

The role of Primary Uptake Compartments on stream Nitrogen cycling

El paper dels Compartiments Primaris en el ciclatge del Nitrogen als ecosistemes fluvials

Marc Peipoch Güell

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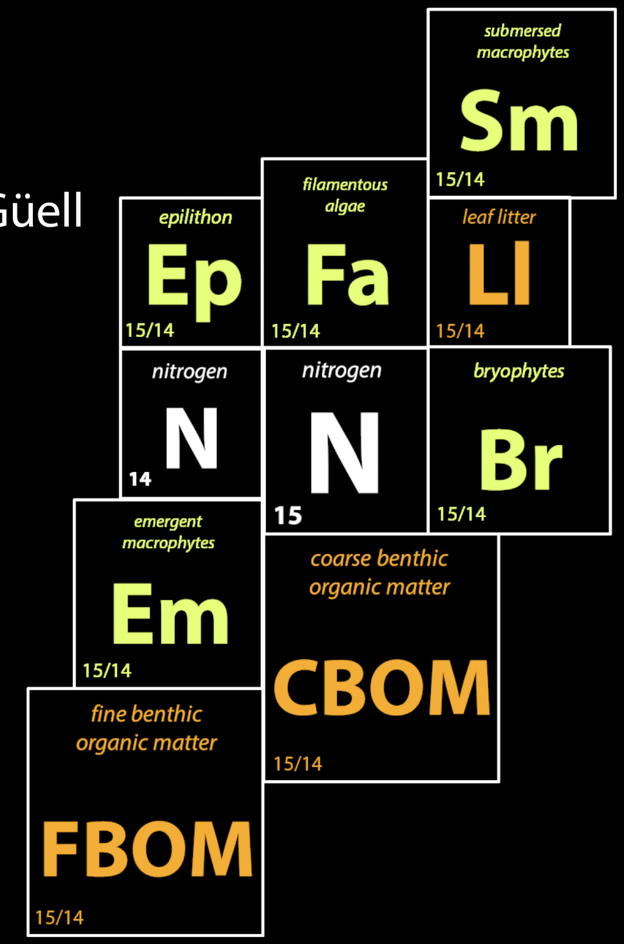


Marc Peipoch - PhD Thesis
The role of Primary Uptake Compartments on stream Nitrogen cycling

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insights from ^{15}N signatures

Marc Peipoch Güell



PhD Thesis
University of Barcelona

Universitat de Barcelona– Departament d'Ecologia

Consejo Superior de Investigaciones científicas (CSIC)

Centre d'Estudis Avançats de Blanes (CEAB)

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Marc Peipoch Güell

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The role of Primary Uptake Compartments on stream Nitrogen cycling

insights from ^{15}N signatures

Memòria presentada per Marc Peipoch Güell
per optar al grau de doctor per la Universitat de Barcelona

Marc Peipoch Güell

Barcelona, Febrer de 2014

Vist i plau
Les directores I tutor de la Tesi

La directora de la tesi	*La codirectora de la tesi*	*El tutor de la tesi*
Dra. Eugènia Martí i Roca	**Dra. Esperança Gacia Passola**	**Dr. Francesc Sabater Comas**
Científica titular al CSIC	Científica titular al CSIC	Professor al Departament d'Ecologia (UB)

Als meus pares,
i a la Susanna

Agraïments

És una quimera intentar agrair sense excepció a tots aquells i aquelles que t'han ajudat a completar un llarg camí com és el de començar una tesi i acabar-la. Però és encara més difícil mostrar-los-hi a tots ells el veritable agraïment, el que va més enllà d'unes bones paraules. A tots aquells que apareixeu en aquesta línies, espero poder-vos anar demostrant en el futur i amb alguna cosa més que aquestes quatre paraules, l'enorme gratitud que sento per la vostra ajuda prestada al llarg d'aquests quatre anys.

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Esperança, no gaire gent pot presumir d'haver tingut dos directors/es de tesi amb un grau d'implicació tant gran i tant similar. Així com amb l'Eugènia, es impossible agrair-te de manera suficient tota la teva confiança, dedicació, i

tot allò que he après de tu durant aquests quatre anys. Amb el teu permís, en el futur intentaré copiar la teva determinació i la teva capacitat de amb menys paraules arribar al mateix destí.

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2009-2011. *Isótopos estables de nitrógeno en ecosistemas fluviales, papel de los componentes bióticos como indicadores de fuentes y procesos del nitrógeno (ISONEF)* Plan Nacional de I+D+I del MCI (ref: CGL2008-05504-C02-02/BOS).

2010-2013. *A metabolically active transient storage model for prediction of nutrient retention in streams*. National Science Foundation (USA) Hydrologic Sciences Program (ref: EAR-0838338).

2012-2014. *Relaciones biogeoquimicas entre los ecosistems riparianos y fluviales en condiciones de clima mediterraneo (MED_FORESTREAM)* Plan Nacional de I+D+I del Ministerio de Ciencia e Innovación (ref: CGL2011-30590-C02-02).

Marc Peipoch

Missoula, 29 de Gener del 2014

Informe del director

Les Dres. Eugènia Martí Roca i Esperança Gacia Passola del Centre d'Estudis Avançats de Blanes (CSIC), directores de la Tesi Doctoral elaborada pel Sr. Marc Peipoch i Güell i que porta per títol "*The role of Primary Uptake Compartments on stream Nitrogen cycling, insights from ^{15}N signatures*".

INFORMEN

Que els treballs de recerca portats a terme pel Sr. Marc Peipoch i Güell 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.

Peipoch, M., E. Martí, and E. Gacia. 2012. Variability in δ^{15}N natural abundance of dissolved inorganic nitrogen and biotic compartments in streams: a meta-analysis. *Freshwater Science*. 31 (3): 1003-1015.

A l'any de publicació d'aquest treball, la revista *Journal of the North American Benthological Society* (*JNABS*) va canviar el seu nom a l'actual de *Freshwater Science*. Per tant, els indicador per l'any 2012 es varen fer considerant encara 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 *JNABS, aquesta* ocupa el 9é lloc de la seva categoria, quedant inclosa en les revistes del 1er quartil.

Peipoch, M., E. Gacia, A. Blesa, M. Ribot, J.L. Riera, and E. Martí. 2013. Contrasts among functional groups of macrophytes in their use of stream water nitrate and ammonium, insights from ^{15}N natural abundance. *Aquatic Sciences*. DOI 10.1007/s00027-013-0330-7

L'índex d'impacte de la revista *Aquatic Sciences* al 2012 (donat que les dades per l'any de publicació encara no són disponibles) va ser de 2.602. 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 97 revistes. Tenint en compte l'índex d'impacte d'*Aquatic Sciences, aquesta* ocupa el 13é lloc de la seva categoria, quedant inclosa en les revistes del 1er quartil.

Peipoch, M., E. Gacia, A, Pastor, M. Ribot, J.L. Riera, F. Sabater, and E. Martí. 2013. Intrinsic and extrinsic drivers of autotrophic N cycling in stream ecosystems: results from a translocation experiment. *Limnology and Oceanography* (en process de revisió)

L'índex d'impacte de la revista *Limnology and Oceanography* al 2012 va ser de 3.405. Aquesta revista està inclosa a la categoria "Limnology". Aquesta categoria té una mediana d'índex d'impacte de 1.425 i inclou un total de 20 revistes. Tenint en compte l'índex d'impacte de *Limnology and Oceanography, aquesta* ocupa el 1er lloc de la seva categoria, quedant inclosa en les revistes del 1er quartil.

Alhora, CERTIFIQUEN

Que el Sr. Marc Peipoch i Güell 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 finançat per la National Science Foundation de USA (*A metabolically active transient storage model for prediction of nutrient retention in streams*), realitzat en col.laboració amb el Dr. Roy Haggerty (Oregon State University, USA).
- 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 anàlisis 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. Marc Peipoch i Güell ha utilitzat o bé té present utilitzar implícita o explícitament aquests treballs per a l'elaboració d'una altra Tesi Doctoral.

Atentament,

Blanes, 24 de gener 2014





Eugènia Martí Roca

Esperança Gacia Passola

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1

General Introduction

1.1 Nutrient cycling in fluvial ecosystems: from the watershed to the stream benthic communities

Most of the nutrients (e.g., carbon, nitrogen, or phosphorous) in stream ecosystems come from their watersheds, principally in dissolved organic and inorganic forms, which are then transported and transformed within the stream channel. Nutrient transformations are mainly controlled by biological processes such as uptake, assimilation, and mineralization that lead to nutrient cycles between biotic and abiotic components of the streams at the same time that nutrients are transported downstream by water flow. Therefore, nutrient dynamics in streams integrates nutrient cycling from the terrestrial watersheds (Bormann and Likens 1967, Vitousek et al. 1979), but also in-stream biogeochemical processes (Alexander et al. 2000, Bernhardt et al. 2003, Hall and Tank 2003). The characteristics of terrestrial watersheds has important implications on streams, for instance, the organic matter dynamics (Fisher and Likens 1973), the resource subsidies across aquatic-terrestrial boundaries (Vitousek et al. 1979, Pinay et al. 1992), or the influx of solar radiation into the stream channels (Minshall 1978). Consequently, most of these influences from the surrounding environment do ultimately determine the structure and function of stream benthic communities (Cummins 1974, Minshall 1978), which in turn are responsible for the nutrient processes within the stream channel (Peterson et al. 2001, Mulholland et al. 2008). Controls of stream nutrient fluxes, therefore, can only be understood with a holistic perspective including the in-stream processes and the external influences on them.

Organisms living on the stream benthos that take up dissolved nutrients from the water column, often referred to as Primary Uptake Compartments (PUCs), are generally composed by both heterotrophs and autotrophs whose

relative abundance is primarily determined by larger-scale constraints (Minshall 1978, Dahm et al. 2003, Lyon and Ziegler 2009). Heterotrophs mostly refer to bacteria and fungi associated to the processing of non-living organic matter, which may obtain nutrients in both inorganic (Sanzone et al. 2001, Cheever et al. 2013) and organic forms (Hall and Meyer 1998). Autotrophs, which must obtain elemental nutrients in dissolved inorganic forms, refer to distinct organisms from diatoms to larger organisms such as bryophytes or vascular plants (Cummins 1974, Allan 1995). Biological uptake, storage, and regeneration by autotrophic and heterotrophic PUCs have an important role in stream nutrient cycling (Valett et al. 1996, Peterson et al. 2001). However, the specific role of each PUC on stream nutrient retention and how this may be affected by different intrinsic biological characteristics and extrinsic environmental conditions are still far from being well comprehended.

1.2 Primary uptake compartments and nutrient cycling

In fluvial ecosystems, PUCs encompass not only organisms with different energy pathways and elemental requirements, but also organisms with a large variety of sizes and structural complexity (Figure 1.1). It is inaccurate to define a unique composition of fluvial PUCs because the distribution and abundance of PUCs vary substantially among streams with different environmental constraints. Fungi can be particularly abundant in leaves, twigs and woods entering the streams (Findlay et al. 2002), while bacteria is generally more strongly associated to fine particles of organic matter (FPOM or FBOM) that are commonly found in stream deposition zones (Bonin et al. 2000, Findlay et al. 2002). Probably, the most ubiquitous compartment in streams is epilithon (i.e., biofilm or periphyton), which is a complex microbial assemblage mostly

composed by algae and bacteria (Lock et al. 1984, Romani and Sabater 1999), and normally dominated by diatoms (Sheath and Cole 1992, Hamilton et al. 2001). The presence and distribution of autotrophic macroorganims in streams are generally less abundant and more heterogeneous than microbial compartments, especially in forested headwaters (*but see* (Grimm 1987, Giorgi et al. 2005, Simon et al. 2007). Variation in the relative composition of filamentous algae, bryophytes, or macrophytes in stream communities is influenced by a large variety of large-scale factors such as turbulence and velocity of stream water (Riis and Biggs 2003, Bowden et al. 2007), temperature (Haslam 1978), surface and hyporheic nutrient availability (Fortner and White 1988), sediment composition (Barko et al. 1991, Svendsen and Kronvang 1993, Madsen and Cedergreen 2002), and light availability (Biggs 1996, Carr et al. 1997, Haury and Aidara 1999).

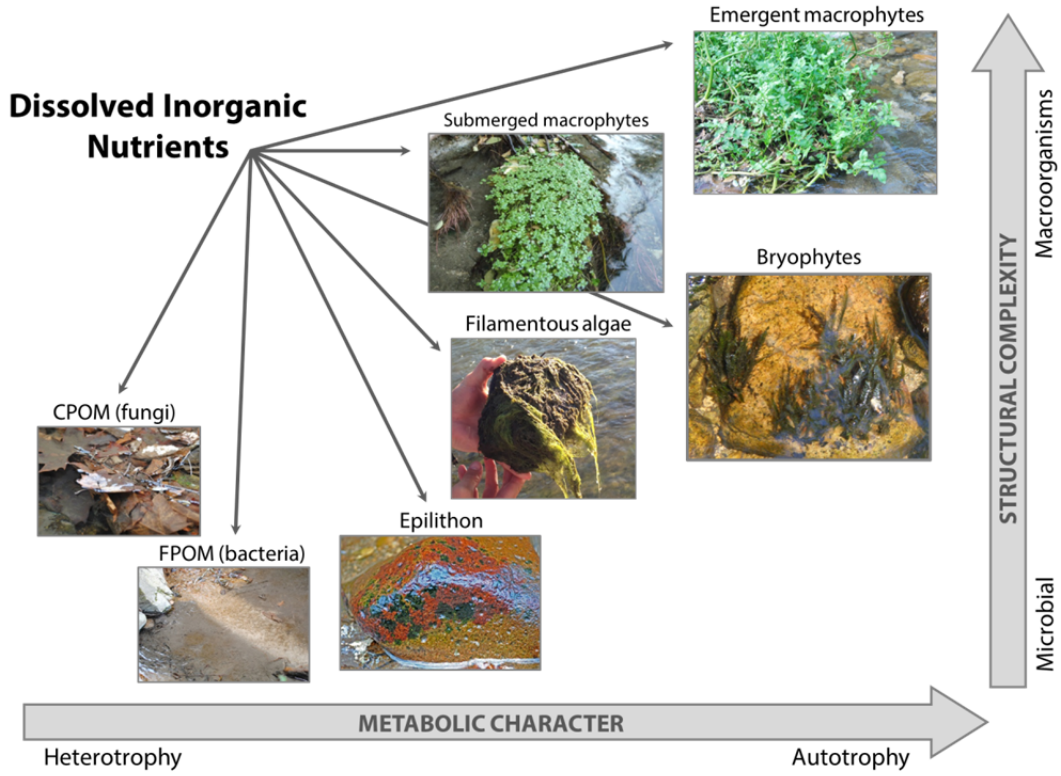


Figure 1.1 Schematic representation of the primary uptake compartments in stream ecosystems in two different axis representing their different energy pathways (*horizontal axis*), from microbial heterotrophs to autotrophs, and their differences in body size and structural complexity (*vertical axis*), from unicellular to large organisms. *CPOM=Coarse Particulate Organic Matter, FPOM=Fine Particle Organic Matter.*

Primary uptake compartments are ultimately responsible for the processing of both organic and inorganic dissolved nutrients in stream ecosystems. Together, autotrophs and microbial heterotrophs make organic energy available for stream consumers at higher trophic levels, and can even provide habitat for epiphytes, invertebrates, and fish as is described for aquatic macrophytes (Allan 1995, Giorgi et al. 2005). Generally, microbial heterotrophic

communities dominate stream metabolism and nutrient cycling, especially in forested headwater streams, which are allochthonous-based ecosystems receiving large inputs of organic matter from the catchment (Fisher and Likens 1973, Minshall et al. 1983, Webster et al. 2003). Notwithstanding, nutrient processes in stream benthos can be also controlled by autotrophic production in streams where riparian vegetation is sparser and sunlight becomes unlimited (Fisher et al. 1982, Grimm 1987, Simon et al. 2007). Therefore, the relative composition of PUCs will change drastically depending on the prevailing environmental conditions of each stream and so will the particular role that each compartment has on the stream nutrient retention. This is especially critical given the large structural and functional differences of stream PUCs (Figure 1.1), since changes in the presence and abundance of PUCs may lead to important consequences for the nutrient cycling of the ecosystem. For instance, streams dominated by microbial communities and by the absence of large organisms are more likely to show fast and tight nutrient cycling given the high uptake efficiency and rapid turnover of stream microbial communities (Tank et al. 2000, von Schiller et al. 2009, Riis et al. 2012). On the contrary, streams where aquatic macrophytes constitute a large portion of the total nutrient uptake may easily exhibit lower uptake efficiency, but longer-term nutrient retention (Simon et al. 2007, Riis et al. 2012). Variation in the relative composition of PUCs among streams can also have implications on the nutrient transport across stream surface and sub-surface environments or on the resource subsidies for stream consumers. Rooted macrophytes on the stream bed are known to act as conduits between the hyporheic and the surface environment because they are able to obtain nutrients from both surface water and sub-surface water in sediments (White and Hendricks 2000), whereas large

stocks of periphyton and filamentous algae are known to provide large amounts of energy for the higher trophic levels compared with aquatic plants (Peterson et al. 1997, Lewis et al. 2000). Overall, large heterogeneity in the relative composition of PUCs across streams merely accentuates the need to fully understand the role of PUCs in stream nutrient dynamics and how this may change under different environmental scenarios.

