Thesis. All the studies mostly focused on uptake of the two DIN species; however, the second and third chapters estimated it at the scale of benthic microbial assemblages (biofilms), while the fourth and fifth chapters estimated it at the whole-reach scale. In the second chapter, we evaluated how assimilatory DIN uptake fluxes (U_{assim}) by benthic biofilms varied under short-term increases (i.e., hours) of either NO_3^- or NH_4^+ concentration. Additionally, biofilm

responses were compared between streams with different ambient DIN concentration. In the third chapter, we evaluated how U_{assim} by benthic biofilms responded to sustained enrichments (i.e., weeks) of either NO_3^- or NH_4^+. This response was assessed in several streams differing in DIN concentration. Here, we additionally explored the interactive effect of one DIN species enrichment to the uptake response of the other DIN species. In the fourth chapter, we explored the in-stream biogeochemical N pathways at reach scale associated with NO_3^- and NH_4^+ uptake in an urban stream receiving NH_4^+-rich inputs from a WWTP effluent. Finally, the fifth chapter aimed to quantify and compare uptake rates of both NO_3^- and NH_4^+ at whole-reach scale, and to evaluate the relative contribution of different benthic primary uptake compartments (PUCs) to whole reach DIN uptake for the two DIN species. To place results into a broader context, we complemented empirical data from our ^{15}N additions in a single stream with existing data on both NO_3^- and NH_4^+ whole-reach uptake in other streams.

In this general discussion section we aim to provide a synthesis overview on both biofilm and whole-reach scale uptake responses to either NO_3^- or NH_4^+ based on the results obtained in the different studies conducted.

6.2 Biofilm N uptake responses to variation on NO_3^- and NH_4^+ availability.

Processes associated with N cycling in ecosystems are mostly driven by biotic activity. In particular, in streams, microbial assemblages developed on benthic habitats are a key biotic compartment, which is ubiquously present in these ecosystems. For this reason, we focused on examining the responses of epilithic microbial assemblages (biofilm) to increases in the availability of the two DIN species, as they can be a key biotic compartment to understand DIN uptake at whole reach scale (Battin et al. 2003; Mulholland and Webster 2010). In the following sections, we discuss results from this PhD Thesis on the effect of variability in the ambient availability of NO_3^- or NH_4^+ as well as on the enrichment (at short-term and under chronic exposure) of the DIN

uptake response for the two DIN species of biofilms. Changes in DIN species availability in streams are subjected to different human land uses (Martí et al. 2010); and thus, epilithic responses can provide understanding on how the DIN response of in-stream microbial communities are subjected to DIN availability changes from human land uses at the catchment scale.

6.2.1. Variability of biofilm DIN uptake rates among streams with different ambient NO_3^- and NH_4^+ availability.

Biofilm U_{assim} measured at ambient DIN concentrations in the different streams within this Thesis (chapters 2, 3 and 5) are represented in Fig. 6.1. Mean U_{assim} (± SE) were 1.18 ± 0.03 and 0.18 ± 0.03 µgN m^{-2} s^{-1} for NO_3^- and NH_4^+, respectively, indicating that benthic biofilms mostly rely on water column NO_3^- to meet their N demand. If we estimate U_{assim} for DIN, by summing U_{assim}-NO_3^- with U_{assim}-NH_4^+, results indicate that U_{assim}-NO_3^- accounted for the largest proportion (73%) of the total U_{assim}-DIN. Apparently, these results may be in conflict to the general preference of microorganisms for NH_4^+ over NO_3^- as an N source due to the lower assimilatory costs associated with NH_4^+ assimilation (Naldi and Wheeler 2002; Hildebrand 2005). However, the higher availability of NO_3^- in or study streams (on average, it represented 94% of the total DIN) likely determines the N demand by biofilms from available sources. This is in agreement with previous studies (Fellows et al. 2006; Newbold et al. 2006; Bunch and Bernot 2012), suggesting that the difference in DIN species availability drives the incorporation of N sources into biotic compartments. However, it is worth noting that biofilm U_{assim}-NH_4^+ accounted for 27% of total biofilm U_{assim}-DIN, regardless that average NH_4^+ only represented 6% of the total DIN. Thus, the assimilation of this DIN species plays a disproportionate role in the total DIN assimilation suggesting the biofilm preference for NH_4^+ uptake.

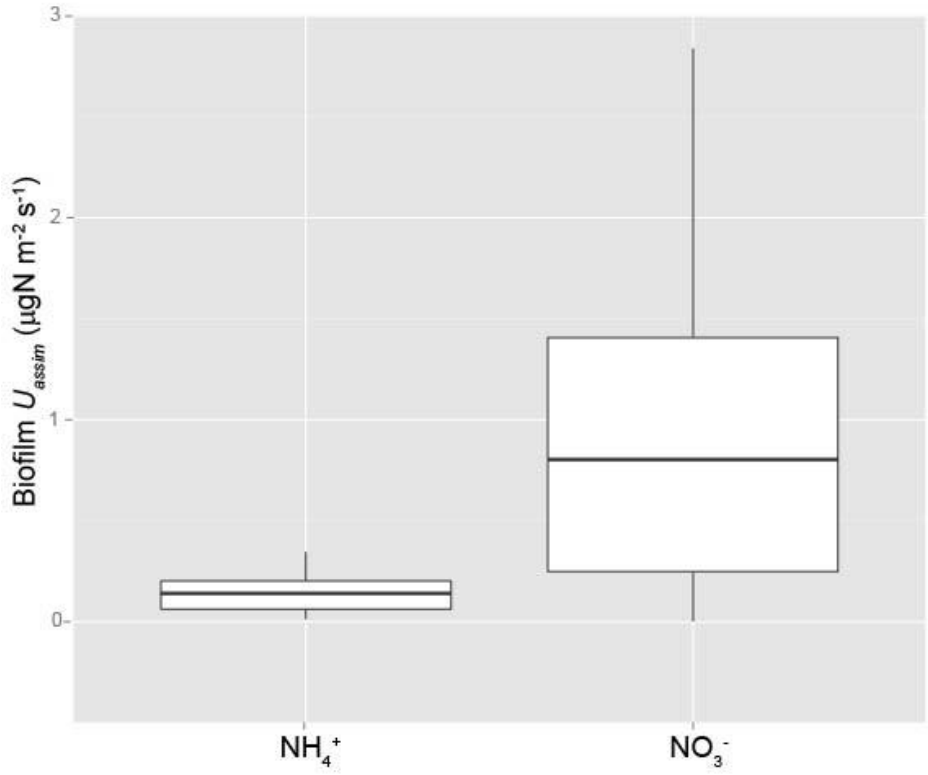


Figure 6.1. Biofilm assimilatory uptake fluxes (U_{assim}; µg N m^{-2} s^{-1}) for NH_4^+ and NO_3^- measured at ambient DIN concentrations in the different study streams from chapters 2, 3 and 5. Points represented the observations. Boxplots display the median, 25th and 75th percentiles (upper and lower hinges). The upper whisker extends from the hinge to the highest value that is within 1.5×*IQR* of the hinge, where IQR is the inter-quartile range, or distance between the first and third quartiles. The lower whisker extends from the hinge to the lowest value within 1.5×*IQR* of the hinge.

6.2.2. Biofilm assimilatory uptake responses to experimental increases in either NO_3^- or NH_4^+ availability: acute and chronic DIN enrichments.

Results from chapter 2, indicated that under short-term pulses of either NO_3^- or NH_4^+ (i.e., hours) biofilms were more reactive to changes in NH_4^+ than in NO_3^- concentration, which was in agreement to previous studies (Bunch and Bernot 2012). Overall, short pulses of NO_3^- enrichment did not enhance or even decreased biofilm assimilatory uptake of NO_3^-, whereas biofilms responded in conformity to Michaelis-Menten (MM) kinetics to increases in NH_4^+ concentration. These results suggest that N uptake by

biofilms was already at saturation under ambient NO_3^- concentration, whereas it was below saturation in relation to ambient NH_4^+ concentration. In addition, the MM response to NH_4^+ concentration differed between the two biofilms studied. The greater kinetic response to NH_4^+ concentration in the stream with higher DIN availability may be attributable to enzymatic repression of NO_3^- uptake (Cresswell and Syrett 1979; Gonzalez et al. 2006) or the contribution of different processes such us nitrification to the total uptake (Teissier et al. 2007; O'Bien et al. 2012). These results may have implications on the contribution of biofilms to the downstream DIN export within the context of humanized landscapes. Overall, considering the observed biofilm responses for N uptake, it is expected that increases in NO_3^- inputs to the stream will tend to be exported, while increases in NH_4^+ will be used by biofilms, at least within a certain concentration range. This is in agreement with previous studies that suggest a lack of biofilm response to acute NO_3^- enrichments; which is often observed in agricultural streams (Stanley and Maxted 2008; Lassaletta et al. 2009).

Previous studies addressing the effect of sustained DIN enrichments show that in-stream biotic compartments have a low capacity to take up N from the water column (O'Brien et al. 2007; O'Brien and Dodds 2010). However, those previous studies were mainly conducted at whole-reach scale and rarely considered biofilm responses in front of the two DIN species in the same study (but see Bunch and Bernot 2012). Therefore, results from Chapter 3 contributed to address this knowledge gap. We expected that NO_3^- or NH_4^+ enrichments would enhance biofilm growth; and thus its N demand if in-stream DIN concentration was below saturation level. In addition, we expected that the biofilm uptake fluxes would be higher for NH_4^+ than for NO_3^- enrichments because biofilms have a higher preference for the former DIN species (Dortch 1990). However, results indicated that sustained enrichments of both NO_3^- and NH_4^+ had an inhibitory effect on biofilm N uptake, being higher for NO_3^- than for NH_4^+ uptake. This effect was especially remarkable when biofilms were exposed to sustained enrichments of NH_4^+.

These results suggest a differential effect of DIN enrichments on stream biofilms depending on the DIN species considered. Biofilm exposures to NH_4^+ enrichment may induce some functional and/or structural changes in the biofilms resulting in a lower demand for NO_3^-. A potential explanation is that NH_4^+ enrichments can favor the development of nitrifiers, which is supported by results from previous studies (Bernhardt and Likens 2004). Nitrifying microorganisms have lower growth efficiencies compared to other microbial components of the biofilms (Risgaard-Petersen et al. 2004) and they also have a preferential demand for NH_4^+, which is used as reduction power in anabolic activity. Our results are in line with other studies conducted in NH_4^+-rich environments such as those provided by WWTP inputs, which indicated that increases in NH_4^+ concentration enhance the proliferation of nitrifiers (Mussmann et al. 2013; Merbt et al. 2014) resulting in high rates of nitrification at whole-reach scale (Merseburger et al. 2005; Ribot et al. 2012). Unfortunately, for this study we did not have data on the microbial assemblage composition of biofilms developed under the different DIN enrichments to support this explanation. Further experiments to test potential shifts in biofilm communities developing on either NO_3^- or NH_4^+ may shed some light to explain the underlying mechanisms responsible of decreases in biofilm uptake for NO_3^- in response to chronic NH_4^+ enrichments.

6.2.3. Beyond epilithic biofilms, the role of other in-stream biotic compartments in assimilatory NO_3^- and NH_4^+ uptake.

Data from chapter 5 indicated that biofilms had a low incidence on the total N uptake by biotic compartments (6% and 7% for NO_3^- and NH_4^+, respectively) in the study stream. However, data compilation including results from other studies encompassing $^{15}NO_3^-$ or $^{15}NH_4^+$ tracer additions indicated that biofilm N uptake can account on average to 20.2% (range = 0 – 49.3%) and 17.3% (range = 4.9 – 45.9%) of the total biotic uptake for NO_3^- and NH_4^+, respectively (Mulholland et al. 2000; Tank et al. 2000; Dodds et al. 2000a; Hamilton et al. 2001; Merriam et al. 2002; Ashkenas et al. 2004; Simon et al.

2005; von Schiller et al. 2009; Sobota et al. 2012; M. Peipoch unpubl.; M. Ribot unpubl.). Therefore, epilithic biofilms may potentially play a significant role in N uptake in streams; and thus, the responses to increases in DIN concentration observed in chapters 2 and 3 can have relevant influences on DIN uptake at whole-reach scale.

Streams also provide habitats for microbial assemblages developed on hyporheic sediments, on detrital compartments such as fine benthic organic matter (FBOM), leaves or wood; and for bryophytes and macrophytes. All these biotic compartments also contribute to total DIN assimilatory uptake in streams (Dodds et al. 2000a; Ashkenas et al. 2004; Riis et al. 2012). Furthermore, riparian vegetation developed on the riparian-stream edge can also contribute to in-stream DIN uptake (Schade et al. 2001; Ashkenas et al. 2004; Pastor et al. 2013; Peipoch et al. 2013). In our particular field experiment, biofilms developed on FBOM accounted for the largest portion of the total DIN assimilatory uptake (roughly 40%) for each of the two DIN species. We also found that roots from riparian trees (mostly alder) also contribute to in-stream DIN assimilation, especially in the form of NO_3^-. This is in agreement with previous studies from arid and semiarid regions (Schade et al. 2001; von Schiller et al. 2009).

In addition, our study allowed comparing the assimilatory uptake response of the different biotic compartments in front of the two DIN species, which has scarcely been assessed. Total assimilatory uptake flux of NO_3^- accounted for 17% of total uptake flux for this DIN specie, whereas in the case of NH_4^+, it accounted for the largest part of total NH_4^+ uptake flux (76 %) at whole-reach scale (Chapter 5). In this sense, and similar to results found for biofilms (chapters 2 and 3), we found differential contribution to NH_4^+ and NO_3^- uptake. In general, assimilatory uptake fluxes for NH_4^+ were higher than those for NO_3^-, except for alder roots. In this sense, detrital compartments (i.e., FBOM, leaves and wood) accounted for 84% of the total assimilatory NH_4^+ uptake, whereas they accounted for 60 % of the total assimilatory NO_3^- uptake. In addition, biofilms colonizing leaves were responsible for 35% of

the total assimilatory NH_4^+ uptake, which agrees with previous studies run in well-shaded streams (Mulholland et al. 2000; Merriam et al. 2002; von Schiller et al. 2009). Overall, results indicate that biotic compartments have a high influence on regulating in-stream DIN cycling associated with NH_4^+ availability, but they may not be as effective to regulate NO_3^- availability.

Despite the novel contribution of our results to understand the role of biotic primary uptake compartments on in-stream DIN dynamics, we acknowledge that our results provide a snap-shot picture of the biotic uptake responses to NH_4^+ and NO_3^-. Further research is needed to evaluate how the different compartments react to changes in the availability of the two DIN species, as we examined in particular for epilithic biofilms. In this sense, further research focused on FBOM responses will be very relevant since this biotic compartment is ubiquously distributed in streams and our and previous results indicate that its activity can be important for in-stream DIN uptake.