1.3 Influences of the global change on stream nitrogen cycling: peculiarities of the Mediterranean region.

Nitrogen constitutes one of the most important nutrients for stream biota and sometimes the limiting nutrient regulating primary production (Grimm and Fisher 1986, Lohman et al. 1991). In addition, nitrogen (N) cycle is one of the most altered nutrient cycles at a global scale due to human development. Almost two decades ago, Vitousek (1994) described several alterations of the global N cycle and alerted that human-induced environmental change was already occurring upon that time. At present, these alterations are likely more severe and the need for understanding their consequences becomes more urgent. Stream ecosystems have been defined as highly vulnerable ecosystems given the strong linkage with their watersheds in which most of human activities take place (Howarth et al. 1996). Increased loading of N into stream ecosystems from both nonpoint (e.g., agricultural fertilization) and point sources (e.g., effluents from wastewater treatment plants, WWTP) has become one of the most important environmental problems within the context of global change resulting in alterations of N cycling rates and losses of biological diversity (Vitousek et al. 1997, Carpenter et al. 1998, Galloway et al. 2004). Because PUCs are directly taking up dissolved nutrients from the stream water

column they are strongly and rapidly perturbed by increases in N inputs (Miltner and Rankin 1998), and consequently they are also susceptible to alter higher trophic levels through bottom-up mechanisms (Fisher et al. 1982, Rosemond et al. 1993, Deegan et al. 1997). A better understanding the human impacts on the community PUCs is then critical to predict and mitigate the consequences of human-caused environmental change on the structure and function of stream ecosystems.

In many aspects, the consequences of global change on the structure and function of stream communities may be exacerbated in arid zones such as the Mediterranean region. The Mediterranean zone is a highly populated area in which urban and agricultural practices result in an exceptional deterioration of soil and water quality due to the unique climate, topography, and soil characteristics (Zalidis et al. 2002, Gurluk 2009). Particularly relevant is the strong seasonal and annual variability of the discharge regime which exhibits long periods of low flow and drying (Sabater et al. 1995, Gasith and Resh 1999), causing a decrease in streams' dilution capacity of anthropogenic nutrient inputs from agricultural activities and from WWTP effluents (Marti et al. 2004, Merseburger et al. 2005). Moreover, climate change predictions foresee that dilution capacity of Mediterranean streams will be continually decreasing due to a ~25% precipitation reduction and a ~4^{0}C increase during summers by the end of the present century (Giorgi and Lionello 2008).

Besides the low dilution capacity of Mediterranean streams, human alterations of riparian vegetation in Mediterranean streams and rivers may also contribute to aggravate the consequences of global change on the composition and functioning of PUCs. Riparian characteristics of the Mediterranean region have important consequences on the degree of

heterotrophy versus autotrophy of stream communities. Riparian vegetation in Mediterranean streams has a more prolonged period of litter fall than in deciduous-forest streams, which can last from early summer until winter (Maamri et al. 1994, Gasith and Resh 1999, Acuña et al. 2007). Longer periods of allochthonous inputs into Mediterranean streams would lead to a dominance of heterotrophic PUCs during most of the year. However, previous studies have also indicated that greater light availability in Mediterranean streams due to sparser vegetation combined with low flows and high temperatures during summer may enhance primary production by allowing algal development in epilithic biofilms (Whitton 1970, Sabater and Sabater 1992, Guasch and Sabater 1995). Large temporal variation in the density of riparian canopy in Mediterranean streams can lead to a large variation on the composition of PUCs over seasons. However, this tight and complex linkage between riparian vegetation and PUCs in Mediterranean streams can be rapidly altered by riparian deforestation due to habitat degradation and alteration of the photosynthetically active radiation levels (Sabater et al. 2000, Kelly et al. 2003, Sweeney et al. 2004).

Overall, Mediterranean streams are particularly threatened by the changes in climate and land-use conditions due to their distinctive low dilution capacity. However, given the large seasonal variation of riparian canopy levels, the effects of human-derived nutrient inputs on the structure and function of PUCs may change drastically over time at both regional and local spatial scales.

1.4 Using nitrogen stable isotopes as tracers of nitrogen cycling in primary uptake compartments

The large variety of PUCs in stream ecosystems demands for ecological methods that can trace nutrient cycling through organisms as different as bacteria and plants. Stable isotopes can help solving biogeochemical fluxes among abiotic and biotic compartments of ecosystems since isotopic signatures normally contain sufficient information to trace both source-sink relations and processes. In the words of one of the first reviews on stable isotopes in ecosystems written by Peterson and Fry (1987):

"*Stable isotope tracers are already present and circulating in natural systems and their natural distribution reflects an integrated history of physical and metabolic processes within ecosystems. The major advantage of the stable isotope approach lies in field studies where measurements of existing isotopic distributions show how components of ecosystems are connected. Where clear-cutting, fertilization, burning, or other kinds of ecosystem-level perturbations alter flows of elements, stable isotopes can be used to follow these changes and show which processes or components are most sensitive to perturbation*".

Over these last three decades, stable isotopes have gained an important role in the toolbox of ecologists as evidenced by the rapid progress of ecological studies using stable isotopes analysis (Figure 1.2A). Given their potential capacity to trace element cycling across a broad range of spatial and temporal scales (Gannes et al. 1997, Dawson et al. 2002), most of ecosystem studies using stable isotopes have focused on biologically important elements such as carbon (C) and N (Figure 1.2A). In particular, the relative amounts of the two N stable isotopes (^{14}N and ^{15}N) vary substantially in most ecosystems owing to the isotope mixing of different N pools and the discrimination against the heavier

N isotope (^{15}N) that occur during N cycling through organisms and their surrounding environments (Fry 2008). Ecologists have long harnessed this variation in ^{15}N signatures (i.e., N isotopic ratios) of both organic and inorganic compounds because ^{15}N signatures can provide information about the history and origin of these compounds. For this reason, a great number of studies have used ^{15}N signatures to make inferences about the rates and patterns of N cycling at local (Angradi 1993, Hoffman and Bronk 2006) and global (Hogberg et al. 1996, Houlton and Bai 2009) scales. However, the use of ^{15}N natural abundances to disentangle ecological (Robinson 2001) and physiological (Evans 2001) N processes in ecosystems comes with the challenge to correctly interpret their spatial variation at different scales and the temporal span integrated by these ^{15}N signatures.

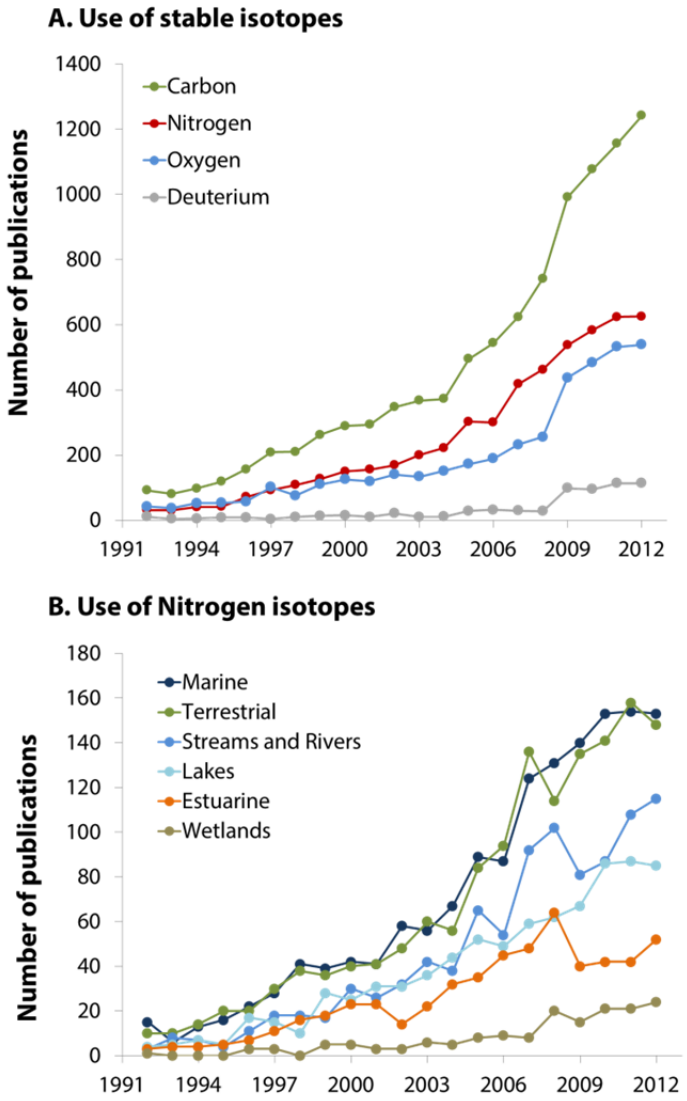


Figure 1.2 Temporal trends on the number of publications using stable isotopes in Thompson Reuters' ISI Web of Knowledge[SM] database from 1992 to 2012. **A)** Publications using stable isotopes of four of the most important elements for the biota. The keywords used as topics were: stable SAME isotop* AND carbon, nitrogen, oxygen, deuterium. **B)** Publications using stable isotopes of nitrogen in the main different ecosystems. The keywords used as topics were: (stable SAME isotop* AND nitrogen OR N15) AND marine, terrestrial OR forest, stream OR river, lake, esturaion OR transition, wetlands.

The increase in the number of studies using N stable isotopes has been particularly remarkable for freshwater ecosystems, especially for running waters (Figure 1.2B). In part, this rapid increase is due to the use of a ^{15}N tracer approach based on the addition of N inorganic compounds, mostly ammonium and nitrate, with high ^{15}N-enrichment levels (Peterson et al. 1997, Mulholland et al. 2000b). These ^{15}N tracer additions have certainly allowed investigating biological rates of uptake, storage, and release in low-order streams at the reach scale. On the contrary, ^{15}N natural abundances of inorganic substances and organism have been essentially used to identify anthropogenic N inputs into different freshwater bodies (Kendall 1998) and to determine the trophic levels of aquatic food webs (Boecklen et al. 2011). Different anthropogenic N sources often have distinct ^{15}N signatures (Kendall 1998), which allows identifying their relative contribution to dissolved inorganic N forms in freshwaters (Mayer et al. 2002, Lefebvre et al. 2007) or to PUCs (Toda et al. 2001, Syvaranta et al. 2008, Kohzu et al. 2009, Pastor et al. 2013). However, ^{15}N signatures of PUCs are known to be highly variable over space and time (Fry 1991, Cabana and Rasmussen 1996). Large variation in ^{15}N signatures of PUCs may be associated to their rapid N turnover and the constantly changing nutrient sources, concentrations, and isotopic signatures in the water column. Because of this variation, several studies have argued that the ^{15}N signatures of primary consumers are better long-term indicators of nutrient status since they integrate the higher temporal variability in ^{15}N signatures of primary uptake compartments (Anderson and Cabana 2006, Diebel and Vander Zanden 2009). Similarly, trophic studies using N isotopes to establish the different trophic levels have principally used primary consumers as isotopic baseline of aquatic food webs to avoid misleading effects derived from the short-term variability in

the ^{15}N signatures of PUCs (Vander Zanden and Rasmussen 2001, Post 2002); however, ^{15}N variation in PUCs may be still relevant since it can confound interpretation of ^{15}N signatures when measuring trophic position of organisms. Consequently, ^{15}N natural abundances of PUCs in stream ecosystems can be categorized as a black-box of isotopic variation because both source-tracing and trophic studies have normally neglected them.

In summary, although variation in the ^{15}N signatures of organisms at the base of fluvial food webs has been recognized, the underlying processes and mechanisms influencing this short-term variability in ^{15}N signatures of PUCs have not been fully examined yet. A better comprehension of the ^{15}N transfer between Dissolved Inorganic Nitrogen (DIN) and PUCs at different spatial and temporal scales may not only help understanding the factors controlling large variation in the ^{15}N natural abundances of PUCs, but also contribute to further understand the N cycling at the base of fluvial food webs. Therefore, the unexplored variation in the ^{15}N signatures of PUCs constituted an initial premise of this thesis, while contending that a proper examination of how different intrinsic and environmental factors influence ^{15}N signatures of DIN and PUCs will contribute to better understand the role of PUCs on stream nutrient retention.

1.5 Objectives and structure of the present thesis

The main goal of this thesis is to investigate how ^{15}N natural abundances can help understanding the role of different primary uptake compartments on stream nutrient retention. To achieve this objective, patterns of variability in the ^{15}N signatures of DIN and PUCs across different spatial and temporal scales, and the ^{15}N transfer between them, have been explored (Figure 2.3).

This thesis is divided in four different studies, written as independent publications, and each one focusses on different spatial scales:

- **Chapter 3.** This initial chapter is a revision of the state of the art on the variation in ^{15}N signatures of DIN and PUCs in ecological studies from the last two decades, and explores intrinsic and environmental factors that might influence such variability at the global scale.

- **Chapter 4.** This chapter examines the DIN transfer from the water column to different riparian macrophyte species to better understand the relevance of stream water N as a source for aquatic macrophytes.

- **Chapter 5.** The aim of this chapter was to investigate intrinsic (structural variation among autotrophs) and extrinsic (variation in nutrients and light availability) controls on the variation in autotrophic N cycling in stream ecosystems.

- **Chapter 6.** The aim of this chapter was to investigate the spatial variability of microbial N uptake at microhabitat scale, the biophysical factors controlling it, and how this variability contributes to explain N cycling at the whole-reach scale.

2

Study design

2. Description of the study design

The study design of this thesis involved the examination of ^{15}N variation at three different spatial scales: the watershed/global scale, the reach scale, and the habitat scale (Figure 2.3). At each level of organization, a common goal of examining how intrinsic (i.e., among PUCs) and extrinsic (i.e., environmental) factors influence ^{15}N signatures of DIN and PUCs is implied, but quite different tools and experimental approaches have been used depending on each scale.

For watershed-scale patterns, a meta-analysis based on a literature review from 1989 to 2009 was performed to examine the global variability in ^{15}N natural abundance of DIN and PUCs in streams and rivers in relation to the environmental factors (i.e., climate, land use, seasonality) that may drive this variability at the large scale **(Chapter 3)**. At the reach scale, attention was focused on the ^{15}N transfer between DIN forms (ammonium and nitrate) and different PUCs. First, to examine the relevance of ammonium and nitrate forms in stream water as N sources for macrophyte riparian species, a field survey was done to explore the variability and relationships between ^{15}N natural abundance of DIN forms and of four different macrophyte species in five streams influenced by inputs from WWTPs and over time within one of these streams has been performed (**Chapter 4**). Secondly, an *in-situ* translocation experiment addressing ^{15}N incorporation into five autotrophic components (biofilm, filamentous algae, bryophytes, submerged and emergent macrophytes) differing in metabolic character and structural complexity was conducted to investigate how rates of N uptake and turnover vary among different autotrophic PUCs under different conditions of nutrient and light availability **(Chapter 5)**. Finally, a ^{15}N-labelled ammonium (^{15}N-NH_4) addition was performed at two different reaches from the same stream, with and

without full riparian canopy, to investigate the mechanisms driving N cycling of autotrophic (epilithon) and heterotrophic (FBOM) microbial assemblages at the habitat scale, and how these mechanisms can drive patterns in stream N uptake at larger spatial scales **(Chapter 6)**.

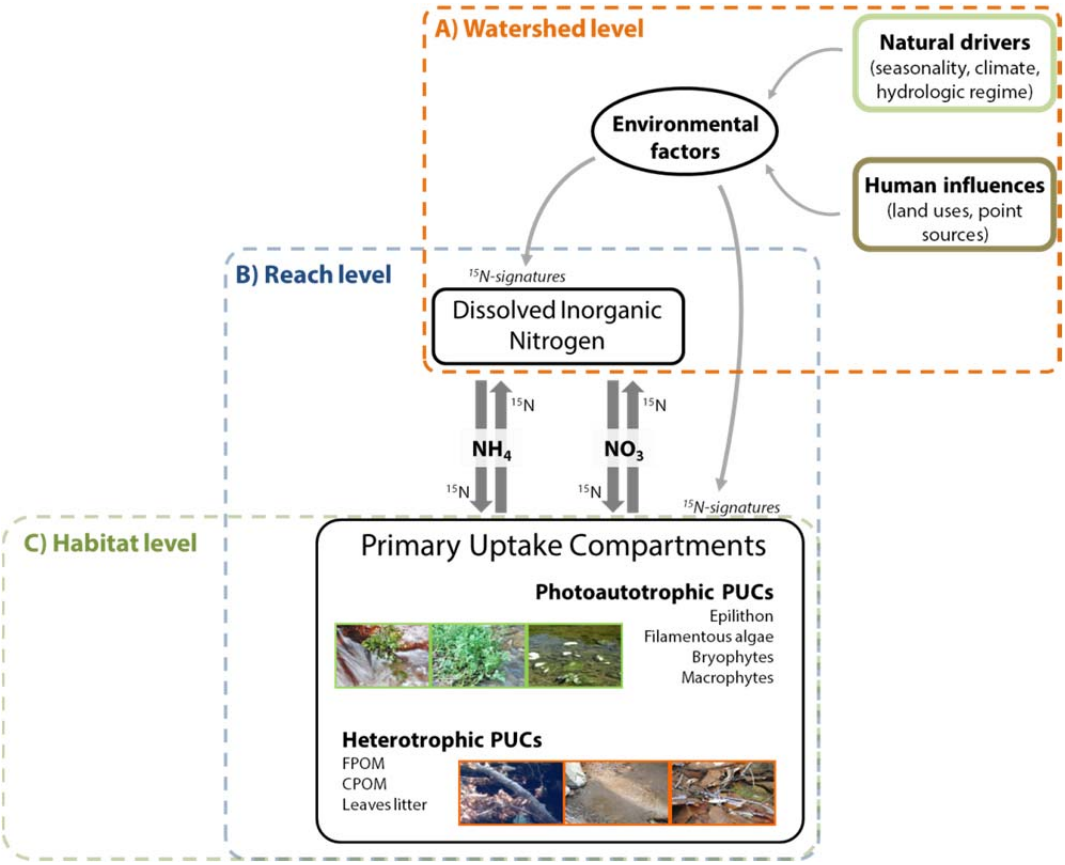


Figure 2.3 General scheme of this present thesis representing the three different scales at which the ^{15}N natural abundances of Dissolved Inorganic Nitrogen (DIN) and Primary Uptake Uptake (PUCs) have been examined. First, at the watershed level **(A)** by using a meta-analysis approach; secondly, at the reach level **(B)** by exploring the ^{15}N transfer from DIN to PUCs across space and over time; and finally at the habitat level **(C)** by using a ^{15}N-labelled N addition.

3

Variability in $\delta^{15}N$ natural abundance of basal resources in fluvial ecosystems: a meta-analysis

3.1 Abstract*

Variation in N stable isotope ($\delta^{15}N$) signatures of basal resources can influence interpretation of trophic relationships in ecosystems, and significant variation in $\delta^{15}N$ signatures has been reported in streams and rivers. However, a comprehensive understanding of the main factors driving $\delta^{15}N$ variability is lacking and confounds the consumer's trophic-level position during $\delta^{15}N$ analysis. We conducted a meta-analysis to examine the variability in $\delta^{15}N$ natural abundance of basal resources and dissolved inorganic N (DIN) in streams and rivers in relation to the environmental factors that may drive this variability. The meta-analysis was based on a literature review over the last 20 y (1989–2009) and contained signatures of $\delta^{15}N$-DIN ($\delta^{15}N$-NO_3 and $\delta^{15}N$-NH_4) and $\delta^{15}N$-basal resources ($\delta^{15}N$-detrital compartments, $\delta^{15}N$-biofilm, $\delta^{15}N$-algae, and $\delta^{15}N$-macrophytes) from >100 rivers or streams. Signatures of $\delta^{15}N$-DIN varied widely (–8.4 – 19.4‰), but we found fewer values for $\delta^{15}N$-NH_4 than $\delta^{15}N$-NO_3, even though NH_4^+ is assimilated rapidly by basal resources. Range of $\delta^{15}N$-basal resources was also broad (–4 – 16‰) within and among compartments. Human land use was the most significant factor explaining variability in $\delta^{15}N$-DIN and $\delta^{15}N$-basal resource signatures. We found significant differences between $\delta^{15}N$ signatures of photoautotrophic (i.e., autochthonous) and detrital (i.e., allochthonous) basal resources. Our results point out the difficulty in defining a baseline $\delta^{15}N$ signature of the food web, and provide a basis to explain confounding results in studies using $\delta^{15}N$ analysis to identify trophic linkages in fluvial food webs.

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*Original publication in Peipoch, M., E. Martí and E. Gacia. 2012. Variability in ^{15}N natural abundance of basal resources in fluvial ecosystems: a meta-analysis. *Freshwater Science*. 31(3): 1003-1015.

3.2 Introduction

Over the past 20 y, C and N stable isotopes have been used extensively to identify trophic relationships in different ecosystems (Michener and Schell 1994, Bluthgen et al. 2003, Premke et al. 2010). The number of stable isotope studies describing stream foodweb linkages has increased exponentially in the last 2 decades. In particular, stable isotopes of C ($\delta^{13}C$) are a powerful tool with which to identify organic-matter flows from basal resources to consumers in stream food webs and to evaluate the role of autochthonous and allochthonous food resources supporting secondary production (Rounick et al. 1982, Hamilton et al. 2004). Stable isotopes of N ($\delta^{15}N$) have been used to establish the trophic position of organisms in food webs as a consequence of the trophic-level enrichment in $\delta^{15}N$ (Minagawa and Wada 1984, Fry 1991). However, unlike $\delta^{13}C$, use of $\delta^{15}N$ in trophic ecology has generated more controversy because of high variability in $\delta^{15}N$ signatures at different trophic levels. Compared to $\delta^{13}C$ signatures, much less is known about the factors influencing such variability although it can confound interpretation of $\delta^{15}N$ signatures when measuring trophic position of organisms (Vander Zanden and Rasmussen 1999, O'Reilly et al. 2002).

Variation in $\delta^{15}N$ of consumers is strongly influenced by variation in $\delta^{15}N$ at the lower levels of the food web; i.e., by variation in $\delta^{15}N$ of basal resources rather than by variable fractionation along the food chain (Kline et al. 1990, Kling et al. 1992, Cabana and Rasmussen 1994). Many investigators using $\delta^{15}N$ signatures to identify foodweb linkages in freshwater ecosystems have pointed out that an appropriate measure of consumer's trophic position can be interpreted only relative to variation in $\delta^{15}N$ of basal resources among and within systems (Cabana and Rasmussen 1996).

The variability in $\delta^{15}N$ of basal resources has been related to isotopic fractionation during uptake processes (Macko et al. 1987, Handley and Raven 1992, Gannes et al. 1997) or to physiological differences among organisms that can lead to differences in N uptake and turnover rates (Cabana and Rasmussen 1996). Moreover, variability in $\delta^{15}N$ of basal resources also can be influenced by variation in the $\delta^{15}N$ of N sources (i.e., dissolved inorganic N [DIN]), which has been associated with human density (Cabana and Rasmussen 1996, Nishikawa et al. 2009), climatic region (Fry 1991), or seasonality (Toda and Wada 1990, Yoshioka et al. 1994). However, this information is related only to discrete studies and particular organisms and is particularly scarce when examining both N sources and basal resources of fluvial ecosystems. Hence, we acknowledge a lack of a comprehensive description and quantification of the variability in the $\delta^{15}N$ signatures of organisms (basal resources) and N sources (DIN) in fluvial ecosystems, even though this knowledge could help provide a better interpretation of the trophic structure of stream food webs.

The objective of our study was to achieve a comprehensive understanding of the variability in $\delta^{15}N$ natural abundance of basal resources and DIN in fluvial ecosystems by exploring the existence of general patterns and examining the intrinsic and environmental factors that might influence such variability. Ultimately, our goal was to provide a basis for a better interpretation of $\delta^{15}N$ signatures in studies describing foodweb linkages of fluvial ecosystems. Our study is based on an extensive review of existing ecological studies in streams and rivers conducted over the last 2 decades that used the natural abundance of N stable isotopes and a meta-analysis of $\delta^{15}N$ data compiled from these studies.

3.3 Methods

3.3.1 Literature search and development of the database

We searched the Thomson Reuters Web of Knowledge database (http://thomsonreuters.com/) for publications in the period from 1989 to 2009 containing the key words *stable isotope*, *stream* and *river*, *nitrate* and *ammonium* (as DIN forms; Table 3.1), and *macrophytes*, *filamentous algae, epilithic biofilm*, *fine particulate organic matter (FPOM)*, *coarse particulate organic matter (CPOM)*, and *leaf litter* (as basal resource compartments; Table 3.1). Combinations of these key words resulted in a set of >850 publications over the 20-y period. Of these publications, 54 contained values of ^{15}N natural abundance for basal resources or DIN (Appendix 3.1). We used this set of publications to generate a database for the meta-analysis of ^{15}N variability of basal resources and DIN. We used $\delta^{15}N$ signature, which is the isotope ratio (^{15}N:^{14}N) of a sample relative to the ratio of a standard (i.e., atmospheric N_2) expressed in parts per mil (‰). This ratio is the most widely used expression for the ^{15}N content of sources and organisms. In instances where data were provided only graphically, we obtained $\delta^{15}N$ signatures by digitization of the graph with GIMP 2.6 free software (Boston, USA). When publications included data from several streams, we introduced data from each stream as a different case in the database. When publications included $\delta^{15}N$ data for the same river or stream from different sampling dates, we introduced these data as different cases. The $\delta^{15}N$ database contained a total of 147 cases, corresponding to 104 different rivers or streams, with 262 signatures of $\delta^{15}N$ natural abundance for different DIN forms and basal-resource compartments.

To examine factors that might contribute to the variability in $\delta^{15}N$ of basal resources ($\delta^{15}N$-basal resources) and DIN ($\delta^{15}N$-DIN), each data case was

complemented with data for several explanatory variables, when available (Table 3.1). We chose these variables as surrogates of different environmental factors that potentially influence $\delta^{15}N$ variability and of intrinsic characteristics of the compartments examined (e.g., form of DIN, compartment type, and C:N ratios of basal resources). Data introduced for each explanatory variable were mostly categorical, except for catchment area, stream discharge, and C:N ratios, which were numerical (Table 3.1).

Table 3.1 Categorical and continuous explanatory variables included in $\delta^{15}N$ database and used in 2 factorial analyses of variances to examine the factors influencing $\delta^{15}N$ variability in both dissolved inorganic N (DIN) and basal resources. The table shows the levels considered for the categorical variables and units for the continuous variables. Climate region was divided in 4 categories following Köppen climate classification (Peel et al. 2007). Urban and agricultural land uses were determined when >25% of the catchment was occupied by those practices, whereas cover under this percentage was considered forested. E. = epilithic, f. = filamentous, CPOM=coarse particulate organic matter, FPOM=fine particulate organic matter.

Variables type	Name	Categories or units
Categorical	Climate region	Equatorial, arid, warm temperate, and continental
	Land uses	Forested, urban, and agricultural
	Sampling season	Winter, spring, summer, and autum
	Stream order	1-7
	Basal resources	E. Biofilm, f. algae, macrophytes, FPOM, CPOM, and leaf litter
	DIN form	NO_3, NH_4
Continuous	Catchment area	km^2
	Discharge	m^3/s
	C:N stoichiometry	Unit less

3.3.1 Analysis of the $\delta^{15}N$ database

We characterized the variability of $\delta^{15}N$ for the different forms of DIN and basal resource compartments with several statistical descriptors (range, mean, median, standard error of the mean [SEM], and variance). We assessed the relative importance of each explanatory variable on the variability of $\delta^{15}N$ with separate analyses of variance (ANOVAs) for basal resources and DIN data. We homogenized the database among streams and rivers by calculating mean $\delta^{15}N$ signatures for those streams having replicated data from a single sampling date. We analyzed only those explanatory variables for which data were available for >95% of the cases (climate region, land use, season, type of basal resources compartment, and DIN form). The variables included in the analysis were all categorical, so we applied a factorial ANOVA to examine which categorical variable accounted for the largest proportion of the variance in $\delta^{15}N$ values. Assumptions of normal distribution and homogeneity of variances of the $\delta^{15}N$ data set were assessed with Kolmogorov–Smirnov and Levene tests, respectively. The model was not balanced (number of cases was not equal among categories), so we used Scheffe's test to make pairwise comparisons among categorical levels within each explanatory variable. We considered results significant when $p < 0.05$. We assessed the relative contribution of each explanatory variable to the total amount of variance explained by the full factorial ANOVA model (all explanatory variables considered) by extracting each variable from the model and comparing the variance obtained to that from the full model. The explanatory variables that were not included in the factorial ANOVA analysis were mostly numerical (i.e., discharge, catchment area, and C:N ratio) except for stream order, which was categorical. Therefore, we assessed their influence on $\delta^{15}N$ variability with univariate linear regression

analysis. We ran statistical analyses in R (version 2.14.0; R Foundation for Statistical Computing, Vienna, Austria).

3.4 Results

3.4.1 Variability in $\delta^{15}N$ for stream basal resources and DIN

Available data for ^{15}N natural abundance of DIN and basal resources are mostly from studies conducted in low-order streams (Figure 3.1A), in warm temperate or continental biogeoclimatic regions (Figure 3.1B), and during summer or spring (Figure 3.1C). Most of the existing $\delta^{15}N$ data are from studies conducted in forested (i.e., relatively pristine) catchments of North America (Figure 3.1D & E).

The range of $\delta^{15}N$ signatures was broad for basal resources (–4 – 16‰) and DIN (–8.4 – 19.4‰), but mean $\delta^{15}N$-DIN was higher for NO_3^- and NH_4^+ than $\delta^{15}N$-basal resources for all compartments (Figure 3.2). $\delta^{15}N$-DIN mostly corresponded to $\delta^{15}N$-NO_3 signatures. $\delta^{15}N$-NH_4^+ data were scarce, but encompassed a broader range. Among basal resources, $\delta^{15}N$ signatures of detrital compartments (leaf litter, CPOM, FPOM) were more variable and had lower means than $\delta^{15}N$ signatures of compartments dominated by photoautotrophic organisms (macrophytes, epilithic biofilm, filamentous algae) (Figure 3.2). Among detrital compartments, $\delta^{15}N$-leaf litter had the narrowest range (–3.9–4.7‰), the lowest variance, and the lowest mean (Figure 3.2), whereas $\delta^{15}N$-FPOM and $\delta^{15}N$-CPOM had similar distributions. $\delta^{15}N$ signatures of compartments dominated by photoautotrophic organisms had similar variances and means (Figure 3.2).

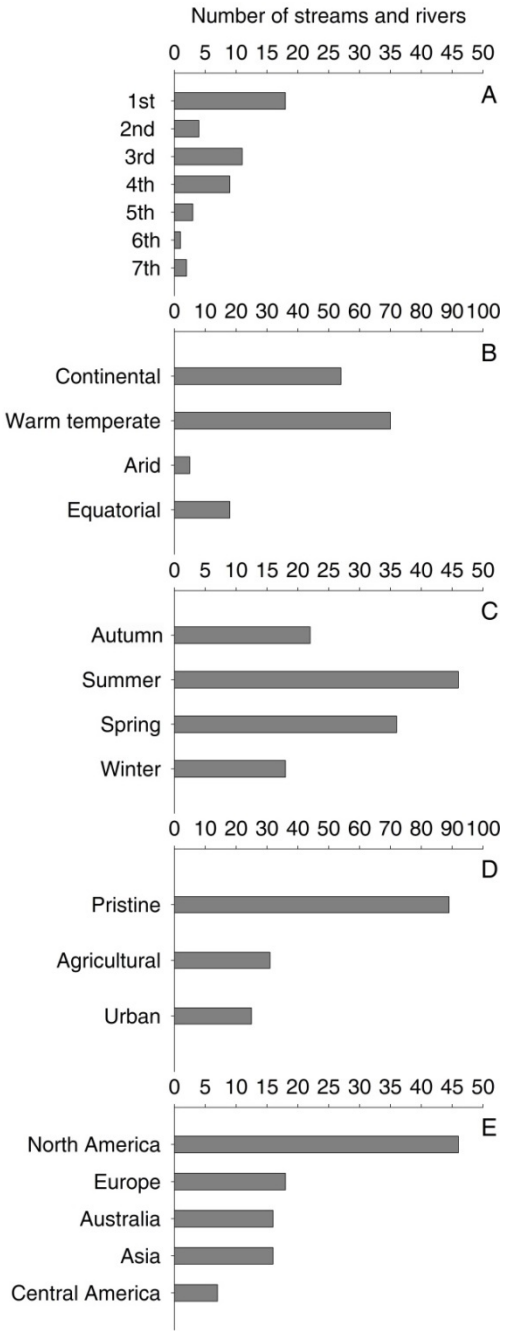


Figure 3.1 Distribution of rivers and streams included in the database by stream order (available for only 48 streams) (A), climate region (B), sampling season (C), catchment land use (D), and continent (F).

3.4.2 Factors influencing $\delta^{15}N$ variability

Variation in δ^{15}N-DIN was not significantly related to catchment area (r^2 = 0.01, n = 59, p = 0.46) or discharge (r^2 = 0.01, n = 46, p = 0.46). Variation in δ^{15}N-basal resources was not related to catchment area (r^2 = 0.01, n = 255, p = 0.09), but was negatively related to discharge, although this factor accounted for a very low proportion of the δ^{15}N variance (r^2 = 0.04, n = 123, p = 0.02). δ^{15}N signatures of basal resources were negatively related to their C:N ratios (r^2 = 0.27, n = 113, $p < 0.001$). In particular, detrital compartments had significantly higher C:N ratios (16.17 ± 2.01‰ [SEM]) and lower δ^{15}N signatures (2.52 ± 0.42‰) than photoautotrophic compartments (8.56 ± 0.7 and 4.63 ± 0.33‰, respectively).

Table 3.2 Results from 2 factorial analyses of variance used to test the relative contribution of the explanatory variables to the variability of δ^{15}N-DIN and δ^{15}N-basal resources. Only variables with significant effect in the analysis are indicated and % total variance explained by each of these variables or by the interaction between them is shown. * = $p < 0.5$, ** = $p < 0.1$, *** = $p < 0.01$.

Explanatory variables	df	Sum of squares	*p*-value	Variability explained
Dissolved inorganic N				
Land use	2	708.9	< 0.001 ***	54.7%
Land use x season	6	225.5	< 0.001 ***	17.4%
Basal resources				
Land use	2	724.3	< 0.001 ***	21.3%
Basal resource ompartment	5	264.9	< 0.001 ***	14.8%
Season	4	157.2	< 0.001 ***	11.0%
Season x compartment	19	267.1	0.015*	9.3%
Climate	3	139.4	< 0.001 ***	3.7%

Land use and its interaction with season accounted for ~72% of the total variance in δ^{15}N-DIN (Table 3.2). δ^{15}N-DIN signatures from rivers or streams draining urban (9.94 ± 0.99‰) and agricultural (7.6 ± 0.47‰) catchments were, on average, 6.6× higher than those from rivers or streams draining forested catchments (2.2 ± 0.60‰). δ^{15}N-DIN did not differ between streams draining urban and streams draining agricultural catchments (Table 3.3).