6.3. Relevance of assimilatory and dissimilatory processes on whole-reach total uptake of NO_3^- and NH_4^+.

The use of ^{15}N stable isotopes (both as natural abundance and as tracer additions) allowed to quantifying the relative relevance of different processes contributing to whole-reach total uptake fluxes for the two DIN species (Chapters 4 and 5). We found that in-stream total NO_3^- uptake was mostly driven by N assimilatory uptake. Dissimilatory uptake pathways of NO_3^- uptake (e.g., denitrification and DNRA) had a very low incidence on the total uptake of NO_3^- (<1%). Therefore, temporary uptake in biotic compartments, rather than permanent removal, dominated whole-reach uptake of NO_3^- in the study stream. On the other hand, total NH_4^+ uptake was equally driven by both assimilatory and dissimilatory (nitrification) uptake (Chapter 5). Results from chapter 4 further indicated that, under high NH_4^+ availability, the contribution of nitrification to total uptake could be even higher. Therefore, both temporary uptake and transformation into NO_3^- contribute to whole-reach uptake of NH_4^+ in the study stream.

To place our empirical results from chapter 5 in a wider context, here we compiled data from previous studies using ^{15}N tracer additions, which explicitly considered both assimilatory and dissimilatory uptake pathways of each DIN species within the same streams. Together, we found 13 and 19 streams with a complete data set for NO_3^- and NH_4^+ respectively (Fig. 6.2).

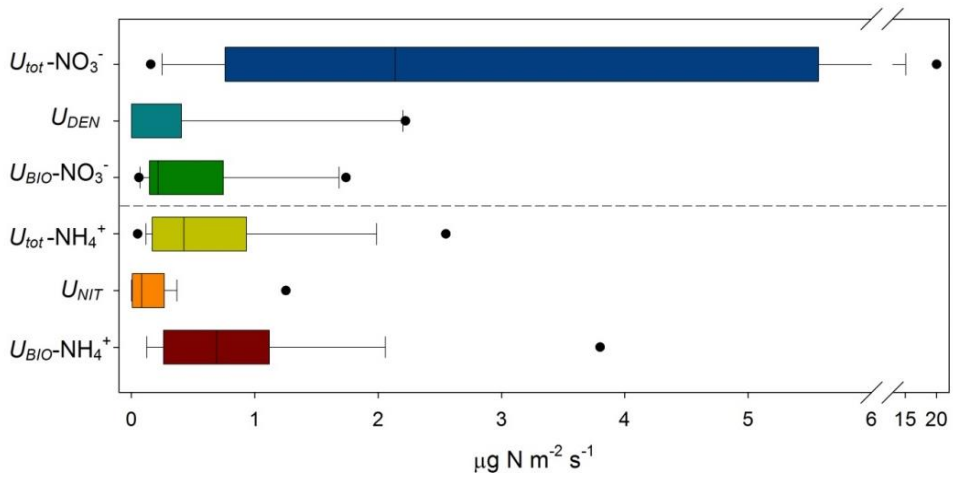


Figure 6.2.Uptake fluxes of the key in-stream N pathways for total NO_3^- uptake (U_{tot}-NO_3^-), denitrification (U_{DEN}), assimilatory NO_3^- uptake (U_{BIO}-NO_3^-), total NH_4^+ uptake (U_{tot}-NH_4^+), nitrification (U_{NIT}) and assimilatory NH_4^+ uptake (U_{BIO}-NH_4^+). Data compiled from published studies using ^{15}N additions. For each DIN species, we only considered those studies in which both assimilatory and dissimilatory N uptake pathways were estimated (n= 13 and 19 for NO_3^- and NH_4^+, respectively). Boxplots display the median, 10th, 25th, 75th and 90th percentiles for each N pathway. Data points represent the 5th/95th percentiles. Data are from: Peterson et al. 2001; Simon et al. 2005; Mulholland et al. 2008; von Schiller et al. 2009; Sobota et al. 2012; Chapter 5 and M. Peipoch unpublished).

In general, total uptake of NO_3^- was clearly higher and more variable than total uptake of NH_4^+. The contribution of denitrification to total uptake of NO_3^- was highly variable among streams (range = 0 - 30%) and averaged 6%. Despite previous research on NO_3^- uptake has mostly focused on denitrification, there are also few available data of assimilatory NO_3^- uptake fluxes (von Schiller et al. 2009; Sobota et al. 2012 and those from Chapter 5). On average, assimilatory uptake of NO_3^- accounts for 24% (range = 2.2 - 92%) to the total uptake of NO_3^-. Contribution of nitrification to total uptake

of NH_4^+ averaged 21% (range = 0 - 59%); and assimilatory uptake of NH_4^+ accounted for 75% of total uptake of NH_4^+ on average (range = 17 - 100%). Therefore, results from our study site for both NO_3^- and NH_4^+ uptake seem to be well bracketed within those from previous studies. It is worth noting at whole-reach scale, the sum of assimilatory and dissimilatory uptake pathways generally accounts for <<100% of the total uptake of NO_3^-; whereas in the case of NH_4^+, they tend to account 100% of the total uptake of this DIN species. The underlying mechanisms responsible for the uncertainty associated with in-stream NO_3^- uptake are not entirely clear and further research is necessary to elucidate them.

Results also indicated that on average assimilatory uptake from biotic compartments accounted for the largest part of total uptake, especially for NH_4^+, which highlights the relevance of temporary retention via assimilatory uptake on in-stream N uptake. On the contrary, dissimilatory processes associated to each DIN species had a low incidence on their respective total uptake. However, it is worth noting that most of the compiled data in Figure 6.2 are from studies conducted in pristine or low-disturbed streams. A study based on 72 streams across USA encompassing a wide gradient of human pressures in their catchments found that on average denitrification accounted 20% of total uptake of NO_3^- (Mulholland et al. 2008), which is higher than the estimated average for the of relative pristine streams considered in Figure 6.2. The same study also reported that increases in ambient NO_3^- concentration enhanced both total uptake of NO_3^- and denitrification. Since data from Mulholland et al. (2008) are available we performed a linear regression analysis, which showed that the contribution of denitrification to the total uptake of NO_3^- also increased with NO_3^- concentration (linear regression of log-log transformed data, r^2= 0.46, p < 0.001). This suggests that the relevance of denitrification to total uptake is higher in those streams with higher NO_3^- concentrations. On the other hand, as indicated above, in streams receiving high inputs of NH_4^+ from WWTPs the contribution of nitrification to total NH4 uptake is very relevant (Merseburger et al. 2005; Merbt et al.

2014, chapter 4). Altogether, these results suggest that dissimilatory processes associated with NO_3^- and NH_4^+ could have a higher incidence on their respective total uptake as the ambient availability of the two DIN species increases.

6.4. Contrast between total NO_3^- and NH_4^+ uptake at whole-reach scale.

Data from Chapters 4 and 5 pointed out that streams tend to be more efficient in taking up NH_4^+ than NO_3^- (i.e., S_w-NH_4^+ < S_w-NO_3^-). In fact, the average distance traveled by NH_4^+ along the stream before being removed from the water column was <100 m, while the distance traveled by NO_3^- was >> 1000 m. This difference between the two DIN sources indicates a higher in-stream bioreactivity over downstream transport for NH_4^+ than for NO_3^- This result is further supported by the data analysis from the literature survey (chapter 5), as well as by results from previous studies in which comparison between the two DIN sources for given streams was not explicitly considered (Ensign and Doyle 2006; Tank et al. 2008). This consistent pattern at whole-reach scale is in agreement with findings at smaller scales of organization, such as microbial assemblages (e.g., benthic biofilms, chapter 3) or single cells (Mccarty 1995; Hildebrand 2005). A plausible explanation provided for the general preference of in-stream biota for NH_4^+ over NO_3^- is that the energetic cost to assimilate NH_4^+ into the cells is lower than that to assimilate NO_3^- (Mccarty 1995; Hildebrand 2005). Our results further suggest that controlling factors at small scales of organization seems to be reflected on N cycling patterns of the two DIN species at whole-reach scale.

Despite differences in the uptake efficiency of the two DIN species, uptake fluxes (*U*) for the two DIN species were similar or even larger for NO_3^-, as indicated by results from both our empirical study and the literature surveys (Fig. 6.3). A possible explanation to conciliate these findings with those of N uptake efficiencies is that despite uptake of NH_4^+ is higher relative its concentration in the stream water than it is for NO_3^-, biota meets their N demand with the DIN species that is in higher concentration (i.e., nitrate).

This could also explain why the total uptake fluxes of the two DIN species can be similar, despite NO_3^- concentration often dominates the concentration of DIN in streams. Urban streams receiving NH_4^+-rich inputs from WWTPs seem to be an exception to this trend (Martí et al. 2004; Martí et al. 2010; chapter 4). This can be explained by the aforementioned preference of in-stream biota for NH_4^+ (Kemp and Dodds 2002; Pastor et al. 2013), but also by the fact that high NH_4^+ concentration fuels nitrification (Merseburger et al. 2005; Merbt et al. 2014; Capter 4). Nevertheless, in this case, NH_4^+ uptake from stream water is mostly transformed into NO_3^-, which tends to be exported downstream.

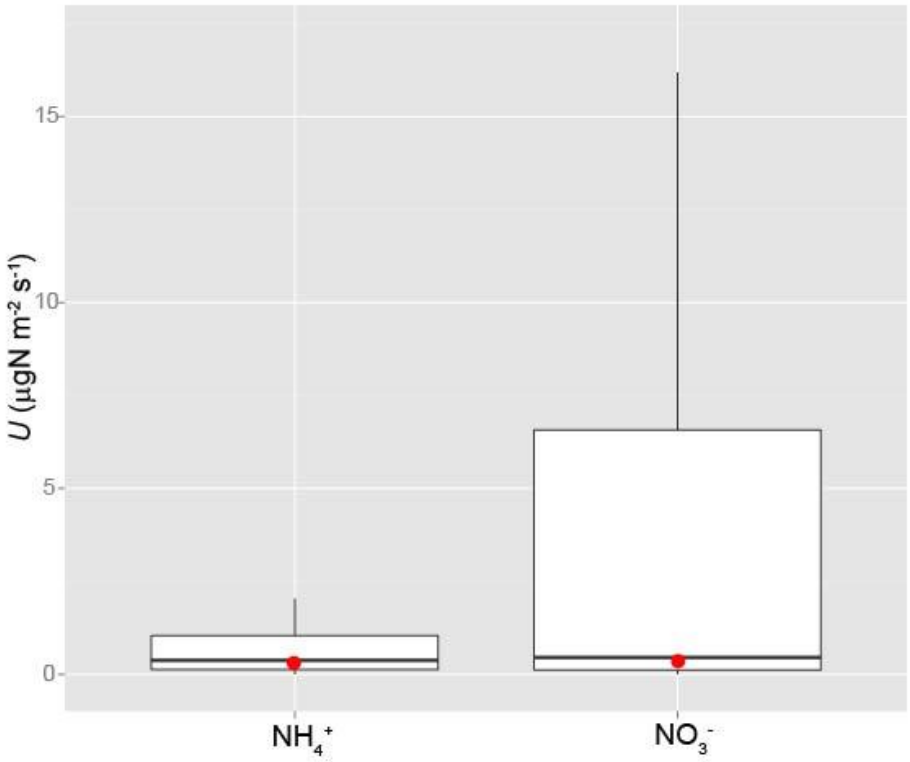


Figure 6.3. Whole-reach total uptake fluxes (*U*; µg N m^{-2} s^{-1}) for NH_4^+ and NO_3^- from literature survey. Boxplots display the median, 25th and 75th percentiles (upper and lower hinges). The upper whisker extends from the hinge to the highest value that is within 1.5×*IQR* of the hinge, where IQR is the inter-quartile range, or distance between the first and third quartiles. The lower whisker extends from the hinge to the lowest value within 1.5×*IQR* of the hinge. Red dots correspond to empirically measured *U* in the study stream from chapter 5.

We also found a higher variability among streams in the uptake fluxes of NO_3^- than of NH_4^+ (Fig. 6.1 and 6.3). Previous studies have indicated that this variability can be associated with the variability in stream discharge (Valett et al. 1996), light availability (von Schiller et al. 2007), in-stream metabolism (Hall and Tank 2003), and ambient DIN concentrations (Dodds et al. 2002; O'Brien et al. 2007). However, these controls have been commonly tested for the uptake variation of one of the two DIN species. Here, we used the data set from the literature survey to examine the influence of DIN concentration on the variability of the uptake for the two DIN species. We found that total uptake of NO_3^- and NH_4^+ increases with increases in the concentration of the respective DIN species, as well as the sum of the uptake fluxes for the two DIN species increases with total availability of DIN (Fig. 6.4).

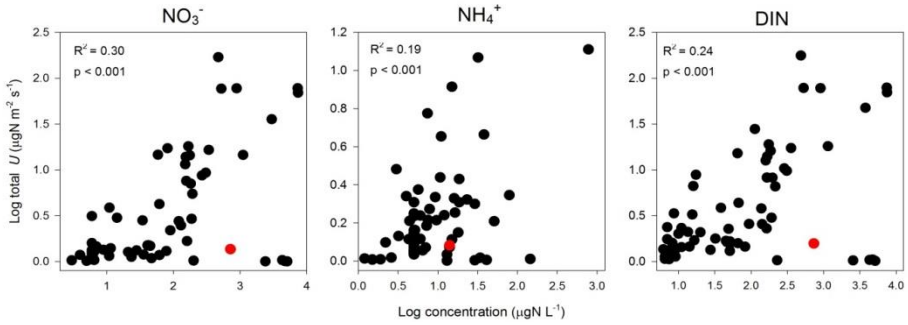


Figure 6.4. Relationships between ambient concentration and whole-reach uptake fluxes (*U*) for NO_3^-, NH_4^+ and DIN (sum of NO_3^- and NH_4^+) from empirical measurements (red dot) as well as from literature survey data (black dots) (from chapter 4). R^2 and p-values for each linear regression of log-log transformed data are shown.

These findings suggest that the bioreactivity of the receiving streams to uptake DIN positively responds to increases in DIN availability. Nevertheless, the fact that the relationship is logarithmic indicates that these increases are not proportional to DIN availability and tend to decrease at higher DIN concentration. This will result in lower DIN uptake efficiency as DIN concentration increases, which uptake is in agreement with results found for a particular biotic compartment (i.e., epilithic biofilms) in chapter 2. Within the

context of widespread in-stream DIN enrichment driven by human activities, these results are relevant because they help to understand the biogeochemical responses of receiving streams to process the two DIN species separately as well as in conjunction, which will ultimately will influence DIN concentration and downstream transport in streams.