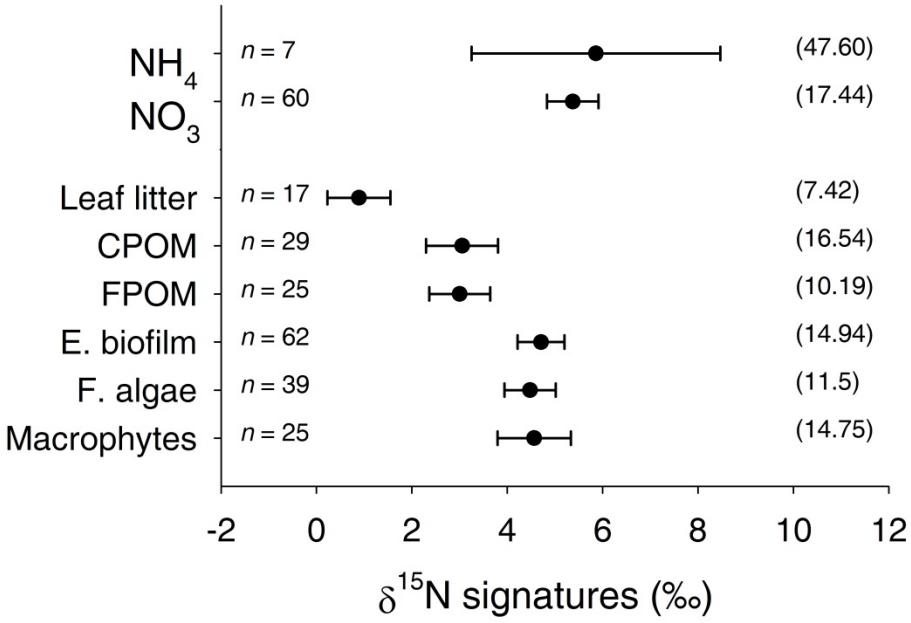


Figure 3.2 Mean (±1 SEM) δ^{15}N signatures of dissolved inorganic N (DIN) and basal resources from streams or rivers compiled from the literature. The available number of δ^{15}N values for each category level is indicated in the left margin of the *y*-axis. Numbers on the right side of the plot are variances. E. = epilithic, f. = filamentous, CPOM = coarse particulate organic matter, CPOM = coarse particulate organic matter.

Land use, compartment type of basal resources, season, climatic region, and the season × compartment type of basal resources together explained ~58% of the total variance in $\delta^{15}N$-basal resources (Table 3.2). Land use accounted for the highest proportion of the total variance in $\delta^{15}N$-basal resources (Table 3.2). $\delta^{15}N$-basal resources signatures from rivers or streams draining urban (6.9 ± 0.57‰) and agricultural (6.5 ± 0.63‰) catchments were, on average, >2× higher than those from rivers or streams draining forested catchments (2.4 ± 0.26‰). This pattern was consistent when considering all $\delta^{15}N$-basal resources together and $\delta^{15}N$ for each basal resource individually (Table 3.3, Figure 3.3). Differences in $\delta^{15}N$ signatures between human-dominated and forested streams were largest for CPOM ($p > 0.05$), smallest for FPOM ($p > 0.05$) and leaf litter ($p < 0.05$), and intermediate for epilithic biofilm, filamentous algae and macrophytes (Table 3.3, Figure 3.3). The variable accounting for the second largest proportion of $\delta^{15}N$-basal resources variance was the type of compartment (Table 3.2). Mean $\delta^{15}N$ of compartments dominated by photoautotrophic organisms was 1.4 to 3.8× higher than that of detrital compartments. Season accounted for 11% of the variance in $\delta^{15}N$-basal resources, and signatures were, on average, substantially higher in autumn (5.6 ± 0.72‰) than in other seasons (summer: 3.7 ± 0.37‰, winter: 2.4 ± 0.77‰, spring: 2.3 ± 0.65‰). $\delta^{15}N$-basal resources from streams in arid regions (7.2 ± 1.36‰) were, on average higher, than those from other climatic regions (equatorial: 4.78 ± 0.51‰, warm temperate: 3.93 ± 0.41‰, continental: 3.1 ± 0.42‰) regardless of the basal-resource compartment considered.

3.5 Discussion

3.5.1 Variability of $\delta^{15}N$-basal resources and $\delta^{15}N$-DIN in fluvial ecosystems

Our literature search showed that authors of a relatively large number of studies included data on natural abundance of N stable isotopes in fluvial ecosystem over the last 2 decades, but only a small fraction of them (54 of 850) reported $\delta^{15}N$ signatures for basal resources or DIN forms. This bias is explained by the fact that most investigators use N stable isotopes to examine food webs, and in these studies, $\delta^{15}N$ signatures for basal resources and DIN are often neglected or are used only to establish the baseline $\delta^{15}N$ value of the system (Minagawa and Wada 1984, Cabana and Rasmussen 1996). We also found that the available data on $\delta^{15}N$-basal resources and $\delta^{15}N$-DIN are not proportionally representative of the broad diversity of rivers and stream ecosystems. Instead entries from small, relatively pristine streams in temperate regions of North America are most abundant. Likewise, basal-resource compartments resources were not proportionally represented in the $\delta^{15}N$ database. $\delta^{15}N$ data for photoautotrophic compartments, especially biofilm, were more abundant than data for detrital compartments. Similarly, $\delta^{15}N$ data for DIN forms were relatively scarce and biased toward $\delta^{15}N$-NO_3 data, whereas $\delta^{15}N$-NH_4 data were very rare. This disparity can be particularly problematic when assessing the coupling between DIN and basal resources because NH_4^+ is the preferred form of DIN for basal resources in streams (Peterson et al. 2001). Temporal variation of stream $\delta^{15}N$ signatures is not well documented in the literature, and most of the $\delta^{15}N$ data are from studies conducted during warm seasons (spring and summer) across climatic regions. Authors of studies of natural abundances of $\delta^{15}N$ signatures generally assume isotopic equilibrium between consumers and their diets. However, our results indicate that seasonal variation of $\delta^{15}N$

signatures for DIN and basal resources may be large enough to produce long-term variation in the isotopic equilibrium between consumers and basal resources. Other investigators also have reported variation in $\delta^{15}N$ at smaller temporal scales. For instance, O'Reilly et al. (2002) showed that abrupt changes in $\delta^{15}N$-DIN signatures produced changes in ^{15}N signatures of basal resources within a few days in a tropical lake. This result suggests that basal resources can be indicators of the short-term variation in $\delta^{15}N$-DIN signatures because of direct DIN uptake and rapid N turnover rates. In contrast, $\delta^{15}N$ signatures of higher trophic levels may integrate this short-term variation of basal resources over longer temporal scales (Cabana and Rasmussen 1996) and may be less variable over time. Changes in $\delta^{15}N$ of DIN and basal resources at different temporal scales may have important implications for studies in which N stable isotopes are used to address the dynamics of trophic interactions and to model stream food webs. Overall, the gaps observed in the existing data highlight the need of future research to broaden our knowledge on the variability of $\delta^{15}N$ signatures to improve interpretation of $\delta^{15}N$ signatures in stream food webs.

Table 3.3 Mean (±1 SEM) N isotopic signatures ($\delta^{15}N$ in ‰) of stream basal resources and dissolved inorganic N collected in the meta-analysis database separated by land use category. Within a row, means with the same letter are not significantly different. * = insufficient data.

Category	Forested	Urban	Agricultural
NO_3^-	5.32 ± 0.77^A	10.38 ± 1.03^B	7.17 ± 0.36^B
NH_4^+	-1.62 ± 6.81^A	7.89 ± 3.01^A	9.73 ± 0.09^A
Macrophytes	2.98 ± 0.6^A	7.96 ± 0.78^B	6.68 ± 0.58^B
F. algae	2.5 ± 0.87^A	6.92 ± 0.77^B	6.58 ± 0.55^B
E. biofilm	3.14 ± 0.39^A	8.11 ± 0.7^B	7.7 ± 2.18^B
FPOM	2.49 ± 0.67^A	4.9 ± 0.35^A	3.83 ± 2.49^A
CPOM	1.39 ± 0.78^A	7.21*	8.99 ± 3.87^A
Leaf litter	1.43 ± 0.49^A	3.36 ± 0.68^B	2.16 ± 0.77^{AB}

Variability in $\delta^{15}N$ signatures was high for both DIN and basal resources in streams and rivers, and most of this variability was related to differences in catchment land use. Streams draining catchments with high agricultural or urban activity had substantially higher $\delta^{15}N$-DIN and $\delta^{15}N$-basal resources than streams draining forested catchments. This pattern was consistent regardless of the compartment of basal resources considered and confirms that high variation of $\delta^{15}N$ signatures among streams is linked to anthropogenic influences. The ^{15}N-enriching effect of DIN sources in human-altered systems seems to be transferred to the different basal resource compartments. Cabana and Rasmussen (1996) showed that $\delta^{15}N$ signatures of DIN and primary consumers in lakes increased with human population density in their catchment. Other studies in fluvial ecosystems had supported this hypothesis, but the variation in $\delta^{15}N$ signatures of basal resources was not considered explicitly in any of the previous studies. Diebel and Vander Zanden (2009) showed that variation in $\delta^{15}N$ signatures of primary consumers was explained

by inputs of inorganic fertilizers, and many investigators have used $\delta^{15}N$-NO_3 signatures as tracers of N sources from different human land uses within the catchment (Chang et al. 2002, Lefebvre et al. 2007). Our results showed that human activity clearly influenced $\delta^{15}N$ signatures of both DIN and basal resources, but neither $\delta^{15}N$-DIN nor $\delta^{15}N$-basal resources signatures clearly distinguished between agricultural *vs*. urban activity in the catchment. This result might be explained by overlapping ranges of $\delta^{15}N$ signatures of DIN sources contributed by different human activities and by the effect of isotopic fractionation associated with N-cycling transformations on $\delta^{15}N$ signatures.

Overall, the high variability observed in $\delta^{15}N$-DIN signatures (essentially represented only for NO_3^-; see above) could be explained in part by the influence of different DIN sources within the catchment, as illustrated by the variability in $\delta^{15}N$-DIN signatures in the Mississippi River (Panno et al. 2006). The range of $\delta^{15}N$-DIN signatures compiled in our study (–8.4–19.4‰) is broad and covers most of the $\delta^{15}N$ signatures of potential DIN sources to streams reported in the literature. For instance, $\delta^{15}N$ signatures from wet deposition (–15–15‰; Kendall et al. (2007)) or soil N (–10–15‰; Broadbent et al. (1980)) are contained relatively well within the range of variation we found for $\delta^{15}N$-DIN. Agricultural N sources that enter streams via diffuse pathways also are within the observed range. $\delta^{15}N$ of fertilizers of biological origin (i.e., guano or animal waste) range from 10 to 20‰ (Kreitler 1979), and $\delta^{15}N$ of synthetic fertilizers (i.e., N industrially fixed from atmospheric N_2) range from –4 to 4‰. In contrast, $\delta^{15}N$-DIN from point sources, such as waste-water treatment plant effluents, tend to exceed the upper limit of the $\delta^{15}N$-DIN range in our study with $\delta^{15}N$-NO_3 signatures up to 30‰ (Toda et al. 2001) and $\delta^{15}N$-NH_4 signatures as high as 28‰ (*unpublished data*). The absence of records for these extreme $\delta^{15}N$-DIN

signatures in streams may be explained by dilution of $\delta^{15}N$-DIN signatures when effluents enter receiving streams.

The variability in $\delta^{15}N$ signatures also has been linked to nutrient concentration because it influences the degree of isotopic fractionation of different N processes (e.g., assimilation, nitrification, or denitrification) (Paerl and Fogel 1994, Cabana and Rasmussen 1996). A 70% increase in N inputs into fluvial ecosystems since the 19th century (Seitzinger and Kroeze 1998) has caused greater availability of bioreactive N, and potentially, intensification of N cycling in terrestrial and fluvial ecosystems. This intensification could lead to higher streamwater $\delta^{15}N$ signatures via greater isotopic fractionation during N-cycling processes (Hogberg 1998, Diebel and Vander Zanden 2009). Studies showing the influence of riparian soil N biogeochemical processes, especially denitrification, on streamwater $\delta^{15}N$ (Houlton et al. 2006) seem to support this last hypothesis.

3.5.2 Foodweb implications of variation in $\delta^{15}N$-basal resources

Basal resources sustain the entire food web. Thus, increases in $\delta^{15}N$ of basal resources in streams affected by human activity may also produce variation in $\delta^{15}N$ signatures of consumers above that caused by stepwise trophic-level enrichment in $\delta^{15}N$ signatures (Kling et al. 1992). Other factors that act within ecosystems also generate variability in $\delta^{15}N$ signatures of basal resources. For instance, Angradi (1994) showed intra-system variation of $\delta^{15}N$ signatures associated with different basal resource compartments, and seasonality affected on $\delta^{15}N$ signatures in 2 eutrophic lakes (Toda and Wada 1990, Yoshioka et al. 1994). We observed variation among compartments and among seasons in our $\delta^{15}N$-basal resources data.

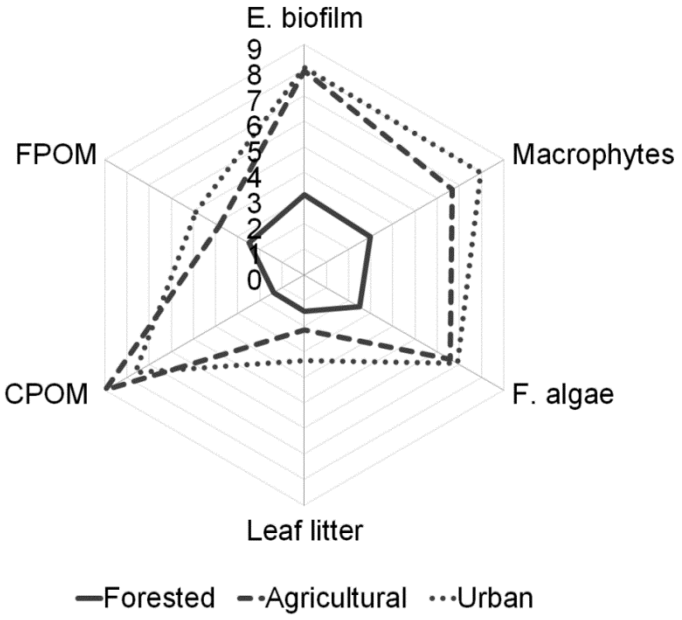


Figure 3.3 Representation of the $\delta^{15}N$ signatures for each basal-resource category from streams and rivers draining the 3 landuse types considered. The lines join the mean of $\delta^{15}N$ for different basal-resource compartments within each landuse category.

$\delta^{13}C$ signatures also can differ significantly between photoautotrophic (autochthonous) and detrital (allochthonous) compartments (Rounick et al. 1982). Autochthonous organic C has lower $\delta^{13}C$ signatures than allochthonous organic C as a result of the $\delta^{13}C$-depleted HCO_3^- source used by photoautotrophic compartments (Rau 1980). In contrast, we found higher $\delta^{15}N$ signatures in photoautotrophic than in detrital compartments. However, unlike $\delta^{13}C$ signatures, mechanisms generating difference in $\delta^{15}N$ between photoautotrophic and detrital compartments are more difficult to ascertain. In principle, organic N in photoautotrophic compartments is derived from on

stream DIN, whereas organic N in detrital compartments is derived from allochthonous and autochthonous material colonized by stream biofilms. This duality poses a major problem when interpreting $\delta^{15}N$ signatures of detrital compartments because samples typically consist of bulk material including detrital organic matter and the microbial communities colonizing it in unknown proportions (Hamilton and Lewis 1992, Hamilton et al. 2004). Thus, description of the mechanisms generating differences in $\delta^{15}N$ among basal resources in a particular stream may be much more complex than a distinction between allochthonous and autochthonous material as described for C stable isotopes.