6.5. Future research questions

The main objective of this Thesis was to increase knowledge on how in-stream processing influences DIN uptake in streams. In particular, results from this Thesis contributes to understand uptake biogeochemical pathways for NO_3^- and NH_4^+. This is relevant in the context of global change, because human activities not only increase availability of bioreactive DIN, but also changes the relative proportion of the two DIN species. Despite research from each particular chapter provided conclusive results, it also opened new questions that would be interesting to address. In this section we suggest some novel research questions that could generate further knowledge to better understand the effects of DIN enrichments and changes in the DIN speciation on in-stream structure (i.e., biofilm community composition) and function (DIN uptake).

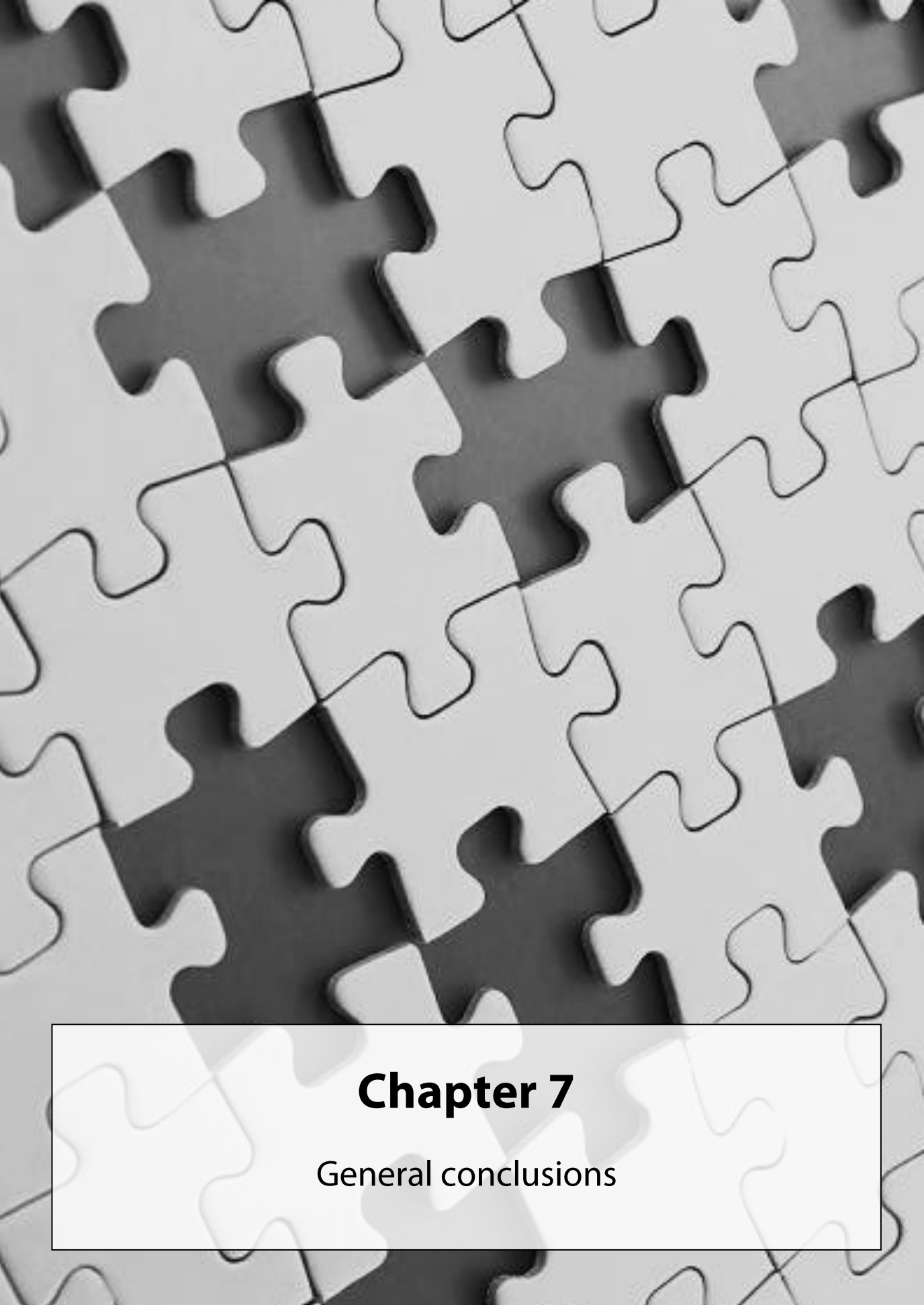
- Overall, results from this Thesis show that biofilms had a clear preference for NH_4^+ as a DIN source, but that they mostly rely on NO_3^- due to a generally higher availability of the later (Chapter 2). The lower assimilatory cost associated to NH_4^+ with respect to that of NO_3^- may explain this fact (Dortch 1990). NH_4^+ diffuses passively though the membrane cell, whereas incorporation of NO_3^- into the cells requires an active pumping and a further reduction to NH_4^+ (Mccarty 1995; Hildebrand 2005). However, even in NH_4^+-rich environments, such as streams receiving WWTP inputs, biofilms may still assimilate NO_3^- from the water column which is an energy-consuming process (Pastor et al. 2013; Chapter 3). Therefore, regardless of the energetic issue, higher biofilm preference or affinity (in terms of uptake kinetics) for NH_4^+ may

be also related to the generally low availability of NH_4^+ across streams and rivers (Collos et al. 2005; Martens-Habbena et al. 2009; Chapter 4). Within this context, a question to be approached is whether biofilms would still have a higher preference and affinity for NH_4^+ with respect to NO_3^- if NO_3^- and NH_4^+ availability were similar, under low and high concentrations of the two DIN species.

- Related to the question above, but extrapolated to the whole- reach scale, another question to be approached is how stream reaches responds to gradual increases in DIN availability and to changes in the the NO_3^-:NH_4^+ ratio (i.e., measuring DIN uptake kinetics at whole-reach scale). There are few studies that have investigated this issue, but results are controversial, and none has contrast responses for the two DIN species in the same stream. Studies have shown that whole-reach NO_3^- uptake responses to NO_3^- enrichments can be linear and positive (Dodds et al. 2000; Earl et al. 2006; O'brien and Dodds 2010), can follow Michaelis-Menten kinetics (Covino et al. 2010; O'brien and Dodds 2010) or can be negative (Earl et al. 2006). On the other hand, NH_4^+ enrichments generally result in positive responses of whole-reach NH_4^+ uptake, and especially follow either linear or Michaelis-Menten models (Dodds et al. 2002) To understand discrepancies in the responses among streams, our results indicates that further studies should consider that whole-reach uptake includes assimilatory and dissimilatory N uptake pathways as well as uptake by different biotic compartments, and that the relative contribution of each pathway to total uptake may differ depending on the DIN concentration.
- Another intriguing result observed in this Thesis was that the sum of all NO_3^- uptake pathways did not account for the total uptake of NO_3^- at whole-reach scale (Chapter 5). The literature survey also points out that this fact is quite common among studies. On the other hand, studies show that assimilatory uptake and nitrification mostly accounts for 100% of total uptake of NH_4^+ at whole reach scale. Improvement of current

methodologies to better account for high in-stream spatial and temporal variability of biomass of primary uptake compartments likely would constraint this uncertainty. On the other hand, a better characterization of microbial assemblages and biogeochemical processes occurring in the hyporheic zone would probably contribute to reduce this uncertainty.

- Finally, results from nutrient diffusing substrata (chapter 3) certainly deserve further investigation. It will be interesting to examine the mechanisms that drive inhibition of DIN uptake under chronic DIN enrichment, especially with NH_4^+, and what causes interactive effects between the two DIN species. In this sense, a closer examination of the composition of microbial assemblages developed under enriched conditions may provide some clue to these questions.



Chapter 7

General conclusions

- At ambient DIN concentrations, benthic biofilms show a clear preference for NH_4^+, but mostly assimilate NO_3^- because it is the predominant in-stream DIN source.
- Benthic biofilms respond differently to enrichments of either NO_3^- or NH_4^+ in terms of assimilatory uptake fluxes. Furthermore biofilm uptake responses vary between acute and chronic DIN enrichments.
- Acute NO_3^- enrichments do not affect biofilm assimilatory NO_3^- uptake fluxes, or even produce an inhibitory effect. On the contrary, acute NH_4^+ enrichments enhance biofilm assimilatory NH_4^+ uptake according to a Michaelis-Menten kinetics model.
- Chronic NO_3^- and NH_4^+ enrichments tend to reduce biofilm assimilatory uptake rates. However, the most relevant effect was observed in NH_4^+ enrichments, which clearly reduced biofilm assimilatory NO_3^- uptake. Presence of nitrifiers may explain this fact because they mostly rely on NH_4^+ to fuel their chemoautotrophic metabolism.
- In our field experiment, biofilms developed on detrital compartments (i.e., FBOM and leaves) accounted for the largest fraction of assimilatory uptake of the 2 DIN species (60 and 84% for NO_3^- and NH_4^+ respectively). In general, most biotic compartments show higher uptake rates for NH_4^+ than for NO_3^-.
- Roots from alder trees accounted for 1/3 of the total assimilation of NO_3^-. This finding highlights the relevance of riparian vegetation in in-stream N uptake through both direct assimilation as well as a source of particulate organic matter, which acts as a colonizing surface for biofilms.
- In general, assimilatory uptake accounts for the largest part of total uptake fluxes at whole-reach scale. Dissimilatory pathways associated with NO_3^- (i.e., denitrification and DNRA) have a low incidence on

total NO_3^- uptake, whereas those associated to NH_4^+ (i.e., nitrification) account for a remarkable fraction of total NH_4^+ uptake.

- Nitrification may have a relevant role on in-stream total uptake of NH_4^+ in both pristine and human-altered streams. On the other hand, the relevance of denitrification on the total uptake of NO_3^- seems to be more constrained to those streams with higher ambient NO_3^- concentration
- At whole reach scale, the study streams are clearly more efficient taking up NH_4^+ than NO_3^- regardless of the ambient NO_3^-:NH_4^+ ratio. However, likely due to the higher NO_3^- availability, uptake fluxes of this DIN species are clearly higher than those of NH_4^+. This pattern is further supported by results from the literature survey indicating that it is a general pattern across streams worldwide.
- Overall, results from this Thesis contribute to increase the scientific understanding of particular processes involved on in-stream DIN cycling and how they are influenced by increases in DIN concentration. A novel contribution is the explicit comparison between NH_4^+ and NO_3^- uptake, which shows a generally higher relevance of in-stream bioreactivity for NH_4^+ than for NO_3^-. In addition, results are relevant within the context of global change and the consequences that this can have on stream ecosystems. Previous studies have shown that human activities within the catchments influence DIN concentration as well as the relative proportion between NO_3^- and NH_4^+. This Thesis shows that increases in each of the two DIN species will result in distinct responses of in-stream N biogeochemical flowpaths and uptake capacity, which ultimately determine the DIN export to downstream ecosystems.

Chapter 8

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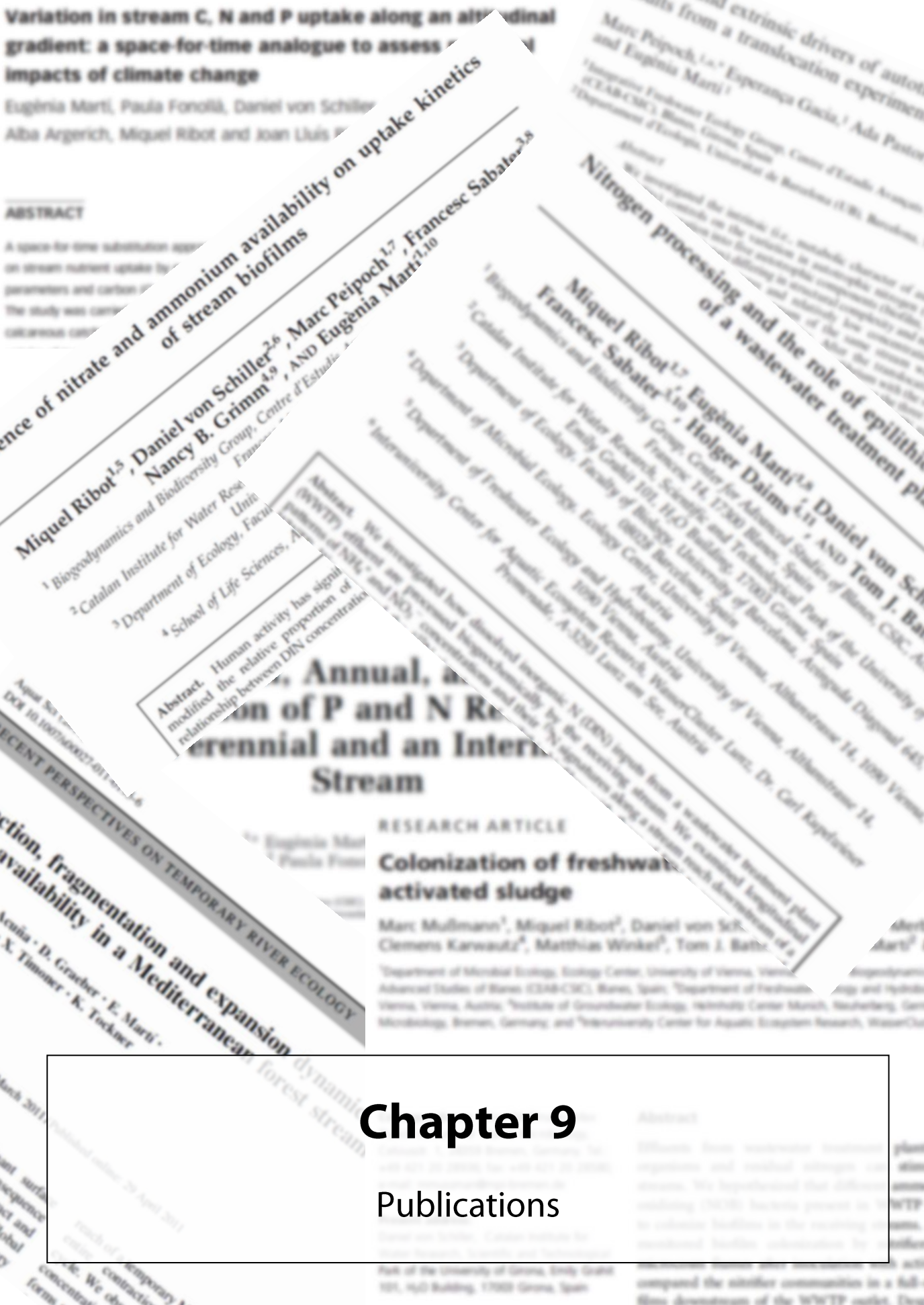

Chapter 9

Publications

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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 on-line 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:

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

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

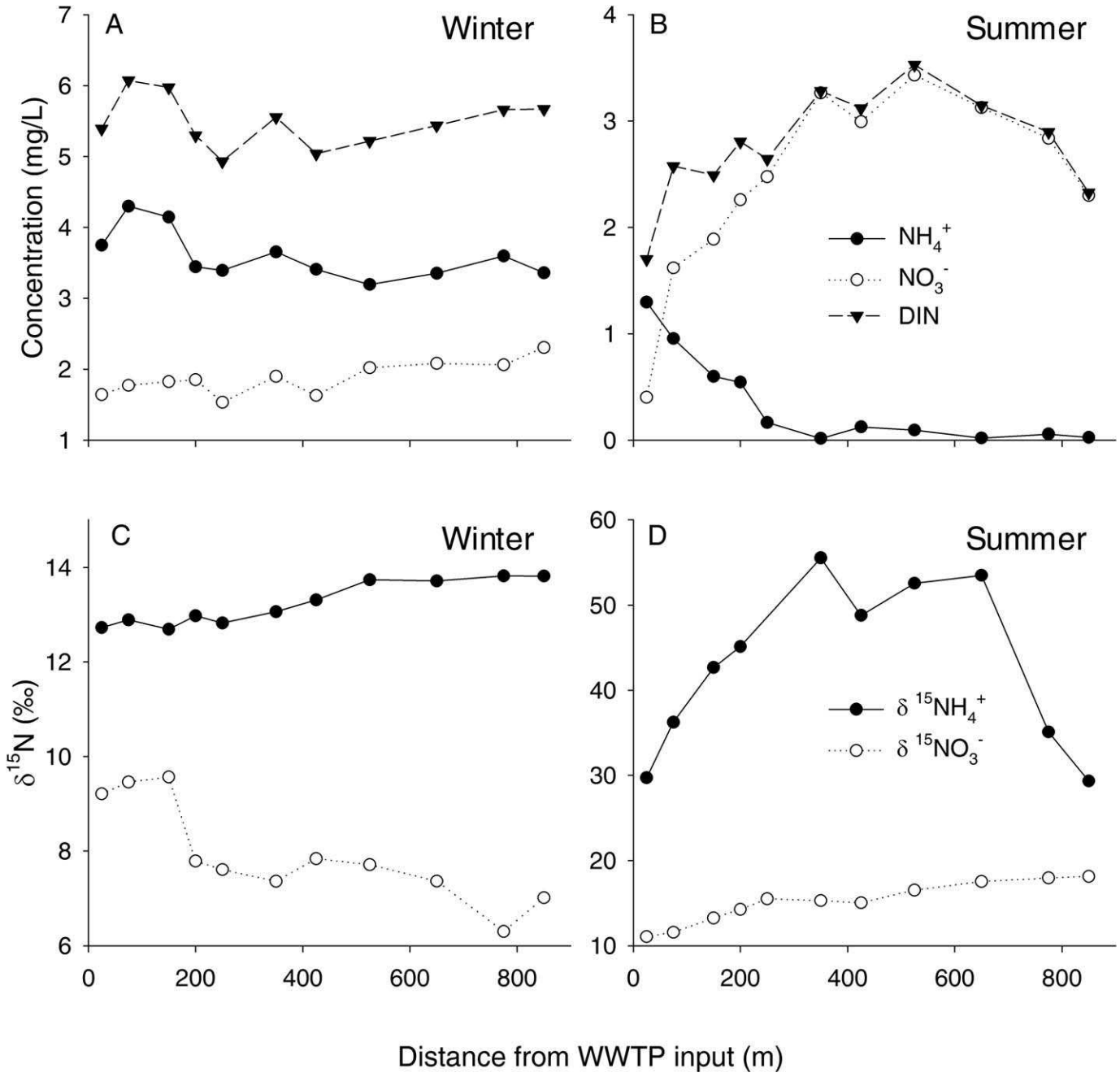

FIG. 1. Variation of ambient concentrations (A, B) and δ^{15}N signatures (C, D) of dissolved N species along the study reach in winter (A, C) and summer (B, D). WWTP = wastewater treatment plant.

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,

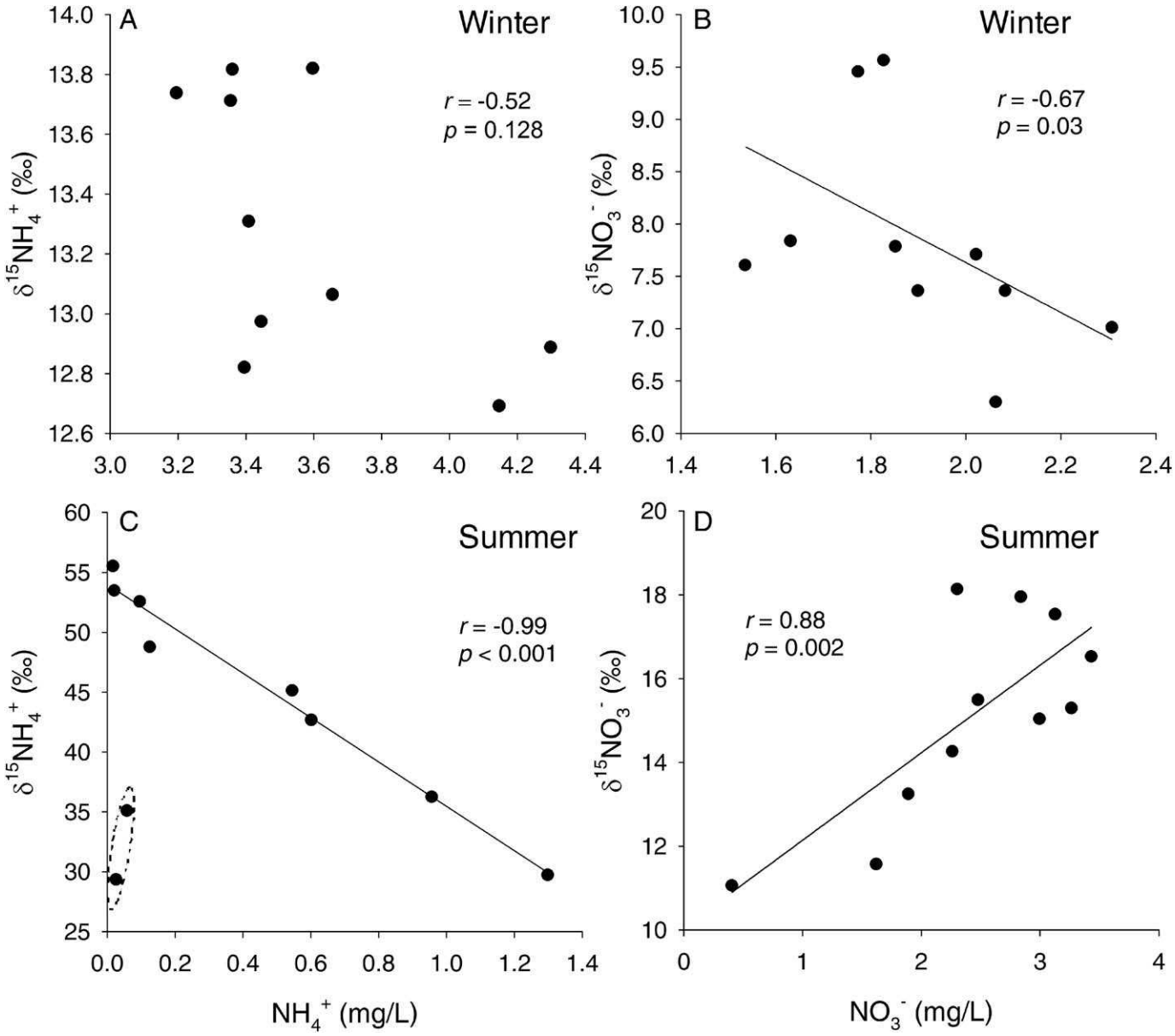

FIG. 2. Relationships between the concentrations of NH_4^+ (A, C) and NO_3^- (B, D) and their respective $\delta^{15}N$ signatures in winter (A, B) and summer (C, D). The dashed ellipse in C indicates 2 outliers of the correlation corresponding with the last 2 sampling sites. Results are for Spearman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

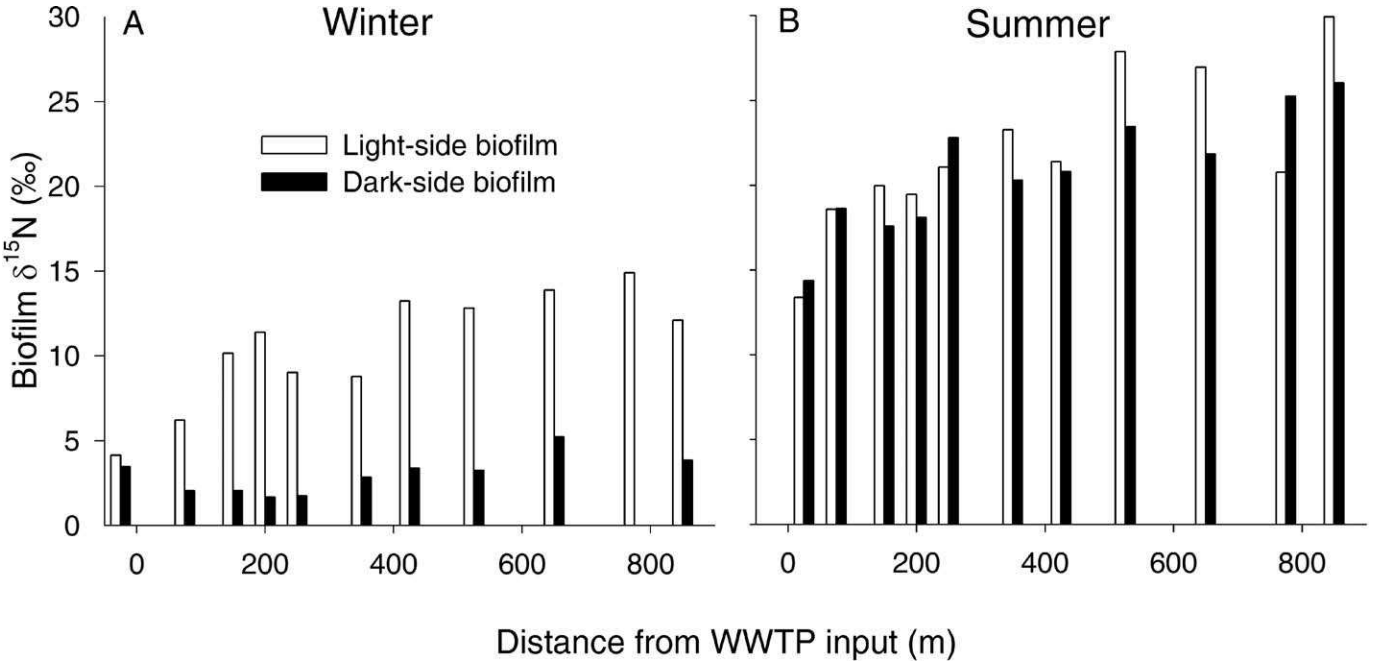

FIG. 3. Variation along the study reach in $\delta^{15}N$ values of biofilm types from the light and dark sides of cobbles measured in winter (A) and summer (B). Negative values for distance indicate the site upstream of the wastewater treatment plant (WWTP) input (0 m).

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 $\delta^{15}N$ 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 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

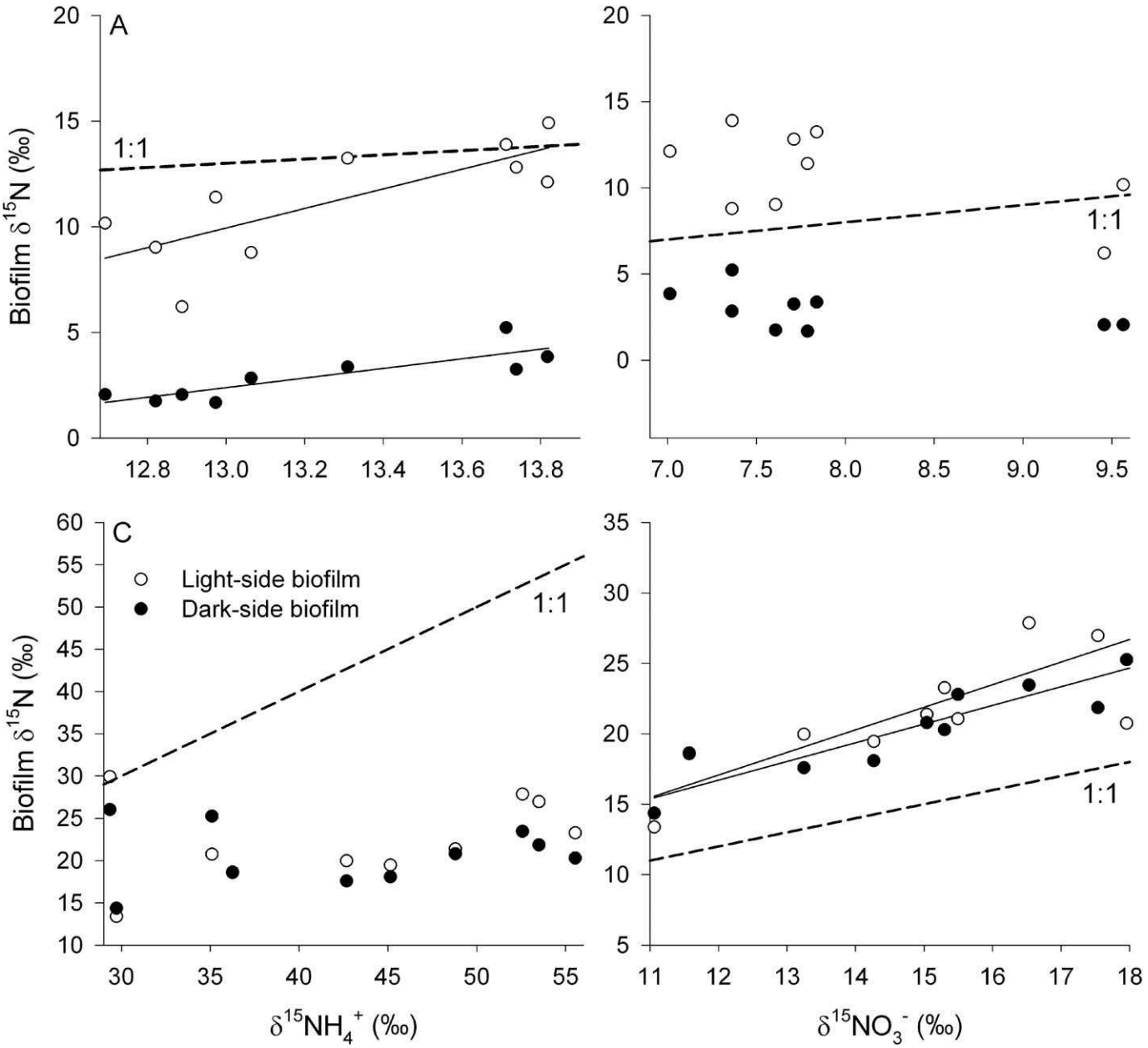

FIG. 4. Relationships between $\delta^{15}N$ signature of NH_4^+ (A, C) and NO_3^- (B, D) and $\delta^{15}N$ signature of the biofilm from the light and dark sides of cobbles in winter (A, B) and summer (C, D). Significant Spearman rank correlations ($p < 0.05$) are indicated by lines. Dashed lines denote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