C:N of terrestrial material that falls into streams normally is 2 to 3× higher than that of aquatic-derived material (Elser et al. 2000, Kendall et al. 2001, Grimm et al. 2003). The result is higher C:N ratios in detrital than in photoautotrophic compartments, as we observed in our study. Allochthonous organic matter entering the aquatic ecosystem rapidly leaches soluble N in its first stages of mineralization (Zeller et al. 2000), and then is colonized by microbial communities that can incorporate DIN from the water (Mulholland et al. 2000b, Gribsholt et al. 2007). Thus, leaf litter, a less mineralized fraction of the detrital organic matter, is expected to have a lower $\delta^{15}N$ signature than stream water. In more advanced stages of mineralization of the organic matter (coarse and fine fraction), the $\delta^{15}N$ signature should become closer to that of stream water because of DIN uptake by microbial communities colonizing this organic matter fraction (Caraco et al. 1998), as we observed in our compiled data. Unlike the photoautotrophic compartments, detrital organic matter ultimately may integrate internal and external sources of N in different proportions, which would result in considerable variation in $\delta^{15}N$ signatures

among detrital compartments (i.e., leaf litter, CPOM, FPOM). Whether differences in $\delta^{15}N$ signatures between photoautotrophic and detrital compartments are relevant for the transfer of $\delta^{15}N$ signatures to the rest of the stream food web will depend on the selective assimilation of N in biofilms by consumers (Hall and Meyer 1998, Tank et al. 2000) and the biochemical quality of detrital organic matter (Findlay and Tenore 1982) rather than the $\delta^{15}N$ signature of the bulk material. Therefore, an appropriate estimate of the importance of microbial biomass and detrital organic matter as a source of organic N for consumers seems to be relevant when addressing detritial pathways in stream food webs and could help explaining confounded results in terms of consumer's trophic level position. In general, our compiled data indicates that studies of stream food webs using N stable isotopes usually are based on samples of bulk material of detrital compartments. Separate measures of microbial $\delta^{15}N$ and detrital organic matter $\delta^{15}N$ should be a major concern in future studies because the observed differences between $\delta^{15}N$ signatures of photoautotrophic and bulk material of detrital compartments are, in some cases, comparable to the common assumption of a 2 to 4‰ increase in $\delta^{15}N$ per trophic level (Minagawa and Wada 1984, Ehleringer et al. 1986, Peterson and Fry 1987), regardless of landuse type considered. Therefore, when differences in $\delta^{15}N$ among basal resources are within the range of trophic-level enrichment, consumer's trophic levels in the stream food web may be underestimated. This issue can be especially relevant in streams where food webs are mostly fuelled by detrital sources. In those cases, calculation of consumer's trophic levels with $\delta^{15}N$ signatures of bulk detritus material will be problematic if detritivores feed specifically on microbial N, which probably has a $\delta^{15}N$ signature closer to the streamwater DIN, and thus, more similar to

photoautotrophic compartments.

In summary, our study provides a broad range of $\delta^{15}N$ values for several basal-resource compartments in streams that can be used to bracket measurements of $\delta^{15}N$ values for consumers in forthcoming studies. In addition, results from the meta-analysis highlight the importance of land use in the catchment as a key driver of the variability in $\delta^{15}N$ signatures of both DIN and basal resources. Variation in $\delta^{15}N$ signatures among streams draining different landuse types seems to be much more pronounced than variation within a single stream, regardless of the compartment considered. Therefore, difficulties would arise primarily when using N stable isotopes to compare food webs across streams without considering values of basal resources because consumers in human-altered streams feed on more ^{15}N-enriched basal resources than their counterparts in forested streams. Our results also show consistent differences in $\delta^{15}N$ between photoautotrophic and detrital compartments that should be taken into account when interpreting foodweb results based on N stable isotopes. Our study has helped elucidate data gaps that could be addressed in future studies to increase our knowledge on the use of N stable isotopes in stream food webs. One of these gaps is the need for additional data on temporal variation of stream $\delta^{15}N$ signatures. Understanding how N isotopic signatures of stream basal resources integrate temporal variation of $\delta^{15}N$-DIN signatures and how this variation is transferred to the stream food web may constitute a major step in the use of N stable isotopes in foodweb studies.

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Appendixes

Appendix 3.1 References for all publications containing values of ^{15}N signatures used in this study. Country (state is also included in streams or rivers from the USA), name of the stream or river, and compartments sampled in each study are also indicated

References	Country	Stream or River	Compartments sampled
1. Angradi 1993	Idahoo (USA)	Henry's Fork	E. biofilm, F. algae, Macrophytes, FPOM and CPOM
2. Angradi *et al.,* 1994	Utah-Arizona (USA)	Colorado	F. algae, Macrophytes, FPOM, CPOM and Leaf litter
3. Aufdenkampe *et al.,* 2007	Peru	Vilcanota–Urubamba, Tambo, Pachitea, Ucayali–Amazonas and Marañón	CPOM
4. Barnes *et al.,* 2008	Connecticut (USA)	Conecticut Creek	Nitrate
5. Benson *et al.,* 2008	Maryland (USA)	Sassafras	Macrophytes
6. Bergfur *et al.,* 2009	Sweden	Rastälven, Sverkestaan, Älgängsan, Strömaran, Penningbyan, Husbyan, Hagaan and Sagan	E. biofilm, FPOM and CPOM
7. Burns and Walker 2000	Australia	Murray	E. biofilm, FPOM and CPOM
8. Buzek *et al.,* 2006	Czech Republic	Jizera	Nitrate
9. Chesman *et al.,* 2009	Australia	Murrumbidgee, Tumut, Goobarragandra and Goodradigbee	E. biofilm
10. Coat *et al.,* 2009	Guadeloupe	Grande-Anse	E. biofilm, F. algae and Leaf litter
11. De Brabandere *et al.,* 2007	Florida (USA)	Chassahowitzka and Homosassa	E. biofilm, Macrophytes and Nitrate
12. Dekar *et al.,* 2009	Arkansas (USA)	Mulberry and Little Mulberry	E. biofilm and CPOM
13. Deutsch *et al.,* 2009	Germany	Elbe	Nitrate and Ammonium
14. Evans-White *et al.,* 2001	Kansas (USA)	Kings Creek	E. biofilm, F. algae, Macrophytes, FPOM, CPOM and Leaf litte
15. Gido *et al.,* 2006	New Mexico (USA)	San Juan	F. algae and FPOM
16. Godwin *et al.,* 2009	Pennsylvania (USA)	Spring Creek	E. biofilm
17. Gustafson *et al.,* 2007	North Carolina (USA)	Neuse	FPOM, CPOM and Nitrate
18. Harrington *et al.,* 1998	Vermont (USA)	Bringo Brook, West Branch, Bethel-Gilead, Peavine, Third Branch and First Branch	F. algae and Nitrate
19. Huang *et al.,* 2007	Taiwan	Hapen Creek	E. biofilm, F. algae, Macrophytes, FPOM and CPOM
20. Jennerjahn *et al.,* 2008	Kerala	Kallada	FPOM and CPOM
21. Kao and Liu 2000	Taiwan	Langyang-Hsi	Macrophytes
22. Kaushal *et al.,* 2006	Colorado (USA)	Blue	F. algae
23. Kohzu *et al.,* 2008	Japan	Lake Biwa tributaries	E. biofilm
24. Lau *et al.,* 2009	China	Tai Po Kau Forest, Shing Mun and Pak Ngau Shek	E. biofilm, F. algae, FPOM and Leaf litter
25. Lefebvre *et al.,* 2007	France	Pleine-Fougières	Nitrate
26. Lofton *et al.,* 2007	North Carolina (USA)	North Buffalo Creek	Nitrate
27. Mantel *et al.,* 2004	China	Tai Po Kau Forest	E. biofilm, F. algae, FPOM and CPOM
28. March and Pringle 2003	Puerto Rico	Espíritu Santo	E. biofilm, F. algae and Leaf litter
29. Miyasaka and Genkai-Kato 2009	Japan	Kuro-kawa	E. biofilm
30. Mulholland *et al.,* 2000b	Tennesse (USA)	Walker Branch	E. biofilm, FPOM and Leaf litter

Appendix 3.1 Continued

References	Country	Stream or River	Compartments sampled
31. Panno *et al.,* 2006	Illinois (USA)	Mississippi	Nitrate
32. Peterson *et al.,* 1993	Alaska (USA)	Kuparuk	E. biofilm and F. algae
33. Piatek *et al.,* 2005	New York State (USA)	Archer Creek	Nitrate
34. Raikow and Hamilton 2001	Michigan (USA)	Eagle Creek	E. biofilm, FPOM and Leaf litter
35. Rasmussen adn Trudeau 2007	Canada	Ste Marguerite, Epinette and Big pool	E. biofilm
36. Reid *et al.,* 2008	Victoria	Pranjip and Joyce's Creek	E. biofilm and F. algae
37. Rezanka and Hershey 2003	Minnesota (USA)	Amity Creek	E. biofilm, F. algae and CPOM
38. Ribot *et al.,* 2012 *in revision*	Spain	La Tordera	E. biofilm, Macrophytes, Nitrate and Ammonium
39. Rock and Mayer 2006	Canada	Oldman	Nitrate
40. Saito *et al.,* 2008	California (USA)	Truckee	E. biofilm
41. Sharpe and Nichols 2007	Tennesse (USA)	Conasauaga	Nitrate
42. Steffy and Kilham 2004	Pennsylvania (USA)	Valley Creek	E. biofilm, F. algae and Leaf litter
43. Stögbauer *et al.,* 2008	Germany	Ruhr	Nitrate
44. Thorp *et al.,* 1998	Kentucky (USA)	Ohio	FPOM and CPOM
45. Toda *et al.,* 2001	Japan	Chikuma	E. biofilm
46. Townsend-Small *et al.,* 2005	China	Lujiang, XitiaOxi, Dongtiaoxi, Yili, Baidu and Liangxihe	Nitrate
47. Udy and Bunn 2001	Australia	Bamboo, Bromfield, Clemenson, Fishers, Tranters, Wrights, Beatrice R., Dirran, Kittabah, Goolagan, Opposum and Djallan	F. algae and Macrophytes
48. Ulseth and Hershfy 2005	North Carolina (USA)	North Buffalo Creek	F. algae, FPOM and Leaf litter
49. Von Schiller *et al.,* 2007	Spain	Santa Fe, Santa Coloma, Gualba and Sant Celoni	E. biofilm, Leaf litter, Nitrate and Ammonium
50. Von Schiller *et al.,* 2009	Spain	Santa Fe, Santa Coloma, Gualba and Sant Celoni	E. biofilm, Leaf litter, Nitrate and Ammonium
51. Wayland and Hobson 2000	Canada	Wapiti	F. algae
52. Williams and Trexlerl 2006	Florida (USA)	Shark and Taylor Slough	E. biofilm, F. algae and Macrophytes
53. Zah *et al.,* 2001	Switzerland	Roseg	E. biofilm
54. Ziegler and Brisco 2004	Arkansas (USA)	Moore Creek and Huey Hollow	F. algae, CPOM and Leaf litter

4

Contrasts among macrophyte riparian species in their use of stream water nitrate and ammonium: insights from ^{15}N natural abundance

4.1 Abstract*

We examined the relevance of dissolved inorganic nitrogen (DIN) forms (nitrate and ammonium) in stream water as N sources for different macrophyte species. To do this, we investigated the variability and relationships between ^{15}N natural abundance of DIN forms and of four different macrophyte species in five different streams influenced by inputs from wastewater treatment plants (WWTP) and over time within one of these streams. Results showed that ^{15}N signatures were similar in species of submersed and amphibious macrophytes and in stream water DIN, whereas ^{15}N signatures of the riparian species were not. ^{15}N signatures of macrophytes were generally closer to ^{15}N signatures of nitrate, regardless of the species considered. Our results showed significant relationships between ^{15}N signatures of DIN and those of submersed *Callitriche stagnalis* and amphibious *Veronica beccabunga* and *Apium nodiflorum*, suggesting stream water DIN as a relevant N source for these two functional groups. Moreover, results from a mixing model suggested that stream water DIN taken up by the submersed and amphibious species was mostly in the form of nitrate. Together, these results suggest different contribution to in-stream N uptake among the spatially-segregated species of macrophytes. While submersed and amphibious species can contribute to in-stream N uptake by assimilation of DIN, macrophyte species located at stream channel edges do not seem to rely on stream water DIN as an N source. Ultimately, these results add a functional dimension to the current use of macrophytes for the restoration of stream channel morphology, indicating that they can also contribute to reduce excess DIN in streams.

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4.2 Introduction

Aquatic plants are an important element of river landscapes from contrasting climatic zones such as temperate (Larned et al. 2006), tropical (Sand-Jensen and Mebus 1996), subtropical (De Brabandere et al. 2007) or Pampean regions (Giorgi et al. 2005, Acuna et al. 2011), but their functional role in fluvial ecosystems has often been neglected. Aquatic plants (generally referred to as macrophytes) are irregularly distributed in stream channels depending on channel morphology, hydrologic regime (e.g., the frequency of flood events; see Riis and Biggs 2003) , water velocity and light availability. In headwater streams, macrophyte development is usually restricted to stream-riparian margins because of water velocity, while lowland streams allow the development of macrophytes within the stream channel because of gentle slopes, slow flowing environments, and a lower degree of riparian canopy.

Although abiotic factors in streams dictate the presence and distribution of macrophytes, they can also modify their surrounding habitat by promoting fine sediment and organic matter accumulation (Clarke 2002, Schulz et al. 2003, Larned et al. 2006), by increasing drag and reducing flow velocity and turbulence (Sand-Jensen and Pedersen 1999). The capacity of macrophytes to create and maintain different stream habitats has been commonly investigated and frequently used in stream restoration programs to stabilize stream beds and margins, and to provide physical habitat for invertebrates and fish (Harrison et al. 2004, Larned et al. 2006, Suren 2009). In addition, macrophytes also have an important demand for nutrients and thus may contribute to in-stream nutrient uptake, as has been reported in other freshwater systems, such as wetlands (Riis and Biggs 2003, Bowden et al. 2007), lakes (Gacia et al. 1994, Marion and Paillisson 2003) or pond fringes (Biggs et al. 2005). Despite this, the

functional role of macrophytes as filters of dissolved nutrients has seldom been considered as a relevant part of stream ecosystem functioning or management, probably because studies of in-stream nutrient uptake have focused mainly on forested headwater streams, where macrophytes are not abundant (but *see* Pelton et al. 1998; Feijoo et al. 2011; Riis et al. 2012) .

Species of macrophytes are distributed within the stream channel across a gradient of water exposure from the main channel to the flood plain according to structural traits related to how they tolerate water flow. These traits allow the differentiation of functional groups of aquatic plants based on their interaction with the stream water environment, including species totally submersed and with floating leaves, amphibious species, and riparian species located in the stream-riparian edge with roots in the reed zone (Bowden et al. 2007). These macrophyte species from different functional groups may thus have different interactions with stream water dissolved inorganic nitrogen (DIN) as their potential source of N (Figure 4.1).

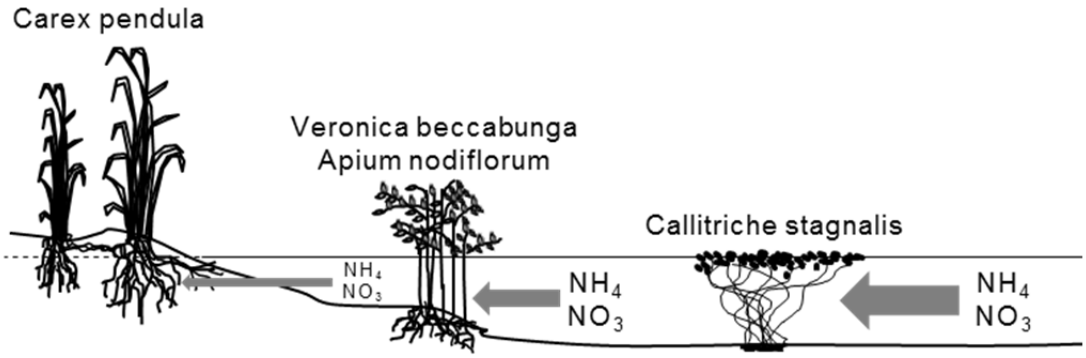


Figure 4.1 Representation of the distribution of different functional groups of macrophytes across a section of the stream channel. There is a gradient of exposure to stream water from the totally exposed *Callitriche stagnalis* to the intermittently exposed *Carex pendula* depending on the stream water level. The thickness of the arrows indicate the expected dependence of each macrophyte species on dissolved inorganic nitrogen forms (NH_4 and NO_3) from the stream water.

Natural abundance of N stable isotopes (^{15}N signatures, expressed as $\delta^{15}N$ in units of ‰) in macrophytes has been used to trace human-derived N inputs into aquatic ecosystems (Costanzo et al. 2001, Benson et al. 2008). Anthropogenic DIN sources are generally characterized by high ^{15}N signatures, and thus elevated ^{15}N signatures in macrophytes can indicate uptake of DIN sources derived from human activities (Cole et al. 2005, Udy et al. 2006, Peipoch et al. 2012). However, factors such as intra-plant isotopic discrimination (Evans et al. 1996), plant age (Evans 2001), or plant stress (Handley et al. 1999) can sometimes strongly influence the variation in plant ^{15}N signatures compared to their N sources. For instance, isotopic discrimination during uptake and assimilation of both ammonium (NH_4) and nitrate (NO_3) can be either small (Evans et al. 1996) or large (Mariotti et al. 1982, Yoneyama et al. 2001) depending on the plant species, although large discrimination effects have

been mostly observed at high concentrations (i.e., 3-100 mg $N\text{-}NH_4$ L^{-1}, ~150 mg $N\text{-}NO_3$ L^{-1}) that are rarely found in natural or even slightly polluted streams (De Brabandere et al. 2007, Cohen and Bradham 2010). While recognizing the influence of these factors on uptake-associated and intra-plant discrimination, plant ^{15}N may still integrate the ^{15}N signatures of its N sources (Evans 2001) since ^{15}N signatures of macrophytes are strongly influenced by ^{15}N-DIN signatures (Kohzu et al. 2008, Pastor et al. 2013).