Acknowledgements

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Influence of nitrate and ammonium availability on uptake kinetics of stream biofilms

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Abstract. Human activity has significantly increased dissolved inorganic N (DIN) availability and has modified the relative proportion of NO_3^- and NH_4^+ species in many streams. Understanding the relationship between DIN concentration and DIN uptake is crucial to predicting how streams will respond to increased DIN loading. Nonetheless, this relationship remains unclear because of the complex interactions governing DIN uptake. We aimed to evaluate how biofilms from 2 streams differing in background DIN concentration would respond to increases in availability and changes in speciation (NO_3^- or NH_4^+) of DIN. We measured DIN uptake by biofilms in artificial flumes in each stream, using separate ^{15}N-NO_3^- and ^{15}N-NH_4^+ additions in a graded series of increasing DIN concentrations. The ambient uptake rate (U) was higher for NO_3^- than for NH_4^+ in both streams, but only U for NH_4^+ differed between streams. Uptake efficiency ($U_{N\text{-}specific}$) at ambient conditions was higher in the low-N than in the high-N stream for both DIN species. A Michaelis–Menten model of uptake kinetics best fit the relationship between uptake and concentration in the case of NH_4^+ (for both streams) but not in the case of NO_3^- (neither stream). Moreover, saturation of NH_4^+ uptake occurred at lower rates (lower U_{max}) in the low-N than in the high-N stream, but affinity for NH_4^+ was higher (lower K_s) in the low-N stream. Together, these results indicate that the response capacity of biofilm communities to short-term increases of DIN concentration is determined primarily by the ambient DIN concentrations under which they develop. Our study also shows that DIN uptake by benthic biofilms varies with DIN availability and with DIN speciation, which often is modified by human activities.

Key words: nitrate, ammonium, biofilm, nitrogen uptake, Michaelis–Menten kinetics, stream, land use, agriculture.

Human activities have significantly increased the concentration of dissolved inorganic N (DIN) in streams (Howarth et al. 1996, Carpenter et al. 1998). Understanding how stream DIN uptake (i.e., the process by which stream biota immobilize DIN from the water column) responds to human alteration of DIN availability has become a research focus for stream ecologists (Mulholland and Webster 2010). Some researchers have studied DIN uptake kinetics (i.e., changes in uptake rates [U] in response to changes in concentration) based on the relationship between whole-reach DIN uptake and DIN concentration by using measurements from different streams spanning a broad range of background DIN concentrations (Dodds et al. 2002, Bernot et al. 2006, Newbold et al. 2006, O'Brien et al. 2007). Other researchers have focused on DIN uptake kinetics within the same stream by following changes in whole-reach uptake in response to short-term DIN

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enrichment (Payn et al. 2005, Earl et al. 2006, Covino et al. 2010, O'Brien and Dodds 2010) or by investigating DIN uptake kinetics in mesocosms (Eppley et al. 1969, Kemp and Dodds 2002, O'Brien and Dodds 2008).

Three mathematical models describe the relationship between DIN uptake and concentration in streams. The first model corresponds to a 1st-order response in which uptake flux (μg N m^{-2} s^{-1}) is directly proportional to concentration of substrate (Dodds et al. 2002). The 2nd model, the efficiency–loss model, follows a power relationship in which U increases but efficiency declines with concentration (O'Brien et al. 2007). The 3rd model follows Michaelis–Menten kinetics and is characterized by saturation of uptake when availability exceeds biological demand (Earl et al. 2006). In general, results from interstream comparisons suggest that the linear and efficiency–loss models best fit the relationship between DIN uptake and concentration (Dodds et al. 2002, O'Brien et al. 2007). Conversely, results from enrichment experiments in the same stream or in mesocosms (i.e., with the same community) suggest that the Michaelis–Menten model best fits DIN uptake kinetics (Payn et al. 2005, Earl et al. 2006, Covino et al. 2010, O'Brien and Dodds 2010).

Human activities also change the relative proportions of the 2 major DIN species: NO_3^- and NH_4^+ (Stanley and Maxted 2008, Lassaletta et al. 2009, Martí et al. 2010). U and kinetics are expected to differ between NO_3^- and NH_4^+ because energetic costs of assimilation associated with NO_3^- are generally higher than those associated with NH_4^+ (Dortch 1990, Naldi and Wheeler 2002). Furthermore, dissimilatory transformations, in which neither compound is incorporated into biomass, contribute to NH_4^+ and NO_3^- uptake. Nitrification (i.e., oxidization of NH_4^+ to NO_3^- by autotrophic or heterotrophic Bacteria and Archaea) will result in apparent NH_4^+ uptake, whereas apparent NO_3^- uptake may include denitrification (i.e., the respiratory process by which bacteria reduce NO_3^- to N_2). These transformations are carried out by different organisms and governed by different controlling factors (Bothe et al. 2007), and thus, may contribute to the expected differences between NO_3^- and NH_4^+ uptake kinetics. Most researchers have investigated NO_3^- or NH_4^+ uptake separately. Thus, we do not know how uptake kinetics differ between these 2 DIN species under similar environmental conditions. In addition, little is known about differences in uptake kinetics of NO_3^- or NH_4^+ of stream biofilms (i.e., the microbial communities that develop on stream substrata) associated with increases in DIN availability. Understanding DIN uptake kinetics of stream biofilms is especially important because biofilms are major contributors to nutrient dynamics in stream networks (Pusch et al. 1998, Battin et al. 2003) and, therefore, may help ameliorate anthropogenic DIN inputs.

We compared U and kinetics for NO_3^- and NH_4^+ between biofilms developed in 2 streams differing in background DIN concentrations. We measured biofilm U in experiments in which we separately added ^{15}N-labeled NO_3^- and NH_4^+ at increasing concentrations to artificial flumes in each stream. We predicted that ambient uptake flux would be higher for NO_3^- than for NH_4^+ and in the high-N than in the low-N stream because of higher availability of NO_3^- with respect to NH_4^+ and the overall higher DIN availability in the high-N stream. In terms of uptake kinetics, we predicted that the Michaelis–Menten model would best fit the relationship between DIN uptake and concentration because DIN uptake is mediated by enzymatic processes. In particular, we expected lower maximum uptake (U_{max}) and ½-saturation constant (K_s) for NH_4^+ than for NO_3^- because of the lower energetic cost of assimilation of NH_4^+ than of NO_3^-. We further expected U_{max} and K_s to be lower in the low-N stream than in the high-N stream because of differences in N affinity between stream biofilms resulting from different histories of nutrient exposure.

Methods

Study sites

Font del Regàs (lat 2°27′00″E, long 41°49′32″N; 929 m asl) is a forested stream situated within the protected area of the Parc Natural del Montseny at the headwaters of the catchment of the river La Tordera. Santa Coloma (lat 2°37′52″E, long 41°52′18″N; 425 m asl) is an agricultural stream situated next to gardening plantations in a lower part of the same catchment. Discharge (mean ± SE) was 56 ± 12 L/s for Font del Regàs and 163 ± 35 L/s for Santa Coloma (biweekly samplings from September 2004–July 2007; MR, DvS, FS, and EM, unpublished data). Concentrations of NO_3^- and NH_4^+ were 181 ± 11 μg N/L and 12 ± 1 μg N/L for Font del Regàs, and 780 ± 44 μg N/L and 19 ± 2 μg N/L for Santa Coloma (biweekly samplings from September 2004–July 2007; MR, DvS, FS, and EM, unpublished data). Hereafter, we refer to Font del Regàs as the low-N stream and to Santa Coloma as the high-N stream.

Channel experiments

We conducted experiments from 3 to 24 July 2007 in the low-N stream and from 23 October to 7 November

2007 in the high-N stream. We placed a set of 6 parallel polyvinyl chloride (PVC) channels (6 m long × 15 cm wide) on the stream bed in a metal structure that held them together and above the stream water (Fig. 1A). Water from an upstream tank fed all channels continuously with a mean (± SE) flow rate of 1.8 ± 0.018 L/min (from measurements done daily throughout the experiments and in each channel). We filled the channels with stream cobbles of similar size and biofilm cover that were collected from the stream bed <50 m upstream from the channel setting. We exposed channels to 5 sequential 24-h fertilization cycles each with an increased concentration (1, 4, 8, 16, and 32× background concentration) of either NO_3^- or NH_4^+ ($n = 3$ channels each; Fig. 1A, B). We released solutions of NO_3^- (as $NaNO_3$) or NH_4^+ (as NH_4Cl) to the corresponding channels at a constant rate from a 3-output carboy (1/channel). We maintained a constant head in each carboy with a Masterflex (Vernon Hills, Illinois) L/S battery-powered peristaltic pump. We also added PO_4^{3-} (as $NaH_2PO_4 \cdot H_2O$) proportionally into the solution at each fertilization level to maintain the background stoichiometric ratio between DIN and soluble reactive P (SRP) throughout the fertilization cycles.

We conducted a tracer addition of either $^{15}NO_3^-$ ($n = 3$ channels) or $^{15}NH_4^+$ ($n = 3$ channels) over the last 6 h of each fertilization level to estimate U of biofilms. We added solutions amended with $^{15}NO_3^-$ (as 99% enriched $K^{15}NO_3$) or $^{15}NH_4^+$ (as 99% enriched $^{15}NH_4Cl$) and NaCl as a conservative tracer at a constant rate using a similar setup as described above. We calculated the amount of $K^{15}NO_3$ and $^{15}NH_4Cl$ needed to produce a target $\delta^{15}N$ enrichment of 3000‰ for both DIN species in the channels. To verify plateau conditions, we logged conductivity every 10 s at the end of each channel with a portable WTW conductivity meter (Weilheim, Germany).

Prior to fertilizations, we collected water at the downstream end of each channel for analysis of ambient nutrient concentrations (3 replicates/channel) and $^{15}NH_4^+$ and $^{15}NO_3^-$ signatures (1 replicate/channel). We also collected composite biofilm samples for the analysis of biomass, pigment content, and natural abundance of ^{15}N (1 replicate/channel) by scraping 3 randomly selected cobbles and filtering the biomass onto combusted, preweighed glass-fiber filters (GF/Fs; Whatman, Maidstone, UK). Before completing each fertilization period (when fertilization and ^{15}N addition were running together), we collected another set of water and biofilm samples (3 replicates/channel) for analysis of nutrient concentration and $^{15}NH_4^+$ and $^{15}NO_3^-$ signatures. Then we stopped the additions, emptied the channels, cleaned them, and filled them again with cobbles from the stream to initiate the experiment with a higher fertilization level (Fig. 1B).

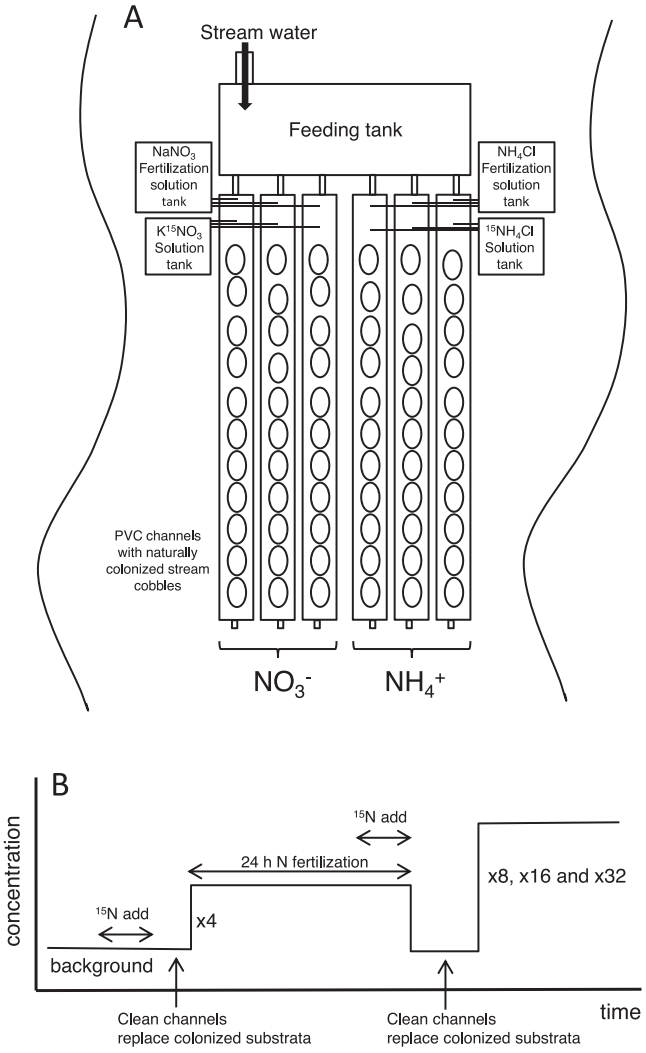

FIG. 1. Scheme of the channel setting used to experimentally approach the objectives of our study. A.—In-situ channel structure. Upstream water supplied the feeding tank, which in turn, fed each polyvinyl chloride (PVC) channel independently. Fertilization and ^{15}N amended solutions for NO_3^- or NH_4^+ reached each single channel independently (3 channels for each dissolved inorganic N [DIN] species). B.—Detail of experimental design to conduct the different fertilization levels (24 h each) and the ^{15}N-tracer additions (add; during the last 6 h of each fertilization treatment) to measure biofilm N uptake for each DIN species (3 channels for each DIN species treatment). For each N fertilization cycle, we used a new set of colonized substrata collected upstream of the channel setting.

We filtered the water samples immediately through combusted GF/Fs into acid-washed, plastic containers and stored them on ice for transportation to the

laboratory. We estimated the cobble surface area by covering it with Al foil and weighing the foil. We stored the filters with biofilm samples on ice in the field and froze (for chlorophyll *a* analysis) or oven-dried them (for ash-free dry mass [AFDM] and ^{15}N analysis) in the laboratory until further processing. We logged photosynthetically active radiation (PAR) every 10 min with a SKP215 quantum sensor (Skye; Powys, UK) connected to a Campbell Scientific data logger (Logan, Utah). We measured temperature at plateau conditions with a WTW 340i portable conductivity meter.