The aim of this study was to gain knowledge on DIN transfer from stream water to different macrophyte species to better understand the relevance of stream water N as a source for macrophytes, and thus their potential role in in-stream N uptake. To achieve this goal, we examined the spatial and temporal variability in ^{15}N natural abundance of four species of macrophytes representative of different functional groups: submersed macrophytes (*Callitriche stagnalis)*, amphibious macrophytes (*Veronica beccabunga* and *Apium nodiflorum),* and riparian macrophytes located at the stream-riparian edge (*Carex pendula),* and then explored how it relates to the variability in the ^{15}N natural abundance of stream water DIN forms (i.e., NH_4 and NO_3). Finally, we used mixing models with ^{15}N signatures of the two DIN forms (NH_4 and NO_3) to assess the most relevant DIN source for those species showing significant correlations with $\delta^{15}N$-DIN values.

We conducted this study in 5 streams receiving effluents from wastewater treatment plants (WWTP) in order to enlarge the range of concentrations of DIN forms (*see* Marti et al. 2004) as well as the range of ^{15}N signatures for both NH_4 and NO_3. Previous studies indicated that in WWTP-receiving streams DIN can exhibit remarkable longitudinal gradients of ^{15}N signatures across relatively short stream distances (i.e., <1 km) due to intense cycling of NH_4 and NO_3 (Toda

et al. 2001, Ribot et al. 2012). In addition, effluents from WWTP may also increase the abundance of aquatic macrophytes in receiving streams (Carr and Chambers 1998, Gucker et al. 2006) by increasing nutrient availability. Therefore, the expected large variation in both DIN concentration and δ^{15}N-DIN values at a reach scale in these streams provided suitable conditions to examine whether or not the ^{15}N of different macrophyte species responded to these changes in the water column, which would suggest they are actively incorporating nitrogen from stream water DIN. We hypothesized that the relevance of DIN in the stream water as an N source for macrophytes would vary among functional groups due to differences in plant-stream interactions and in preferential uptake of NH_4 or NO_3 by the different species. We expected that δ^{15}N of macrophytes would be positively correlated with δ^{15}N-DIN if this was a relevant N source, and that these correlations would gain strength among macrophytes as their degree of interaction with stream water increased (i.e., from riparian to submersed species). Ultimately, these results may have implications for stream management and restoration programs because macrophytes are currently used in restoration of stream channel morphology for their physical properties in substrate stabilization.

4.3 Methods

4.3.1 Study sites

The study was conducted in La Tordera catchment (NE Barcelona, Catalunya, Spain), which has an area of 868.5 km^2 and is dominated by siliceous bedrock. This catchment is subjected to a large variety of land use practices, although urban activities are predominant in the catchment (von Schiller et al. 2008a, Caille et al. 2012). The seasonal hydrologic regime of the streams in this basin

typically shows high and permanent flow from late autumn to early spring and low or intermittent flow during summer. This temporal hydrologic variability results in a large variation in the relative contributions of WWTP effluents to stream water DIN within this catchment (Merseburger et al. 2005), and also in ^{15}N-DIN signatures of the receiving stream (Merbt et al. 2011, Ribot et al. 2012). Our study was based on a correlative approach in which we examined both spatial and temporal variation in the relationships between ^{15}N signatures of NH_4, NO_3, and macrophytes.

To assess spatial variation, we selected five stream reaches (ca. 1 km) within La Tordera catchment that were located downstream of inputs from urban WWTPs effluents. These streams were similar in terms of channel geomorphology, light availability and hydrologic regime. However, they differed in their dilution capacity of the WWTP input (i.e., stream discharge relative to WWTP outflow), in their nutrient loads, and in the contribution of WWTP inputs to in-stream NH_4 and NO_3 concentrations (Table 4.1). The study reaches were sampled once in autumn (October, 6 to 26) 2009. To avoid high discharge typical from spring and early summer in this Mediterranean climate, we waited for medium flow and mild temperatures to guaranty active leaf growth, the presence of *Callitriche stagnallis* and a significant but not excessive ratio of WWTP input to stream flow. These conditions were met in October 2009.

To assess temporal variation, we sampled seasonally one of the reaches (Sta. Maria de Palautordera) on 4 different dates from August 2010 to May 2011. We also included additional ^{15}N data for DIN and macrophytes from a previous monitoring (January 2008 and September 2008). Therefore, we had a data set consisting of longitudinal variation (see details below) within a reach for 5

streams on a single sampling date and for 1 stream on 6 different sampling dates.

4.3.2 Field sampling

On each sampling occasion, eight to 10 equidistant sampling stations were selected along a 670-850 m reach located immediately downstream of the WWTP inputs to maximize the gradients in DIN concentration and δ^{15}DIN signal. At each station, we measured longitudinal variability in physical and chemical variables and in δ^{15}N of NH_4, NO_3 and macrophyte species. We measured conductivity, water temperature, and dissolved oxygen concentration using WTW (Weilheim, Germany) 340i portable probes. Discharge was estimated using NaCl slug additions at the bottom end of each study reach and on each sampling date in SMP (Gordon et al. 2004). Surface water samples were collected at each sampling station from the stream thalweg for the analysis of nutrient concentrations (3 replicates per station, 45 mL each) and δ^{15}N of NH_4 and NO_3 (1 L sample per station). All water samples were immediately filtered in the field through pre-ashed Albet (Barcelona, Spain) FVF glass fiber filters (0.7 µm pore size) into pre-washed plastic containers, stored on ice, and brought to the laboratory. Samples for the analysis of ^{15}N-NH_4 were immediately processed (see *Laboratory analyses* section), while filtered samples for nutrient concentrations and ^{15}N-NO_3 analyses were stored at −22 ºC until further processing.

At each reach station and sampling date, the four macrophyte species were sampled when present. The presence and abundance of the each of the selected species at a particular site varied in response to the large inter-annual variability in rainfall and temperature that is characteristic of Mediterranean climates. Phenologically, all species were active at the time of sampling either

because they are perennial (*Carex pendula*) or because they are annual but may grow until late fall under mild Mediterranean weather (*Callitriche stagnalis*). The two hemicryptophytes, *Veronica beccabunga* and *Apium nodiflorum* have biennial rhizomes and leave emergence is subjected to inter-annual variation. In the study area, *V. beccabunga* behaves more as an annual while *A. nodiflorum* shows perennial growth. In addition to phenology, *C. stagnalis* has fine roots and is thus susceptible of being washed away after torrential rains frequent under Mediterranean climate. Indeed, the interaction between emergence phenology and the occurrence of flooding events have been described as an important driver of spatial and temporal variation in plant composition of floodplain environments (Blom and Voesenek 1996). *Carex pendula* is a flood-resistant species that grows along stream banks. Typically, it exhibits waterlogged roots in the reed zone and perennating rhizomes beneath the soil surface. We only sampled individuals that were close to the stream channel, which were more likely to take up water and DIN from the stream. For each species and at each reach station, we collected leaves from 3 to 6 randomly selected plants to generate a composite sample. Samples were rinsed with water, immediately stored on ice in the field and brought to the laboratory for further processing.

4.3.3 Laboratory analyses

Stream water samples were analyzed for $N\text{-}NH_4$ and $N\text{-}NO_3+NO_2$ concentrations following standard colorimetric methods (APHA 1995) on a Bran+Luebbe AA3 Autoanalyser (Nordersted, Germany) at the Institute of Marine Sciences (ICM-CSIC, Barcelona, Spain). The ^{15}N content of stream water NH_4 and NO_3 was determined following procedures adapted from Holmes et al. (1998) and Sigman et al. (1997), respectively. For the analysis of $^{15}N\text{-}NH_4$

signatures, samples were amended with 3 g L^{-1} of MgO and 50 g L^{-1} of NaCl and a Teflon filter packet. The Teflon filter packets consisted of a pre-combusted Whatman GF/D glass fiber filter (1 cm diameter), which was previously acidified by adding 25 µl of K_2HPO_4 to trap the diffusing NH_3, and sealed within a packet formed by two pieces of Teflon tape. Immediately after adding the packets, the samples were tightly capped and incubated at 40 °C in a shaker table over three weeks. For ^{15}N-NO_3 analysis, NH_4 was initially removed by boiling the samples with 3.0 g of MgO and 5.0 g of NaCl. The resulting samples (0.1 L) were then transferred into acid-washed HDPE plastic bottles that were amended with 0.5 g MgO, 0.5 g Devarda's alloy and a Teflon filter packet. Immediately after adding the Teflon filter packets, samples were tightly capped and incubated at 60°C for 48 h to reduce NO_3 to NH_4, and then incubated in a shaker table for seven days at room temperature. At the end of the incubation, filter packets from ^{15}N-NH_4 and ^{15}N-NO_3 samples were removed from the bottles, placed in scintillation vials, and dried in a desiccator until further processing. Further details on these methods can be found in Von Schiller et al. (2009).

For determination of ^{15}N content of macrophytes, all samples were dried at 60 °C, ground with a MM 200 mixer mill (Retsch, Germany), and then subsamples (3 – 4 mg) were weighed on a Mettler-Toledo (Greifensee, Switzerland) MX5 microbalance. All samples (NH_4, NO_3 and macrophytes) for ^{15}N analysis were encapsulated in tins and analyzed at the UC Davis Stable Isotope Facility (California, USA). The stable isotope signatures of N for both the water samples and macrophytes were determined using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). All ^{15}N signatures are expressed in

terms of delta values (δ), which are parts per thousand (‰) differences in the $^{15}N/^{14}N$ ratio between the sample and a standard (atmospheric N_2).

4.3.4 Statistical analysis

We characterized the variability of $\delta^{15}N$ values over space (i.e., among streams) and time (i.e., within SMP stream) for the DIN forms and the different macrophyte species by examining the mean, the range (i.e., minimum and maximum values), and the coefficient of variation (calculated from atom ^{15}N % values instead of $\delta^{15}N$ to avoid negative values, following procedures described in Fry (2008)). To describe how $\delta^{15}N$ values of NH_4, NO_3, and macrophyte species varied across streams, and among sampling dates within a stream, we used univariate permutational analysis of variance (using 999 permutations; hereafter referred to as pANOVA) using PRIMER v6 (Clarke and Gorley 2006) with the PERMANOVA+ add-on (Anderson 2001). We examined spatial and temporal variability separately; thus we conducted one pANOVA with macrophyte and DIN species (5 levels) and stream (5 levels) as fixed-effects factors and another pANOVA with macrophyte and DIN species (6 levels) and sampling date (6 levels) as fixed-effects factors. We performed pANOVA tests on reach-averaged $\delta^{15}N$ values of each macrophyte and DIN species at each stream or sampling date to handle the unbalanced data set. We also examined how spatial and temporal variation of macrophytes $\delta^{15}N$ was related to $\delta^{15}N$-NH_4 and $\delta^{15}N$-NO_3 using Spearman rank correlations. The relationships were examined with data from each reach (either from different streams or sampling dates) separately, as well as with data from all streams or sampling dates together. Results were considered significant at $p < 0.05$. Finally, in those cases where $\delta^{15}N$ values of macrophytes were significantly correlated to both $\delta^{15}N$-NH_4 and $\delta^{15}N$-NO_3 values, we estimated the relative proportions of NH_4 and NO_3

taken up by these macrophytes. This was done by fitting five mixing models differing in the isotopic discrimination factors considered: assuming no discrimination (mixing model 1), a single discrimination factor for NH_4 and NO_3 (mixing model 2), separate discrimination factors for NH_4 and NO_3 (mixing model 3), separate discrimination factors for NH_4 and NO_3 depending linearly on nutrient concentration (mixing model 4), and separate discrimination factors for NH_4 and NO_3 depending on concentration with a Monod equation (mixing model 5). A detailed description of these models can be found in Pastor et al. (2013). Models were fitted by maximum likelihood estimation using the 'bblme' R package (Bolker 2012) and compared using the Akaike information criterion corrected for small sample size (AICc). The goodness-of-fit of each model was examined by comparing observed and predicted values of $\delta^{15}N$ for macrophytes using simple linear regressions. All statistical tests were done using R 2.14.0 (R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/) except for the pANOVA tests.

4.4 Results

4.4.1 Physical and chemical characteristics of the streams

Stream water temperature was similar among streams, while discharge and dissolved oxygen concentration showed larger variation (Table 4.1). DIN concentration varied among streams, ranging from 2.1 to 6.8 mg L^{-1} as well as the relative proportion of DIN as NH_4 and NO_3 (Table 4.1). Seasonal variation of stream water temperature in SMP followed the climatic pattern of this Mediterranean region with high values in summer and minimum in winter (Table 4.2). Stream discharge was low during summer and autumn dates and increased during the spring dates (Table 4.2). Dissolved oxygen concentration

was relatively high on all sampling dates except in summer 2008, while DIN concentration in the receiving stream varied widely among all dates (Table 4.2). Significant temporal shifts in the relative dominance of the two DIN forms were found at the different sampling dates (Table 4.2).

Table 4.1 Geographic, physical and chemical characteristics of the five study sites in Fall 2009. Values are mean ± SE of data from eight to 10 sampling stations along the 670-850 m reaches. Discharge corresponds to a single measure at the bottom of the stream reach.

Stream	GUA	BRE	SMP	COL	CEL
Latitude 41º N	43′43″	44′14″	41′7″	51′6″	41′31″
Longitude 2º E	30′34″	34′3″	27′33″	40′9″	30′23″
Discharge (L s^{-1})	4.6	5.2	23.4	37.6	69.4
Temperature (°C)	18.7 ± 0.1	18.7 ± 0.4	19.8 ± 0.1	18 ± 0.1	21 ± 0.2
O_2 (mg L^{-1})	7.2 ± 0.4	5.4 ± 0.1	8.2 ± 0.3	8.7 ± 0.1	6.4 ± 0.2
NH_4-N (mg N L^{-1})	1.3 ± 0.4	0.9 ± 0.2	0.2 ± 0.1	0.9 ± 0.04	1.5 ± 0.1
NO_3-N (mg N L^{-1})	1.1 ± 0.2	5.9 ± 0.6	2.2 ± 0.1	1.5 ± 0.05	0.6 ± 0.1
NH_4/NO_3	2.1 ± 0.9	0.2 ± 0.1	0.1 ± 0.03	0.6 ± 0.05	3 ± 0.5

4.4.2 Variability in ^{15}N signatures of DIN forms and macrophyte species

Natural abundance of $\delta^{15}N$ for the two DIN forms and for the macrophyte species showed high spatial variability within and among stream reaches (Table 4.3; Figure 4.2). Both stream (two-way pANOVA, Pseudo-$F_{(4,20)}$ = 7.58, P < 0.01) and species (two-way pANOVA, Pseudo-$F_{(4,20)}$ = 17.86, P < 0.01) had a significant effect on isotopic signatures. Values of $\delta^{15}N$ of DIN forms and macrophytes were significantly lower in GUA than in the other streams (Pair-wise test, P < 0.05; Table 4.3), while $\delta^{15}N$-NH_4 was significantly higher than $\delta^{15}N$ values of NO_3, *Apium nodiflorum*, and *Carex pendula* (Figure 4.2). Variability of $\delta^{15}N$-NH_4 values was consistently higher (i.e., higher coefficients of variation; CV) than that for $\delta^{15}N$-NO_3 (Table 4.3; Figure 4.2). $\delta^{15}N$ values of macrophytes

were generally bracketed between those of NH_4 and NO_3, except in BRE, where they were lower than $\delta^{15}N$ values of the two DIN forms (Table 4.3).

Table 4.2 Physical and chemical characteristics in SMP at the different sampling dates. Values are mean ± SE of data from eight to 10 sampling stations along the 670-850 m reach. Discharge corresponds to a single measure at the bottom of the stream reach.

Sampling date	Winter 2008	Summer 2008	Summer 2010	Autumn 2010	Winter 2011	Spring 2011
Discharge ($L\ s^{-1}$)	73.3	13.6	24	17.9	93	342.9
Temperature (°C)	10.4 ± 0.1	22.6 ± 0.6	22.8 ± 0.4	13.6 ± 0.2	9.3 ± 0.3	16.9 ± 0.5
O_2 ($mg\ L^{-1}$)	9.9 ± 0.1	4.9 ± 0.3	8.7 ± 0.1	10.2 ± 0.1	11.7 ± 0.1	10.4 ± 0.1
NH_4-N ($mg\ N\ L^{-1}$)	3.6 ± 0.1	0.4 ± 0.1	0.1 ± 0.02	2.3 ± 0.3	0.5 ± 0.02	0.2 ± 0.03
NO_3-N ($mg\ N\ L^{-1}$)	1.9 ± 0.1	2.4 ± 0.2	1.5 ± 0.1	2.1 ± 0.1	2.3 ± 0.03	1.5 ± 0.2
NH_4/NO_3	1.9 ± 0.1	0.4 ± 0.3	0.1 ± 0.01	1.2 ± 0.2	0.2 ± 0.01	0.2 ± 0.02

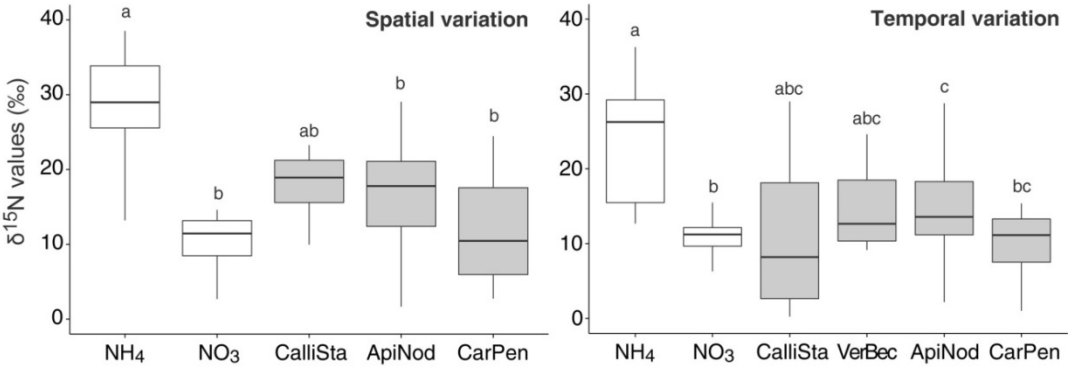


Figure 4.2 Box plot of δ15N values of NH4, NO3, Callitriche stagnalis (CalliSta), Veronica beccabunga (VerBec), Apium nodiflorum (ApiNod) and Carex pendula (CarPen) from the study streams in fall 2009 (spatial variation) and from all different sampling dates in SMP (temporal variation). Letters indicate significant differences from two-way pANOVA test ($p < 0.05$).

Temporal variation in $\delta^{15}N$ values of NH_4, NO_3 and macrophytes in SMP was high (Figure 4.2). The two-way pANOVA on data from the different sampling dates showed that both sampling date (Pseudo-$F_{(5,26)} = 7.31$, $P < 0.01$) and the macrophytes and DIN species (Pseudo-$F_{(5,26)} = 11.03$, $P < 0.01$) had a significant effect on $\delta^{15}N$ values. In winter 2008, $\delta^{15}N$ values of macrophytes were significantly lower and showed lower variation than in both summer dates (Pair-wise test, $P < 0.05$ for 2008; Pair-wise test, $P < 0.05$ for 2010) regardless of the species considered (Table 4.3). Similar to the results from spatial variability, $\delta^{15}N$-NH_4 values were significantly higher than those for NO_3 and macrophytes species, except for *Callitriche stagnalis* and *Veronica beccabunga* (Figure 4.2). Variation in $\delta^{15}N$-NH_4 values was higher and variation of $\delta^{15}N$-NO_3 values was lower than the ^{15}N variability of any species of macrophytes considered (Table 4.3; Figure 4.2). Values of $\delta^{15}N$ for macrophytes were generally bracketed between $\delta^{15}N$ values of the two DIN forms, but clearly displaced towards $\delta^{15}N$-NO_3 (Table 4.3; Figure 4.2). Overall, *Callitriche stagnalis* was the only species

showing values of $\delta^{15}N$ similar to those of NH_4 and NO_3 for both spatial and temporal variation (Figure 4.2).

Table 4.3 Mean (‰), minimum (Min) and maximum (Max) values, and coefficient of variation (CV) of $\delta^{15}N\text{-}NH_4$, $\delta^{15}N\text{-}NO_3$ and $\delta^{15}N$-macrophytes in each study stream during fall 2009 and at SMP on different sampling dates.

Compartment	**Study streams**															
	GUA			BRE			SMP			COL			CEL			
	Mean	Min-Max	CV	Mean	Min-Max	CV	Mean	Min-Max	CV	Mean	Min-Max	CV	Mean	Min-Max	CV	
Ammonium	18.3	−1.9 to 35	12.9	22.3	11.1 to 38.5	9.1	34.2	24.2 to 36.4	4.2	28.9	28.6 to 30	0.5	33.6	30.8 to 35.4	1.6	
Nitrate	3.6	−2.4 to 6.9	3.7	13.1	7.7 to 14.6	2.1	12.2	10.4 to 13.5	1.1	8.6	7.9 to 9.4	0.6	12.2	10.4 to 13.5	1.1	
Callitriche stagnalis							20.7	17.9 to 23.2	2.1				15.1	10 to 15.8	3.1	
Apium nodiflorum				9.0	1.7 to 29	8.2	18.2	6.4 to 24	5.4	21.5	17.7 to 24.2	2	18.4	14.1 to 22.4	2.6	
Carex pendula	6.6	3.6 to 8.3	1.8	4.7	2.8 to 6.2	1.1	12.7	10.2 to 20.7	4.4	16.5	7.1 to 21	5.1	19.2	11.5 to 24.5	4.2	

Compartment	**Sampling dates in SMP**																	
	Winter 2008			Summer 2008			Summer 2010			Autumn 2010			Winter 2011			Spring 2011		
	Mean	Min-Max	CV	Mean	Min-Max	CV	Mean	Min-Max	CV	Mean	Min-Max	CV	Mean	Min-Max	CV	Mean	Min-Max	CV
Ammonium	13.2	12.7 to 13.8	0.4	45.1	29.3 to 55.5	8.8	17.3	15.2 to 25.6	3.7	28.9	26.3 to 31.9	2.0	30.0	28.5 to 31.6	1.1	26.2	20.6 to 27.9	11.1
Nitrate	7.7	6.3 to 9.6	1.0	15.4	11.6 to 18.1	2.1	11.9	11.6 to 14.8	1.0	10.0	9.6 to 10.6	0.3	11.8	11.2 to 12.1	0.3	9.8	9.1 to 12.5	1.9
Callitriche stagnalis	2.1	−0.5 to 4.9	1.7	18.4	10.6 to 29	6.4										8.1	8.1 to 8.2	0.1
Veronica beccabunga	10.3	9.2 to 12.5	1.1	18.5	12.8 to 24.6	3.8												
Apium nodiflorum	8.0	6.3 to 14.8	3.1	22.6	14.7 to 28.7	4.1	15.6	8.7 to 25.1	5.2	12.0	2.2 to 13.3	3.7	16.2	12.3 to 20	3.0	13.3	9.4 to 16.9	3.3
Carex pendula							11.5	1 to 23.5	6.9	12.6	7.2 to 14	2.6	11.3	4.1 to 15.4	5.0	9.8	6.1 to 14.9	2.7

4.4.3 Relationships between ^{15}N signatures of DIN and macrophytes

Considering data within each stream reach separately, we did not find any significant correlation between $\delta^{15}N$ of the macrophyte species and $\delta^{15}N$ of the two DIN forms, with the exception of $\delta^{15}N$ for *Apium nodiflorum*, which was correlated to $\delta^{15}N$-NH_4 in BRE (Spearman correlation, r= 0.95, $P < 0.01$). When $\delta^{15}N$ data from all five study streams were pooled together to enlarge the ranges of both DIN concentration and isotopic signal, there was again a significant positive correlation between $\delta^{15}N$ for *Apium nodiflorum* and $\delta^{15}N$-NH_4 (Spearman correlation, $r = 0.59$, $P < 0.001$; Figure 4.3). No other significant correlations were found among reaches.

Considering data for each sampling date separately, we found positive significant correlations between $\delta^{15}N$-NO_3 and $\delta^{15}N$ of *Callitriche stagnalis (r*= 0.9, $P < 0.001$), *Veronica beccabunga (r*= 0.89, $P < 0.001$), and *Apium nodiflorum (r*= 0.72, $P < 0.001$) in the summer of 2008 when the range of $\delta^{15}N$ values within the reach was the broadest (Table 4.3). When $\delta^{15}N$ data from all sampling dates in SMP were pooled, the $\delta^{15}N$ of all the macrophyte species, except for *Carex pendula,* exhibited significant and positive correlations with one or both $\delta^{15}N$-NH_4 and $\delta^{15}N$-NO_3 values (Figure 4.4). No clear influences of either NH_4 or NO_3 concentrations on the correlation between DIN and macrophytes $\delta^{15}N$ values were observed (Figure 4.4). However, we found that low NH_4 concentrations (corresponding to summers 2008 and 2010) coincided with a high variation of $\delta^{15}N$-NH_4, whereas high NH_4 concentration (corresponding to winter 2008) coincided with low variation of $\delta^{15}N$-NH_4 (Figure 4.4).

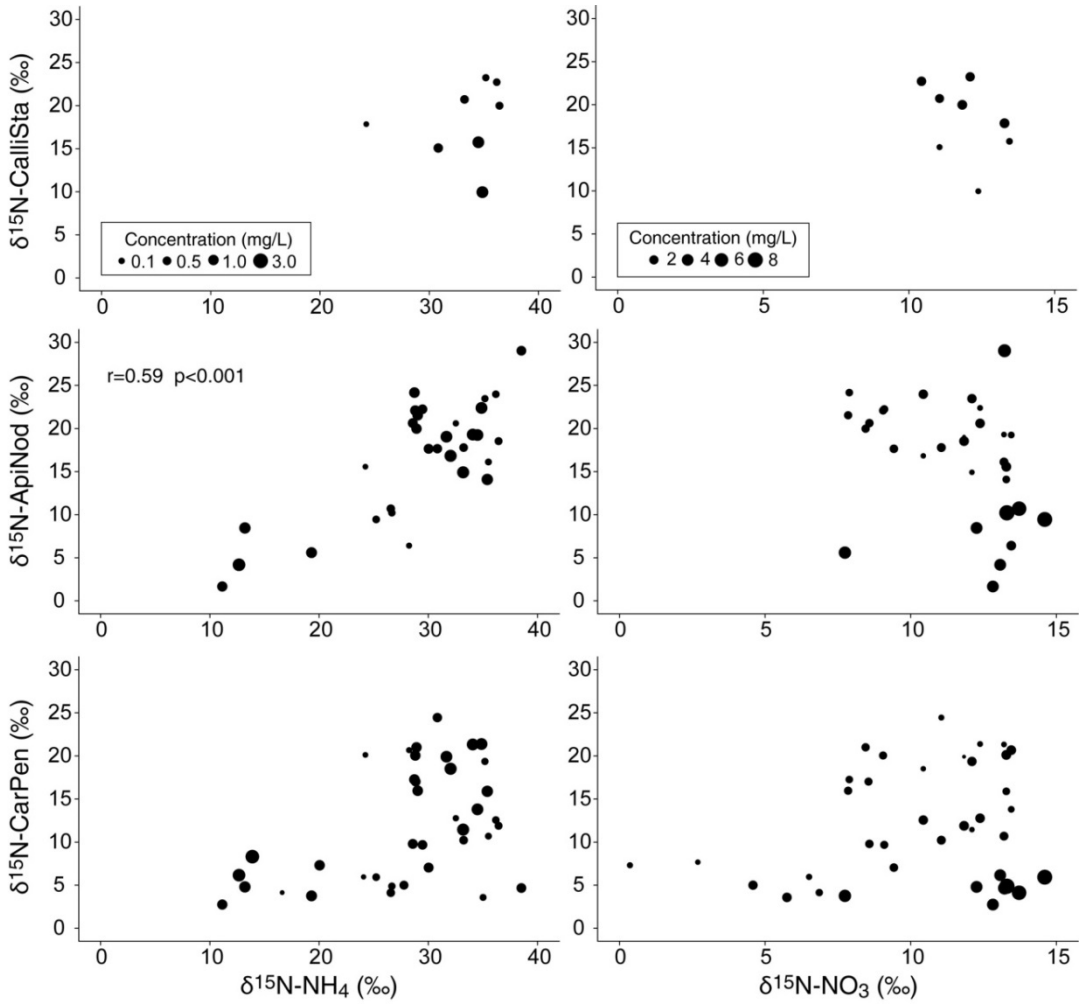


Figure 4.3 Relationships between $\delta^{15}N$ values of DIN (NH_4 and NO_3) and *Callitriche stagnalis* (CalliSta), *Apium nodiflorum* (ApiNod) and *Carex pendula* (CarPen). Each panel includes all $\delta^{15}N$ data from the five study streams. The Spearman's rank correlation coefficient is shown in the top left corner of each panel for all significant correlations. Size of circles is proportional to NH_4 or NO_3 concentration respectively.

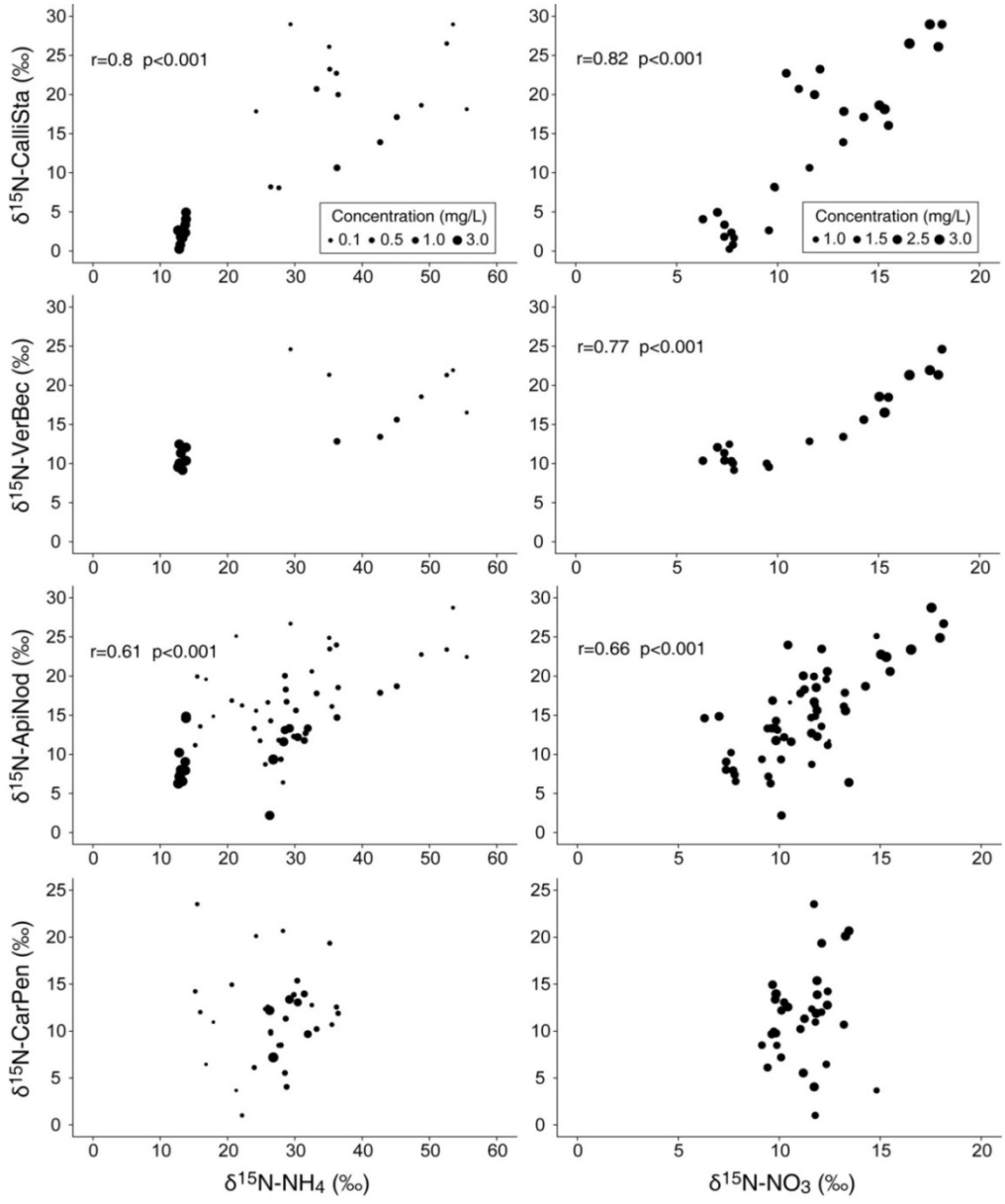


Figure 4.4 Relationships between $\delta^{15}N$ values of DIN (NH_4 and NO_3) and *Callitriche stagnalis* (CalliSta), *Veronica beccabunga* (VerBec), *Apium nodiflorum* (ApiNod) and *Carex pendula* (CarPen). Each panel considers all $\delta^{15}N$ data from different sampling dates in SMP. The Spearman's rank correlation coefficient is shown in the top left corner of each panel for all significant correlations. Size of circles is proportional to NH_4 or NO_3 concentration respectively.

4.4.4 Relative proportion of NH_4 and NO_3 taken up by macrophytes

We examined the proportion of the two DIN forms that accounted for the ^{15}N signals of those macrophyte species showing significant correlations with ^{15}N of both DIN forms (i.e., *Callitriche stagnalis* and *Apium nodiflorum*). The results from the models indicated that the relative proportion of NO_3 taken up by *Callitriche stagnalis* and *Apium nodiflorum* was much higher than the proportion of NH_4 (Table 4.4). Specifically, the estimated proportion of NO_3 taken up by *Callitriche stagnalis* and *Apium nodiflorum* ranged between 60.4 and 83.5%, and between 63.7 and 73.5%, respectively (Table 4.4). For both macrophyte species, the best-fit models (i.e., lower AICc values) were those assuming a relationship between isotopic discrimination and nutrient concentration (i.e., models 2-5), although for *Callitriche stagnalis* all fitted models showed a high prediction power (Table 4.4).

Table 4.4 Results from the five mixing models performed with temporal $\delta^{15}N$ data of *Callitriche stagnalis* and *Apium nodiflorum*. Values shown in the table are the Akaike Information Criterion corrected for small sample size (AICc), the number of parameters estimated (df), the variability explained by each model (r^2 of linear regression between observed and fitted values), the estimated relative proportions of NH_4 and NO_3 taken up by macrophytes (pNH_4 and pNO_3, respectively), and the *p*-value for the estimated parameter (pNH_4 was estimated and tested $H_0: pNH_4 = 0$; pNO_3 was parameterized as 1 - pNH_4).