Laboratory analyses

We analyzed water samples for concentrations of NO_3^-, NH_4^+, and SRP on a Bran+Luebbe (Norderstedt, Germany) TRAACS 2000 autoanalyzer with standard colorimetric methods (APHA 1995). We processed water samples for analysis of $^{15}NO_3^-$ and $^{15}NH_4^+$ with the NH_3-diffusion technique (Sigman et al. 1997 and Holmes et al. 1998, respectively). To measure $^{15}NO_3^-$, we amended a known volume of sample with 3 g of MgO and 5 g of NaCl and boiled it to remove the NH_4^+. We then added 0.5 mg MgO and 0.5 mg Devarda's alloy to reduce the NO_3^- to NH_4^+, and treated the remaining sample as for $^{15}NH_4^+$. To measure $^{15}NH_4^+$, we amended a known volume of sample with 3 g/L of MgO and 50 g/L of NaCl and a Teflon filter packet containing a 1-cm-diameter combusted Whatman GF/D fiber glass filter acidified with 25 μL of 2.5 M $KHSO_4$ (to trap the volatilized NH_3), and incubated it on a shaker at 40°C for 4 wk. Once the incubation was completed, we removed the filter packets and placed them in a desiccator for 4 d. We encapsulated filters in tins and stored them until ^{15}N analysis.

We oven-dried filters with biofilm samples at 60°C until they reached a constant mass. To estimate the biofilm AFDM (g/m^2), we weighed subsamples on a Sartorious MC1 analytical balance (Göttingen, Germany) and combusted them at 500°C for 5 h. We measured biofilm chlorophyll *a* content (μg/cm^2) following McIntire et al. (1996). We submerged frozen filters in a known volume of 90% volume/volume acetone and kept them in the dark at 4°C overnight. We sonicated the filters for 5 min and centrifuged them for 10 min at 4000 rpm. We measured the absorbance of the resultant supernatant at 664, 665, and 750 nm before and after acidification with a Shimadzu ultraviolet (UV) spectrometer (Tokyo, Japan). To determine the ^{15}N signature of biofilms, we weighed 1-cm-diameter subsamples to the nearest 0.001 mg on a Mettler-Toledo MX5 microbalance (Greifensee, Switzerland) and encapsulated them in tins. We sent the samples for analysis at the University of California Stable Isotope Facility (Davis, California). We measured the N content (as % dry mass) and the abundance of the heavier isotope, expressed as the ^{14}N:^{15}N ratio compared to that of a standard (N_2 from the atmosphere) using the notation of $\delta^{15}N$ 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).

Calculation of U *and data analysis*

We used independent *t*-tests to explore differences in ambient nutrient concentrations, biofilm AFDM, and biofilm chlorophyll *a* content between streams.

To calculate the uptake rates of NO_3^- and NH_4^+, we first calculated the amount of ^{15}N tracer contained in biofilm ($^{15}N_{biofilm}$; μg N/m^2) with the equation:

$$^{15}N_{biofilm} = B_{biofilm} N (MF_i - MF_b)/100 \quad [1]$$

where $B_{biofilm}$ is the biofilm biomass as dry mass per unit area, N is the biofilm N content expressed as % dry mass, MF is the molar fraction of ^{15}N in biofilm at plateau conditions (MF_i) and at background conditions (MF_b).

We estimated the biofilm U (μg N m^{-2} s^{-1}) for NO_3^- or NH_4^+ with the equation (adapted from von Schiller et al. 2007):

$$U = \frac{^{15}N_{biofilm}}{T_{addition}\left(^{15}N_{flux}/N_{flux}\right)} \quad [2]$$

where $^{15}N_{biofilm}$ is the amount of ^{15}N tracer in biofilm biomass from eq. 1, $T_{addition}$ is the duration of the ^{15}N addition (6 h), $^{15}N_{flux}$ is the ^{15}N flux (as either NO_3^- or NH_4^+) at plateau conditions in the channel water, and N_{flux} is the total N flux (as NO_3^- or NH_4^+) at each fertilization level in the channel water based on concentration and channel flow rate (μg N/s). We then calculated the biomass-specific U ($U_{N\text{-}specific}$; d^{-1}) for biofilm communities and DIN species as a surrogate of N uptake efficiency by dividing biofilm U (μg N m^{-2} s^{-1}) by the N content of dry mass (μg N/m^2).

To compare U and $U_{N\text{-}specific}$ for NO_3^- and NH_4^+ at ambient conditions within and between streams, we used a 2-way analysis of variance (ANOVA) with DIN species (NO_3^-, NH_4^+) and stream (low-N, high-N) as factors. We used post hoc Tukey Honestly Significant Difference tests after significant ANOVAs ($p < 0.05$)

TABLE 1. Mean (± SE) water temperature, photosynthetically active radiation (PAR), background nutrient concentration for both dissolved inorganic N (DIN) species, soluble reactive P (SRP), and biofilm ash-free dry mass (AFDM) and chlorophyll *a* for both study streams during the experiments. Nutrient data from biweekly samplings from September 2004–July 2007 also provided (in parentheses).

Variable	Low-N stream	High-N stream
Water temperature (°C)	15.4 ± 0.1	11.0 ± 0.2
PAR (mol m^{-2} d^{-1})	9.5 ± 3.4	1.4 ± 0.3
NO_3^- (μg N/L)	222 ± 2 (181 ± 11)	400 ± 27 (780 ± 44)
NH_4^+ (μg N/L)	15 ± 1 (12 ± 1)	8 ± 1 (19 ± 2)
SRP (μg P/L)	11 ± 0.3 (4 ± 0.5)	3 ± 0.3 (15 ± 2.6)
DIN:SRP (molar)	48 ± 1 (192 ± 32)	394 ± 32 (429 ± 106)
AFDM (g/m^2)	0.9 ± 0.1	4.3 ± 0.3
Chlorophyll *a* ($\mu g/cm^2$)	0.3 ± 0.03	2.6 ± 0.2

to further examine the effects of stream and DIN species on U and $U_{N\text{-}specific}$.

To explore the relationship between U and concentration of each DIN species at the different levels of fertilization, we determined the fit of our experimental data to the 3 mathematical models described in the introduction. The 1st-order response model followed the equation:

$$U = a + bC \quad [3]$$

where U is assumed to increase linearly with DIN concentration (C) and a and b are a constant and the slope, respectively. The Michaelis–Menten model followed the equation:

$$U = \frac{U_{\max} C}{K_s + C} \quad [4]$$

where C is the DIN concentration, U_{max} is the maximum U, and K_s is the concentration at which ½ U_{max} is reached. K_s is an indicator of the biofilm affinity for DIN. High values indicate lower affinity than low values. The efficiency–loss model followed the equation:

$$U = aC^b \quad [5]$$

where U is assumed to increase with DIN concentration (C) as a power law with exponent $b < 1$.

The parameters a and b from each mathematical model (for the Michaelis–Menten model, U_{max} corresponds to a and K_s corresponds to b), were calculated based on the Gauss–Newton algorithm, an iterative process that seeks the values of the parameters that minimize the sum of the squared differences between the observed and predicted values of the dependent variable. We estimated the confidence intervals (CIs; 95%) for each coefficient by the generic function *confint* powered by R software (version 2.14.0; R Development Core Team, Vienna, Austria). The default method assumes asymptotic normality, and requires that suitable *coef* and *vcov* methods be available. The default method can be called directly for comparison with other methods. We used the Akaike Information Criterion (AIC) to estimate Akaike weights (w_i), which yield the relative likelihood of each model given a particular data set. Within the set of candidate models for the data, we selected the model with the highest w_i.

We conducted all statistical tests with R. When necessary, data were log(x)-transformed before analysis to meet assumptions of homogeneity of variance and normality (Zar 1996).

Results

Environmental conditions differed substantially between the 2 study streams during the experiments (Table 1). Mean water temperature and PAR were 1.4 and 7× higher, respectively, in the low-N stream than in the high-N stream. Consistent with the long-term trend (i.e., biweekly sampling), mean NO_3^- concentration was 2× higher in the high-N than in the low-N stream (t-test, $p < 0.001$; Table 1). In contrast to the long-term trend, mean NH_4^+ concentration was 2× higher in the low-N stream than in the high-N stream (t-test, $p < 0.001$; Table 1). Mean SRP concentration was 4× lower and mean DIN:SRP ratio was 8× higher in the high-N than in the low-N stream (t-test, $p < 0.001$). Mean biofilm AFDM and chlorophyll *a* content were higher (5 and 9×, respectively) in the high-N than in low-N stream (t-test, $p < 0.001$).

DIN species, stream, and the DIN × stream interaction affected both U and $U_{N\text{-}specific}$ at ambient concentrations (ANOVA, all $p < 0.01$). U_{NO3-} (3.1 ± 0.6 μg N m^{-2} s^{-1} in the low-N stream, 4.1 ± 0.8 μg N m^{-2} s^{-1} in the high-N stream) was higher than U_{NH4+} (0.3 ± 0.02 μg N m^{-2} s^{-1} in the low-N stream, 0.06 ± 0.01 μg N m^{-2} s^{-1} in the high-N stream) in both

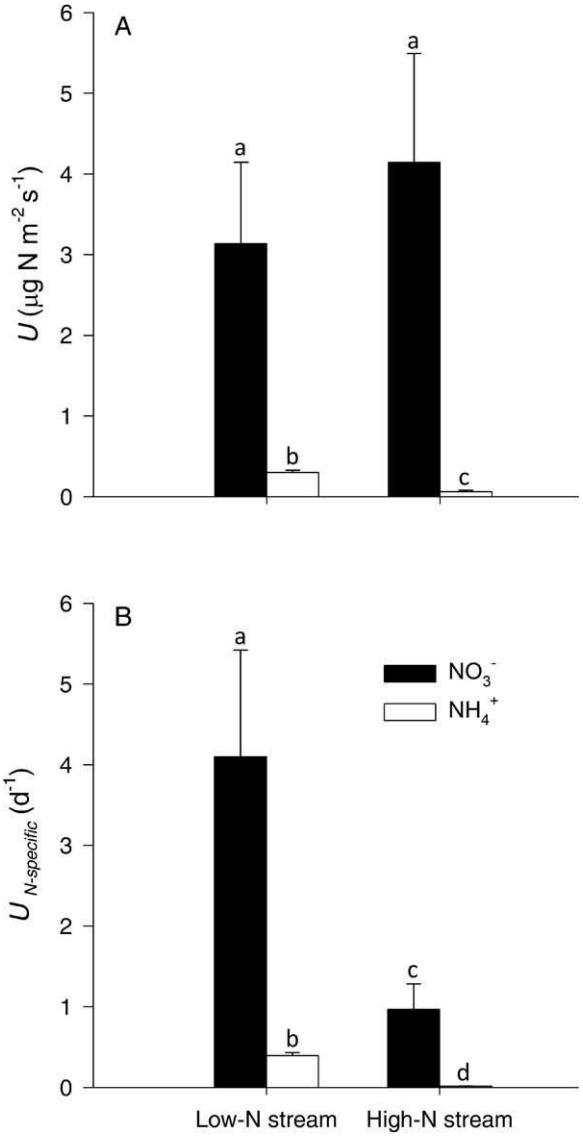

FIG. 2. Mean (±1 SE; $n = 3$) uptake rate (U) (A) and biomass-specific N uptake rate ($U_{N\text{-}specific}$) (B) at ambient concentrations for the 2 dissolved inorganic N species (NO_3^- and NH_4^+) and study streams. Bars with the same letters are not significantly different ($p > 0.05$) based on post hoc Tukey Honestly Significant Difference test.

streams (Fig. 2A). U_{NH4+} differed between streams (Tukey HSD test, $p = 0.001$), whereas U_{NO3-} did not (Tukey HSD test, $p = 0.636$). $U_{N\text{-}specific}$ for NO_3^- (4.1 ± 0.8 d^{-1} in the low-N stream, 1.0 ± 0.2 d^{-1} in the high-N stream) was higher than $U_{N\text{-}specific}$ for NH_4^+ (0.4 ± 0.02 in the low-N stream, 0.01 ± 0.002 in the high-N stream) in both streams (Fig. 2B). In contrast to U, $U_{N\text{-}specific}$ for both NO_3^- and NH_4^+ differed between streams (Tukey HSD test, $p < 0.001$).

Uptake responses to increases in DIN concentration differed substantially between DIN species and streams (Fig. 3A–D). The relationship between U and NO_3^- concentration differed between streams, but uptake kinetics did not fit Michaelis–Menten model in either stream (Fig. 3A, B). In the low-N stream, AIC analysis indicated that the relationship between U and NO_3^-concentration better fit a 1st-order model with a negative slope (Table 2). Conversely, in the high N-stream, 95% CIs for b in all 3 models contained 0, indicating no significant fit, and AIC analysis resulted in no clear model selection (Table 2).

U for NH_4^+ varied with increases in NH_4^+ concentrations (Fig. 3C, D). The AIC analysis indicated the Michaelis–Menten model as the best fit for the relationship between U and NH_4^+concentration in both streams (Table 2). However, uptake kinetic parameters differed between streams. U_{max} and K_s were lower in the low-N than in the high-N stream, and 95% CIs did not overlap (Table 2).

Discussion

We evaluated the response of biofilm U to changes in DIN concentration, and tested whether this response varied among DIN species. We used an experimental approach that combined nutrient fertilizations and ^{15}N-tracer additions in situ in artificial flumes. We predicted that U and uptake kinetics would depend on DIN species (NO_3^- vs NH_4^+) and ambient DIN concentration in the stream (low-N vs high-N). Our results supported these predictions only partially. U was higher for NO_3^- than for NH_4^+ in both streams, but only U_{NH4+} differed between streams, with lower values in the high-N stream. In addition, $U_{N\text{-}specific}$ at ambient conditions was higher in the low-N stream for both DIN species. In terms of uptake kinetics, the Michaelis–Menten model best fit the relationship between U and concentration in the case of NH_4^+ (for both streams), but not in the case of NO_3^- (neither stream). Moreover, saturation of NH_4^+ uptake occurred at lower U_{max} in the low-N stream than in the high-N stream, but affinity for NH_4^+ was higher (lower K_s) in the low-N stream.

Biofilm DIN uptake in streams of contrasting DIN availability and speciation

U of epilithic biofilm for both DIN species under ambient conditions in our study were similar to values reported from previous studies using whole-stream ^{15}N-tracer additions (Mulholland et al. 2000, Tank et al. 2000, Hamilton et al. 2001, Merriam et al. 2002, Ashkenas et al. 2004, von Schiller et al. 2009, Sobota et al. 2012). This result indicates that values of

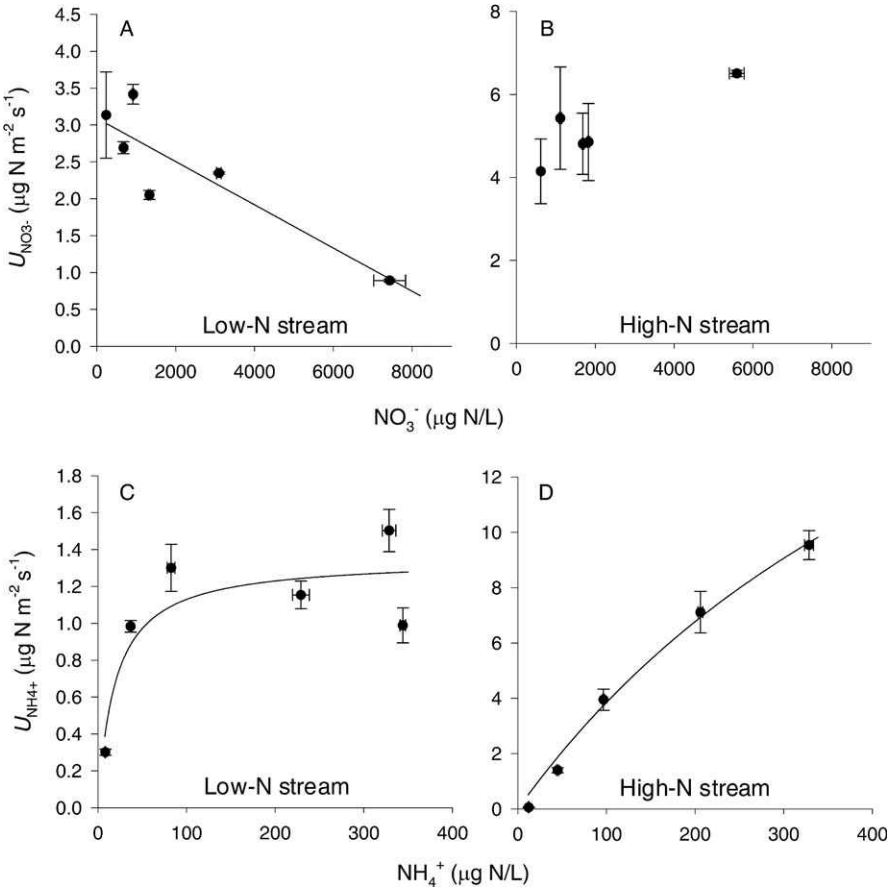

FIG. 3. Mean (±1 SE; $n = 3$) uptake rates (U) for NO_3^- (U_{NO3-}) (A, B) and NH_4^+ (U_{NH4+}) (C, D) in the low-N (A, C) and high-N (B, D) streams. The first point in each panel corresponds to U measured at ambient concentration. Lines represent the selected regression model from Akaike Information Criterion analysis (see Table 2 for regression statistics).

U in our channel experiments were representative of natural field conditions.

Ambient U_{NO3-} was 10× higher than U_{NH4+} in both streams, even though NH_4^+ is theoretically an energetically less costly DIN source and, thus, was expected to be preferentially assimilated over NO_3^- (Dortch 1990, Naldi and Wheeler 2002). Estimated values of the relative preference index (RPI) were ~1 in the 2 streams. This index was proposed by Dortch (1990) as a means to determine the preference for NH_4^+ over NO_3^- (values < 1) or for NO_3^- over NH_4^+ (values > 1). The RPI value of ~1 in our study suggests that biofilms in the 2 streams have no preference for either DIN species. Thus, the observed higher U_{NO3-} than U_{NH4+} was mostly attributable to the higher concentrations of NO_3^- than of NH_4^+.

Ambient U_{NO3-} did not differ between streams, but U_{NH4+} was 10× lower in the high-N than in the low-N stream. Higher NO_3^- availability relative to NH_4^+ availability in the high-N stream may have favored uptake of NO_3^- over NH_4^+ in the high-N stream, as suggested by other authors (Fellows et al. 2006, Newbold et al. 2006, Bunch and Bernot 2012). Furthermore, at low NH_4^+ concentration, the presence of NO_3^- can favor NO_3^- assimilation (Geisseler et al. 2010). Expression and biosynthesis of assimilatory nitrate reductase (the enzyme responsible for NO_3^- assimilation processes) is induced by NO_3^- and NO_2^- and suppressed by NH_4^+ (Gonzalez et al. 2006). Thus, the concurrence of high NO_3^- and low NH_4^+ concentration at ambient conditions in the high-N stream may have led to lower NH_4^+ assimilation rates than in the low-N stream.

Differences in nitrification, which can contribute to NH_4^+ uptake in biofilms, are another potential explanation for the differences in U between streams. If nitrification rate were constrained by the low substrate (NH_4^+) availability in the high-N stream, then we would expect the contribution of nitrification to total NH_4^+ uptake to be lower in that stream. In both streams, $\delta^{15}NO_3^-$ increased during plateau conditions in the channels where we did $^{15}NH_4^+$

TABLE 2. Mean (95% CI) for statistical parameters of linear ($U = a + bC$), Michaelis–Menten ($U = aC/b + C$), and efficiency–loss ($U = aC^b$) models used to evaluate the model that best fit the relationship between uptake rate (U) and dissolved inorganic N (DIN) concentration (C) for both streams and DIN species (NO_3^- and NH_4^+). The Akaike Information Criterion (AIC) was used to estimate Akaike weights (w_i), which give the relative likelihood of each model. The highest relative likelihoods are marked in bold. For the Michaelis–Menten model, a corresponds to the maximum uptake rate (U_{max}; μg N m^{-2} s^{-1}) and b corresponds to the ½-saturation constant (K_s; μg N/L).

Model	Low-N stream				High-N stream			
	a	b	AIC	w_i	a	b	AIC	w_i
NO_3^-								
Linear	**3.1 (2.7–3.5)**	**−0.00029 (−0.0004 to −0.00018)**	**33.4**	**0.97**	4.3 (3.1–5.5)	0.00040 (–0.000023–0.00082)	55.1	0.36
Michaelis–Menten	2.1 (1.6–2.6)	−85.8 (−131.9 to −7.6)	48.0	0	6.5 (4.8–9.2)	384 (−36.5–1282)	55.6	0.28
Efficiency–loss	11.9 (5.3–27.1)	−0.2 (−0.4 to −0.1)	48.1	0.03	1.3 (0.3– .6)	0.2 (−0.010–0.4)	55.1	0.37
NH_4^+								
Linear	0.8 (0.5–1.0)	0.0016 (0.00029–0.0029)	17.3	0	0.3 (−0.5–1.1)	0.030 (0.025–0.034)	45.1	0.03
Michaelis–Menten	**1.3 (1.2–1.5)**	**17.1 (7.8–34.9)**	**2.6**	**0.98**	**28.0 (17.4–113)**	**628 (307–3449)**	**38.9**	**0.77**
Efficiency–loss	0.4 (0.2–0.7)	0.2 (0.093–0.3)	10.9	0.02	0.082 (0.030–0.2)	0.8 (0.7–1.0)	41.7	0.19

additions, a result indicative of nitrification (2.6 ± 0.5‰ and 1.9 ± 0.9‰ in the low-N and the high-N streams, respectively). Based on these $\delta^{15}NO_3$ increases, we estimated the contribution of nitrification to total biofilm NH_4^+ uptake for each fertilization cycle. This contribution ranged from 0.2 to 7.6% in the low-N stream, whereas it was <0.2% in the high-N stream. These results contrast with findings from Bernhardt et al. (2002), who found a higher contribution of nitrification to total NH_4^+ uptake in high-NO_3^- streams of Hubbard Brook (New Hampshire, USA). They hypothesized that when assimilatory processes switch to NO_3^- uptake (i.e., in high-NO_3^- streams), competition between nitrifiers and heterotrophs is ameliorated, resulting in higher nitrification rates. Our data do not support this mechanism because nitrification rate was probably lower in the high-N than in the low-N stream. Instead, we suggest that combination of lower NH_4^+ assimilation and lower nitrification by biofilms in the high-N stream explains the differences in U_{NH4+} between streams.

$U_{N\text{-}specific}$ values indicate that the biofilm from the high-N stream took up both NO_3^- and NH_4^+ from the water column less efficiently than the biofilm from the low-N stream. Lower uptake efficiencies often occur in streams with high DIN concentrations because of saturation of assimilative processes (O'Brien et al. 2007). Thus, our results suggest functional differences in the way DIN is cycled within biofilm communities grown under low- and high-N conditions, which in turn, may lead to differences in the uptake kinetics for both DIN species between stream types.

Biofilm DIN uptake kinetics

Contrary to expectations from nutrient kinetic theory, increases in NO_3^- availability did not enhance biofilm U_{NO3-}. In the high-N stream, addition of NO_3^- had no effect on biofilm U, suggesting that uptake capacity of biofilm assemblages probably was saturated at the ambient NO_3^- concentration. Earl et al. (2006) suggested that when N is not limiting in streams, a 0-order mathematical model (i.e., constant rate with slope = 0) is more applicable than a higher-order model, a suggestion in concordance with our results in the high N-stream. Alternatively, the lack of biofilm uptake response to increases in NO_3^- concentration might be explained by tight coupling of NO_3^- uptake to availability of other nutrients (Fairchild et al. 1985, Sterner et al. 1992). Schanz and Juon (1983) suggested that P is a potentially limiting element at DIN:P > 20 (others have suggested a transition from N to P limitation at DIN:P ≈ 16–17; Redfield 1958, Grimm and Fisher 1986). We added

SRP in the fertilization solutions to maintain background DIN:P, but ratios were well above the potential P-limitation thresholds, especially in the high-N stream (394 ± 32 μg P/L). In this sense, NO_3^- uptake in the high-N stream may have been constrained by P insufficiency. However, if P were the limiting nutrient, then increases in P availability should alleviate P limitation and, thus, enhance NO_3^- uptake. We think this alternative explanation is unlikely because previous nutrient-limitation bioassays in the high-N stream failed to show P limitation (von Schiller et al. 2007).

Increases in NO_3^- availability in the low-N stream produced a decrease in biofilm *U*, indicating a possible inhibitory effect of high NO_3^- concentrations on biofilm uptake in this stream. Inhibitory effects on the uptake of NH_4^+ or NO_2^- at high concentrations have been reported in the literature (usually associated with nitrification processes; Kim et al. 2006, Vadivelu et al. 2007). However, as far as we know, no previous evidence exists for inhibition of NO_3^- uptake at high NO_3^- concentrations. However, inhibitory effects of long-term NO_3^- enrichment have been reported for periphyton growth in nutrient-diffusing substrate experiments (Bernhardt and Likens 2004), and a few investigators have shown potentially toxic effects of NO_3^- on freshwater animals and plants (Camargo and Alonso 2006, Lambert and Davy 2011). Our experiments do not allow us to identify the mechanisms underlying observed patterns but do provide evidence that a short-term, sharp increase in NO_3^- concentration may be inhibitory.

Michaelis–Menten kinetics described biofilm uptake responses to increases in NH_4^+ concentration in both streams. Values of K_s were higher than ambient concentrations of NH_4^+ in both streams, so we conclude that biofilm uptake for this DIN source was below saturation at ambient concentrations (Tilman 1982). Therefore, biofilms were able to respond positively to short-term increases in NH_4^+ concentration within a certain range in the 2 streams. Bunch and Bernot (2012) also compared uptake responses of microbial communities to NH_4^+ and NO_3^- enrichments. They observed that responses to NH_4^+ were immediate and pronounced, whereas responses to NO_3^- were delayed and more variable. They suggested that preference for NH_4^+ as a DIN source by microbial communities dictates stronger and more rapid uptake responses to changes in NH_4^+ than in NO_3^- concentration.

Our results agree with those by Bunch and Bernot (2012) in showing rapid response to increases in NH_4^+. However, the values of RPI of ~1 in our study indicated no clear preference for NH_4^+ over NO_3^-, at least under ambient conditions. An alternative explanation for the difference in the kinetic responses between NO_3^- and NH_4^+ involves enzymatic responses to short-term changes in availability. Increased availability of NH_4^+ in NH_4^+-amended channels may have triggered repression of NO_3^- reductase and increased biofilm NH_4^+ uptake to meet N demand (Gonzalez et al. 2006). This mechanism could explain the positive biofilm NH_4^+ uptake response to increases in NH_4^+ concentration even though uptake responses for NO_3^- indicated that biofilm demand for this DIN species was saturated at ambient conditions. Previous investigators have found a Michaelis–Menten response of nitrification rates to increases in NH_4^+ concentration within a range of NH_4^+ concentrations similar to that used in our study (Koper et al. 2010). Nitrification probably was substrate-limited at the relatively low NH_4^+ concentrations in the 2 study streams, which would produce a positive response to increased NH_4^+ concentration that conforms to a Michaelis–Menten model. However, our a posteriori calculations of nitrification contribution to the whole-channel uptake suggest that nitrification is only a minor contributor to observed kinetics of NH_4^+ uptake. We suggest that a combination of several mechanisms best explains the different kinetic responses of NH_4^+ and NO_3^- in the study streams.

NH_4^+ uptake kinetics fit the Michaelis–Menten model in the 2 streams, but the kinetic parameters (K_s and U_{max}) clearly differed between streams, supporting our predictions. NH_4^+ U_{max} of the biofilm in the high-N stream was 21× higher than U_{max} of the biofilm in the low-N stream. The high-N stream had higher biofilm biomass and more photoautotrophic organisms (as indicated by chlorophyll *a* content) than the low-N stream, a result that could explain the higher U_{max} observed in the high-N stream. However, U_{max} weighted by N content of biofilm dry mass, a surrogate measure of uptake efficiency, was only 4× higher in the high-N stream. Therefore, biofilms were relatively more efficient in NH_4^+ uptake in the low-N than in the high-N stream, a result that is in agreement with uptake results measured at ambient DIN conditions.

In contrast, biofilms showed a higher affinity (lower K_s) for NH_4^+ in the low-N stream than in the high N-stream. Higher affinities for substrate often are attributed to exposure of microorganisms to lower ambient concentrations (Collos et al. 2005, Martens-Habbena et al. 2009). This explanation may not apply to our study if we consider only ambient NH_4^+ concentration, which was similar and low in the 2

streams. However, when discussing nutrient limitation, it is more appropriate to consider total DIN concentration, which was 2× lower in the low-N than in the high-N stream, because biofilms can meet their N demand by uptake of either DIN species. Alternatively, differences in NH_4^+ affinity between streams could be caused by boundary-layer constraints arising from differences in biofilm structure (Dodds et al. 2002). In support of this idea, the higher AFDM content per unit area in the high-N stream implies thicker biofilms and limitation of diffusion of DIN to all cells in the biofilm (Stewart 2003, Teissier et al. 2007). Limitation by diffusion has been demonstrated for uptake of inorganic C and nitrification activity in model biofilms, with both processes restricted to the surface layer of the biofilm (Gieseke et al. 2005). As a result, the thickness of the biofilm in the high-N stream may contribute to an increase in the range of NH_4^+ concentrations within which U_{NH4+} responds positively. Constraints resulting from diffusion limitation in thicker biofilms operate for both N assimilation and nitrification and, thus, can amplify the range of NH_4^+ concentrations that can be reached before saturation occurs because the 2 processes may have different kinetics.

We cannot rule out differences in environmental conditions, such as light availability and temperature, between the 2 streams as potential causes of differences in biofilm uptake kinetics for NH_4^+. We tried to conduct experiments in streams with similar environmental conditions, but a large flood in the high-N stream forced us to postpone the experiment until the biofilm communities recovered fully. As a result, temperature and light availability were higher in the low-N than in the high-N stream during the experiments and could have enhanced biofilm activity and kinetic responses in the low-N stream. However, the effect of temperature on nutrient uptake kinetics is unclear, and Smith (2011) found no evidence of sensitivity of Michaelis–Menten parameters to temperature. Light availability was higher in the low-N stream, but biofilm chlorophyll *a* content was 9× higher in the high-N than in the low-N stream. Thus, this factor could not have caused the observed kinetic differences, at least for the photoautotrophic component of the biofilms. Thus, observed differences in biofilm uptake kinetics between streams seem to be more influenced by differences in DIN concentrations and relative proportions of DIN species than by differences in other environmental factors.

Conclusions

Biofilm uptake responses to short-term changes in DIN concentration in the 2 Mediterranean streams investigated during the study period depended on ambient conditions, including DIN concentrations, where biofilm developed, and the DIN species considered. Under short pulses of increased DIN concentration, the stream biofilms in our study were more reactive to changes in NH_4^+ than to changes in NO_3^- concentration, but ambient U_{NO3-} far exceeded ambient U_{NH4+}, largely because NO_3^- was present at much higher concentrations. The greater kinetic response to NH_4^+ may be attributable to repression of enzymes associated with NO_3^- uptake or the contribution of a different process (nitrification) to total uptake. Lack of response to NO_3^- suggests this species was present in saturating concentrations. Our results contrast with findings from laboratory-scale experiments, in which NO_3^- kinetics conformed to the Michaelis–Menten model (Eppley et al. 1969, Kemp and Dodds 2002, Maguer et al. 2011). In our study, stream biofilm communities were able to respond to increases in NH_4^+ concentration, which is an energetically cheaper N source than NO_3^- and is the substrate for nitrification. However, we found clear differences between streams in biofilm responses to NH_4^+ that probably arose from differences in biofilm characteristics, interactions with other N species, such as NO_3^-, or adaptive changes in affinity.

Human activities associated with different land uses may enrich adjacent streams with DIN and alter the proportion of DIN species in the streams. Thus, streams draining catchments dominated by agricultural practices tend to be NO_3^- enriched, whereas streams draining urbanized catchments are often NH_4^+ enriched (Stanley and Maxted 2008, Lassaletta et al. 2009, Martí et al. 2010). Given widespread changes in land use, our results have implications for understanding and managing N losses to downstream ecosystems. The N species that reach stream ecosystems potentially could be retained by in-stream biofilm communities (NH_4^+) or exported downstream with the subsequent enrichment of receiving waters (NO_3^-).

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Appendix 1

Data base from literature survey of in-stream NO_3^- and NH_4^+ uptake metrics (Chapter 5)

Appendix 1

Reference	Stream	Q	width	depth	velocity	NO_3^-	NH_4^+	S_w-NO_3^-	V_f-NO_3^-	U-NO_3^-	S_w-NH_4^+	V_f-NH_4^+	U-NH_4^+
		$L\ s^{-1}$	m	m	$m\ s^{-1}$	$\mu gN\ L^{-1}$	$\mu gN\ L^{-1}$	m	$mm\ min^{-1}$	$\mu gN\ m^{-2}\ s^{-1}$	m	$mm\ min^{-1}$	$\mu gN\ m^{-2}\ s^{-1}$
Chapter 5	COL	4	4.6	0.05	0.03	666	13	1719	0.04	0.36			
Chapter 5	COL	4	4.5	0.06	0.03	772	13				66	0.86	0.21
Mulholland et al 2008	Q. Bisley	13	3.2	0.10	0.04	171	3	1000	0.23	0.67			
Merrian et al 2002	Q. Bisley	14	4.7	0.11	0.03	148	4				19	9.89	0.62
Mulholland et al 2004	Walker Br.	0	0.9		0.02	26	4	36	0.73	0.32			
Mulholland et al 2000	Walker Br.	9	3.1			28	4				28	6.17	0.46
Mulholland et al 2008	Sycamore Cr.	21	3.7	0.03	0.23	58	2	185	1.87	1.80			
unpublished	Sycamore Cr.	43	5.8	0.08	0.29	9	6				43	15.31	1.03
Mulholland et al 2008	Mack Creek	31	6.7	0.05	0.09	63	6	1667	0.16	0.17			
Ashkenas et al 2004	Mack Creek	57	5.1	0.16	0.08	59	2				55	11.98	0.41
Mulholland et al 2008	South Kings Cr.	13	2.4	0.07	0.08	9	1	161	2.08	0.31			
Dodds et al 2000	South Kings Cr.	16	2.4	0.15		2	3				56	18.11	2.03
Dodds et al 2002	Kings Creek	14						281	1.91	7.12			
Dodds et al 2002	Kings Creek	20									166	3.67	16.98
Hall and Tank 2003	Ditch Creek	231	5.8	0.14	0.28	5	2	824	2.90	0.24	249	9.60	0.35
Hall and Tank 2003	Spread Creek	87	5.5	0.10	0.16	13	1	105	9.00	2.00	75	12.60	0.25
Hall and Tank 2003	Two Ocean Lake out	144	4.1	0.13	0.28	10	3	2341	0.90	0.15	383	5.50	0.30
Hall and Tank 2003	Pilgrim Cr. Channel 1	46	4.1	0.06	0.20	5	1	518	1.30	0.11	280	2.40	0.02
Hall and Tank 2003	Pilgrim Cr. Channel 2	12	2.5	0.04	0.11	5	1	480	0.60	0.05	144	2.00	0.02
Hall and Tank 2003	Lizard Creek	25	2.5	0.11	0.10	6	1	1500	0.40	0.04	429	1.40	0.02
Hall and Tank 2003	Bailey Creek	118	5.4	0.10	0.21	5	2	771	1.70	0.15	819	1.60	0.04
Hall and Tank 2003	Glade Cr. tributary	149	3.0	0.15	0.33	5	0	764	3.90	0.33	324	9.20	0.03
Bernot et al 2006	RAB	63				5100	40	1404	3.01	0.00	372	1.93	0.01
Bernot et al 2006	BUSK	2				4300	12	904	0.61	0.03	245	1.30	0.01
Bernot et al 2006	SAND	7				200	28	804	4.80	0.02	240	2.40	0.01
Bernot et al 2006	RED	17				2400	144	1906	0.61	0.00	672	0.92	0.03

Bernot et al 2006	COBB	575				4700	33	2207	2.40	0.01	780	2.63	0.04
Tank et al 2008	Snake River	12000	41.0			5	5	2500	7.40	0.59	2000	9.30	0.73
Martí and Sabater 1996	SOLANA	21	3.9	0.07	0.14	181	15	161	2.00	6.05	71	4.54	1.14
Martí and Sabater 1996	RIERA MAJOR	58	3.3	0.19	0.26	471	14	49	21.45	168.29	34	30.91	7.21
Gücker and Pusch 2006	Erpe-D	164	2.4	0.46	0.15			20456	0.20	0.01	2936	1.41	0.00
Gücker and Pusch 2006	Erpe-P	511	3.7	0.77	0.18			11880	0.69	0.10	9624	0.85	0.00
Gücker and Pusch 2006	DMB-D	23	0.9	0.27	0.09			49597	0.03	0.01	3368	0.44	0.00
Gücker and Pusch 2006	DMB-P	22	1.0	0.25	0.09			47460	0.03	0.01	3746	0.36	0.00
Simon et al 2005	East tributary	15	1.4	0.11	0.15	5	4	240	3.24	0.23	68	14.44	0.78
Simon et al 2005	North tributary	23	1.5	0.15	0.20	3	3	216	5.28	0.18	60	22.14	1.19
Rasmussen et al 2011	S1	26	1.7	0.11		10	6	48	22.17	2.86	18	59.20	4.96
Rasmussen et al 2011	S2	31	1.6	0.10		5	10	31	32.88	2.12	37	27.05	3.51
Rasmussen et al 2011	S3	43	4.9	0.12		8	11	140	2.93	0.34	81	5.40	0.74
Rasmussen et al 2011	S4	22	1.9	0.07		5	5	169	2.39	0.12	82	1.25	0.31
Martí et al 2009	PUI	10	1.9	0.03		89	4	400	1.50	1.18	56	10.60	0.38
Martí et al 2009	PAU	8	1.8	0.05		129	12	385	0.30	1.47	625	0.20	0.08
Martí et al 2009	REN	3	1.6	0.04		35	17	385	0.20	0.19	85	1.10	0.41
Martí et al 2009	BIS	7	1.5	0.05		20	28	357	0.70	0.27	135	1.70	1.00
Martí et al 2009	URM	14	2.6	0.06		153	13	62	5.70	12.81	370	1.00	0.18
Martí et al 2009	CAM	7	1.4	0.02		890	14	59	6.20	76.50	238	1.50	0.30
Merserburger et al 2011	TOR Up	171	6.8	0.12	0.27	1110	37	3027	1.62	13.50	670	9.00	3.62
Merserburger et al 2011	TOR Down	228	5.8	0.14	0.39	2990	780	4158	1.08	34.67	2585	2.76	11.90
Merserburger et al 2011	GUR Up	49	2.6	0.12	0.27	7430	50	1946	1.74	68.33	1662	2.16	0.62
Merserburger et al 2011	GUR Down	101	3.5	0.09	0.39	7330	78	7464	0.78	76.67	2016	1.14	1.22
Mineau et al 2011	Baldwin	94	3.3	0.27		6	7	373	4.58	0.46	288	5.93	0.64
Mineau et al 2011	Squaw	228	3.0	0.29		268	18	2678	1.72	7.68	793	5.81	1.69
Mineau et al 2011	Willow	152	2.6	0.21		120	18	3973	0.88	1.75	979	3.55	1.04
Mineau et al 2011	City	24	1.5	0.09		44	6	1439	0.65	0.48	522	1.79	0.18

Mineau et al 2011	Deep	22	3.4	0.17		11	5	170	2.25	0.39	104	3.68	0.31
Hoellein 2012a	State Control	60	2.3	0.15	0.158	167	7	308	6.42	17.07	242	7.87	0.88
Hoellein 2012a	State Restored	52	2.1	0.15	0.153	175	10	563	4.81	13.36	188	9.45	1.75
Hoellein 2012a	Shane Control	38	2.3	0.10	0.151	156	9	589	2.44	6.57	273	4.29	0.64
Hoellein 2012a	Shane Restored	38	1.7	0.14	0.189	150	8	391	4.40	10.47	171	8.07	1.16
Hoellein 2012a	Walton Control	38	2.0	0.15	0.092	340	15	439	3.26	15.49	330	4.16	0.80
Hoellein 2012a	Walton Restored	34	2.3	0.10	0.136	192	22	871	2.10	4.46	222	4.44	1.10
Hoellein et al 2012b	Tawhekarere	9	1.3	0.06	7.42	58	6	30	14.09	13.59	81	5.15	0.53
Hoellein et al 2012b	Hinehopu	17	1.6	0.08	8.17	61	5	208	3.18	3.22	435	1.52	0.14
Hoellein et al 2012b	Unnamed	98	2.0	0.23	13.00	524	5	345	8.67	75.77	169	17.65	1.37
Hoellein et al 2012b	Te Wairoa	150	2.6	0.37	9.36	81	31	286	12.02	16.18	166	20.65	10.69
Hall et al 2002	Bear Brook	12	2.7	0.08	0.05		4				101	2.84	0.18
Bernhardt et al 2002	Bear Brook	12	2.7	0.08	0.05	40		360	0.75	0.50			
Hall et al 2002	Cascade Brook	2	4.0	0.06	0.01		4				19	1.62	0.11
Bernhardt et al 2002	Cascade Brook	2	4.0	0.06	0.01	4		92	0.33	0.02			
Hall et al 2002	Cone Pond Outlet	4	1.7	0.07	0.04		4				47	4.31	0.22
Bernhardt et al 2002	Cone Pond Outlet	4	1.7	0.07	0.04	46		1375	0.11	0.08			
Hall et al 2002	Hubbard Brook	89	9.9	0.09	0.09		4				243	1.39	0.15
Bernhardt et al 2002	Hubbard Brook	89	9.9	0.09	0.09	78		2355	0.23	0.30			
Hall et al 2002	Paradise Brook	5	2.4	0.10	0.02		4				105	1.28	0.08
Bernhardt et al 2002	Paradise Brook	5	2.4	0.10	0.02	187		211	0.62	1.91			
Hall et al 2002	W2 stream	1	1.5	0.04	0.02		4				9	3.04	0.33
Bernhardt et al 2002	W2 stream	1	1.5	0.04	0.02	4		214	0.21	0.01			
Hall et al 2002	W3 stream	6	2.1	0.06	0.05		4				90	1.97	0.13
Bernhardt et al 2002	W3 stream	6	2.1	0.06	0.05	42		235	0.72	0.50			
Hall et al 2002	W4 stream	4	2.0	0.08	0.03		4				19	3.78	0.45
Bernhardt et al 2002	W4 stream	4	2.0	0.08	0.03	305		77	1.63	8.29			
Hall et al 2002	W5 stream	2	1.9	0.04	0.02		4				12	4.73	0.28

Bernhardt et al 2002	W5 stream	2	1.9	0.04	0.02	2		57	0.91	0.03			
Hall et al 2002	W6 stream	2	1.8	0.04	0.03		4				25	3.00	0.22
Bernhardt et al 2002	W6 stream	2	1.8	0.04	0.03	23		255	0.32	0.12			
Hall et al 2002	West Inlet	1	1.2	0.06	0.01		4				5	10.81	0.72
Bernhardt et al 2002	West Inlet	1	1.2	0.06	0.01	2		64	0.85	0.03			