	Callitriche stagnalis						*Apium nodiflorum*					
	AICc	df	r^2	$p\,NH_4$	$p\,NO_3$	*p*- value	AICc	df	r^2	$p\,NH_4$	$p\,NO_3$	*p*- value
Mixing Model 1	188.20	2	0.78	16.5	83.5	**<0.01**	480.12	2	0.36	26.5	73.5	**<0.001**
Mixing Model 2	181.26	3	0.74	39.6	60.4	**<0.001**	481.22	3	0.38	32.3	67.7	**<0.001**
Mixing Model 3	183.97	4	0.74	39.6	60.4	**<0.001**	483.44	4	0.38	32.3	67.7	**<0.001**
Mixing Model 4	171.74	4	0.82	22.4	77.6	0.42	474.56	4	0.45	36.2	63.8	**<0.001**
Mixing Model 5	180.75	6	0.80	31.7	68.3	**<0.001**	480.63	6	0.43	36.3	63.7	**<0.001**

4.5 Discussion

This study shows high variation in ^{15}N signatures of macrophytes in streams receiving inputs from WWTPs and reveals that this variability is significantly related to the variation of ^{15}N-DIN signatures, except for the riparian species (i.e., *Carex pendula*) growing on the stream bank. This coupling suggests a transfer of the ^{15}N-DIN signature to submersed and amphibious species, and supports evidence that these macrophyte species rely on stream water DIN as an N source. In the study streams, the range of observed $\delta^{15}N\text{-}NO_3$ values in WWTP-receiving streams (from −2.4 to 18.1‰) almost overlapped the range encompassed by a compilation of values from human altered streams world-wide (4.6 – 19.4‰; in Peipoch et al. (2012)), and that of $\delta^{15}N\text{-}NH_4$ clearly expanded the upper limit reported in the same study (2 – 11.9‰). The intensive N processing (especially nitrification and denitrification) within the batch reactors of the WWTP is subjected to isotopic discrimination and results in high ^{15}N-DIN signatures in the effluent and in a subsequent increase of ^{15}N signatures in receiving streams as observed in our data and in previous studies (Gammons et al. 2010, Merbt et al. 2011). The variability in concentration and ^{15}N signatures of the two DIN forms found in this study, both across streams and over time within a stream, could be partly explained by differences in the relative contribution of the WWTP effluent to the DIN load among receiving streams or over time within a stream. However, in-stream processes in the receiving streams can also contribute importantly to enlarge the variability of ^{15}N signatures of the two DIN forms, especially in those seasons when microbial biological activity in streams is higher (Ribot et al. 2012). In fact, in our study the largest variability in $\delta^{15}N$-DIN within the SMP reach coincided with base flow conditions in the stream (i.e., summer), when the relative contribution of the

effluent and the influence of in-stream processes on DIN are both maximized. Because biogeochemical processes affecting concentration and ^{15}N signatures of DIN are the same in the WWTP and within the stream, it is reasonable to expect that they could be somehow exposed to similar seasonal variation.

Macrophyte species showed remarkable spatial and temporal variability in their $\delta^{15}N$ values. As with $\delta^{15}N$-DIN signatures, the observed ranges of $\delta^{15}N$ for the study macrophytes clearly expanded the upper limit of those previously reported for freshwater macrophytes affected by anthropogenic disturbances (up to 20‰, 8‰, and 15‰ in Finlay and Kendall (2007), in Benson et al. (2008), and in Kohzu et al. (2008), respectively). However, patterns of variability in $\delta^{15}N$ values of macrophytes differed among species. Variation of ^{15}N signatures of *Callitriche stagnalis* was tightly coupled to that of $\delta^{15}N$-DIN, but this coupling was less pronounced in species of macrophytes located away from the stream channel (i.e. *Carex pendula*). Amphibious species rooted in the stream sediments (i.e. *Apium nodiflorum* and *Veronica beccabunga*) are known to use other additional N sources from hyporheic water and groundwater (Svendsen and Kronvang 1993, Madsen and Cedergreen 2002) that may have distinct ^{15}N signatures, which can explain why ^{15}N variation in *A. nodiflorum* and *V. beccabunga* was less coupled to $\delta^{15}N$-DIN variation. Moreover, DIN uptake and translocation via the rhizome system of *Carex pendula* could also be an important factor influencing its $\delta^{15}N$ variation as it has been observed for other species of *Carex* (Brooker et al. 1999).

In general, both the spatial and temporal $\delta^{15}N$ variability of all macrophytes exceeded that of $\delta^{15}N$-NO_3, but not that of $\delta^{15}N$-NH_4. However, $\delta^{15}N$ values of selected macrophytes were consistently more similar to $\delta^{15}N$-NO_3 than to $\delta^{15}N$-NH_4 values. Previous studies have also found small differences between $\delta^{15}N$-

NO_3 and $\delta^{15}N$ of macrophytes in streams where NO_3 was the dominant DIN source ($\Delta\delta^{15}N$; from 0 to 6‰ in Kohzu et al. 2008 and from 1.9 to 3.6‰ in De Brabandere 2007). However, these studies did not measure stream water $\delta^{15}N$-NH_4 signatures even though aquatic plants are known to assimilate both DIN forms (Taiz and Zeiger 2010). In this study, the large data sets of paired ^{15}N signatures for the two DIN forms and for macrophytes allowed examining in detail how $\delta^{15}N$ values of the two DIN forms and of macrophytes are related. Although no clear relationships were found along single stream reaches, when we considered data from different sites or from different dates within a stream, we found significant relationships between the ^{15}N signatures of DIN and those of macrophytes. Correlations between $\delta^{15}N$ values of DIN forms and macrophyte species were strongest for *Callitriche stagnalis*, showing the relevance of stream water DIN as a N source for submersed species. Previous studies have demonstrated that nutrient uptake through floating leaves of *Callitriche* genus, and other submersed species, can be sufficient to satisfy their N demand (Madsen and Cedergreen 2002). Nichols and Keeney (1976) revealed that availability of DIN in the water column enhanced foliar uptake to supplement root uptake in submersed macrophytes such as *Myriophyllum spicatum*. Considering the high DIN concentration of the study streams, it is likely that N uptake through leaves occurred in *Callitriche stagnalis,* which explains why ^{15}N signatures of *Callitriche stagnalis* were strongly coupled to ^{15}N signatures of the two DIN forms. Surprisingly, amphibious species showed similar relationships to those for submersed macrophytes, which was not expected considering the conspicuous structural and physiological differences between species from these two functional groups. Nitrogen uptake through leaves of *Veronica beccabunga* and *Apium nodiflorum* is unlikely because in

these particular streams their leaves are rarely submersed within the stream water (M. Peipoch, *personal observation*). However, their roots are shallow and commonly observed above the streambed, which could explain the significant relationships with ^{15}N-DIN signatures. Further studies examining the uptake and translocation of ^{15}N by roots and shoots of amphibious macrophytes may provide evidence on how they incorporate the ^{15}N signature of stream water DIN into their leaves, and thus confirm the in-stream DIN reliance by amphibious species to fulfill their N demand. Finally, the lack of significant correlations between δ^{15}N values of DIN and *Carex pendula* suggest that species located at the stream-riparian margin may rely more on other N sources, such as hyporheic and/or groundwater rather than on stream water DIN. Variation in the strength of the relationships between ^{15}N signatures of DIN and macrophyte species is in agreement with the degree of stream water exposure and structural traits of the different functional types of macrophytes. The less complex structure and higher water exposure of submersed and amphibious species may allow these species to better integrate the short-term variability of ^{15}N-DIN signatures and thus increase the chances of observing correlations between ^{15}N signatures of DIN and macrophytes. On the other hand, the more intermittent water exposure and the existence of perennating buds in *Carex pendula* may result in long N turnover compared to submersed and amphibious species (Blom and Voesenek 1996), and lead to a lower capacity by this species to integrate the short-term variability of ^{15}N-DIN signatures.

Overall, the relationships observed between δ^{15}N values of DIN and macrophytes in this study suggest that macrophyte species can contribute differently to in-stream N uptake depending upon their functional group.

Recent studies using nutrient additions of ^{15}N-labelled NH_4 in streams have demonstrated how submersed and amphibious macrophytes can have a relevant contribution in stream N uptake in macrophyte-rich streams (Simon et al. 2007, Riis et al. 2012). Results from our study support these previous findings, but also indicate that this contribution might be mostly restricted to macrophytes that develop within the stream channel, whereas those macrophytes that develop along stream-riparian edges seem to have a lower influence on in-stream N uptake. In addition, the consistent displacement of $\delta^{15}N$ values of submersed and amphibious species towards ^{15}N-NO_3 signatures provides strong evidence that the contribution of NO_3 to the ^{15}N pool of these macrophytes in the study streams is greater than that of NH_4. This is further confirmed by the results from the mixing models using isotopic signatures, which indicate that in these particular WWTP-influenced streams the contribution of submersed *Callitriche stagnalis* and *Apium nodiflorum* to in-stream N uptake is predominantly in the form of NO_3. The relative proportions of each DIN source taken up by macrophytes were quite consistent among mixing models with and without discrimination, and with different equations for sources mixing. Preferences in the inorganic N form used by plants have involved some controversy in the past, and although some studies have shown a preference for NH_4 (Cedergreen and Madsen 2002), other studies indicate that this may not be general (Melzer 1999, Gherardi et al. 2013), which is in accordance with our findings. The correlative approach used in this study is not sufficient to reveal which mechanisms are controlling the relative proportions of each DIN source taken up by macrophytes in these particular WWTP-influenced streams. However, it is worth mentioning that average NH_4 and NO_3 contribution to macrophytes N uptake estimated from the different models

was similar to the proportion of these two DIN sources found in water. These results, therefore, suggest that the proportion of each DIN source taken up by macrophytes in these WWTP-influenced streams may be influenced by their concentration in the water, which is in agreement with the greater predictive power (i.e., higher r^2 values) for mixing models considering DIN concentration observed in this study. Studies showing how higher NO_3:NH_4 ratios in stream water stimulate the uptake rates of NO_3 by the biota support our results (O'Brien et al. 2007, von Schiller et al. 2009).

In conclusion, our results contribute towards understanding ^{15}N variation in aquatic macrophytes of streams impacted by WWTP effluents, and more importantly, highlight the potential influence of macrophytes on in-stream N uptake and how it may vary according to different species of aquatic plants. In this regard, this study indicates that direct assimilation of stream water DIN is occurring by submersed and amphibious species and, in the study streams, it seems to be mainly in the form of NO_3. In contrast, our results also indicate that macrophytes species at the stream-riparian edge most likely rely on N sources other than those provided by stream water DIN. The functional attributes of different macrophytes can be of interest to water managers when evaluating the use of macrophytes to restore channel morphology of high nutrient-loaded streams since this study shows that macrophytes, especially the species developing in the stream channel, not only influence channel morphology but they can also contribute to retain excess DIN in streams with high loads.

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5

Intrinsic and extrinsic drivers of autotrophic N cycling in stream ecosystems: results from a translocation experiment

5.1 Abstract

This study investigated the intrinsic (i.e., metabolic character of autotrophs) and extrinsic (i.e., nutrients and light availability) controls on the variation in autotrophic nitrogen (N) cycling in stream ecosystems. We examined the ^{15}N isotopic incorporation into five autotrophic components (biofilm, filamentous algae, bryophytes, submerged and emergent macrophytes) differing in structural complexity and metabolic character. The experiment consisted on the translocation of the autotrophs from a stream site with depleted ^{15}N signatures and relatively low concentration of dissolved inorganic N (DIN) to three reaches downstream of the same stream with higher ^{15}N-DIN signatures and DIN concentration, and different light availability. After the translocation, autotrophs showed an asymptotical increase in their ^{15}N signatures, achieving an isotopic equilibrium with the stream water, from which we calculated N uptake and turnover rates for each autotrophic compartment at the three reaches. Differences in DIN uptake rates were greater across reaches than among autotrophs, suggesting that autotrophic DIN uptake at both organism and community level is principally controlled by extrinsic factors (e.g., DIN concentration and light incidence). Uptake rates were highest when both DIN and light availability were also highest. Submerged macrophytes clearly showed the highest N uptake rates measured in this study, whereas biofilm, filamentous algae, and bryophytes showed similar intermediate N uptake rates. In contrast, variation in N turnover rates was larger among the different autotrophs than among the study sites, suggesting a stronger control by intrinsic rather than extrinsic factors.

5.2 Introduction

Dissolved nutrients in streams are retained and transformed principally by organisms living on the stream benthos (Peterson et al. 2001). These benthic assemblages are generally composed by both autotrophs and heterotrophs whose relative abundance is primarily determined by larger-scale factors such as stream-riparian interactions, hydrologic regime, or resource subsidies (Minshall 1978, Dahm et al. 2003, Lyon and Ziegler 2009). Heterotrophs mostly refer to bacteria and fungi associated to non-living organic matter, which may obtain nutrients in both inorganic (Sanzone et al. 2001, Cheever et al. 2013) and organic forms (Hall and Meyer 1998). Autotrophs, which must obtain elemental nutrients in dissolved inorganic forms, refer to organisms of very different size and structural complexity, from diatoms or filamentous algae to bryophytes and vascular plants (Cummins 1974, Allan 1995). Because metabolic rate and biomass production of organisms consistently scales with body size (Ernest et al. 2003, Brown et al. 2004), stream autotrophs can differ substantially in the amount of energy and matter required for their growth and activity.

In many ways, energy flow and nutrient cycling in streams are tightly coupled either at the organism or ecosystem level since both energy and nutrients are needed for biomass production (Mulholland et al. 1997, Fellows et al. 2006). Consequently, variation in size and structural complexity among autotrophs may not only lead to differences in energetic requirements but also to variation in the rates of nutrient cycling among autotrophs. This means that larger organisms, such as vascular plants, need greater amounts of energy and elements to sustain growth than diatoms or filamentous algae, which are in turn energetically more efficient than higher-order organisms (Dodds et al. 2004, Allen et al. 2005). Previous research has indeed observed large variation

in nutrient uptake rates and turnover among stream autotrophs; most of the times by using tracer additions of isotopically-labelled nutrient compounds (Newbold et al. 1983, Mulholland et al. 2000b). However, nutrient cycling rates of different autotrophs seem to vary across streams with different canopy cover and/or nutrient availability (*see* Ashkenas et al. 2004, Simon et al. 2007, Riis et al. 2012). As a result, variation in nutrient cycling among autotrophs and across streams is large and difficult to predict. Therefore, the interplay between intrinsic (i.e., metabolic character) and extrinsic (i.e., nutrient and/or light availability) factors influencing autotrophic nutrient cycling may probably explain the lack of consistent patterns among autotrophs and across different streams. In this study, we investigated how rates of N uptake and turnover vary among different stream autotrophs developed under different nutrient and light conditions. To approach this objective, we conducted an in situ translocation experiment in which autotrophs from relative pristine sites were moved to sites with higher ^{15}N signatures and DIN availability to then follow the temporal patterns of stream water ^{15}N isotopic incorporation into autotrophs.

Ecologists have long harnessed variation in N isotopic ratios (i.e., ^{15}N signatures) of both organic and inorganic compounds because ^{15}N signatures can provide information about the history and origin of the N compounds in the biota. For this reason, a great number of studies have used ^{15}N signatures at natural abundance level to make inferences about the rates and patterns of nitrogen cycling at organism (Angradi 1993, Hoffman and Bronk 2006) and ecosystem (Hogberg et al. 1996, Houlton and Bai 2009) levels. However, the use of ^{15}N natural abundances to disentangle ecological (Robinson 2001) and physiological (Evans 2001) N processes comes with the challenge to correctly

interpret the specific temporal span integrated by these ^{15}N signatures. In streams, this interpretation remains unclear although the ^{15}N natural abundance of autotrophs may contain information on different N processes that take place either inside or outside these organisms. Therefore, we contend that understanding the dynamics of ^{15}N transfer from water to autotrophs will provide knowledge on the interaction between intrinsic and extrinsic factors (here, nutrient and light availability) that ultimately determine the role of autotrophs in stream nutrient cycling.

Variability in ^{15}N signatures among stream benthic organisms is known to be high (Peipoch et al. 2012) and to follow patterns of variability in ^{15}N signatures of DIN (Pastor et al. 2013). In addition, the ^{15}N signatures of autotrophs can change rapidly over time due to the constantly changing nutrient sources, concentrations, and isotopic signatures that may affect them through N uptake (*see* O'Reilly et al. (2002)). Here, the in situ translocation experiment allowed focussing on the time span of the stream water ^{15}N incorporation into stream autotrophs to estimate rates of both DIN uptake and N turnover. Moreover, these estimates were done under three scenarios differing in nutrient concentrations and incident light. Two reaches were located downstream the inputs of a wastewater treatment plant (WWTP) and they showed high concentrations of both NO_3 and ammonium (NH_4), but differed in the incident light on the stream reach. The other reach was located upstream the WWTP inputs and characterized by high nitrate (NO_3) concentration. This translocation experiment was designed to generate large differences between the ^{15}N signatures of autotrophs and DIN, mostly derived from the WWTP inputs (Ribot et al. 2012), allowing monitoring the ^{15}N transfer from water to these different organisms until they reached an isotopic equilibrium. Moreover, remarkable

changes in DIN concentrations caused by the WWTP inputs and differences in light availability among sites provided us the opportunity to evaluate influences of nutrient and light availability at a relatively small scale (< 1 km) and within a single stream.

5.3 Methods

5.3.1 Study sites

The study was conducted in the main course of La Tordera river, a middle-order stream from NE Spain (41ºN 41'7'' – 2ºE 27'33''), from May 9th to June 14th 2011. At the study sites, this river drains a relatively small forested catchment (80.7 Km^2) dominated by siliceous lithology. We conducted the study in a 1km-reach of 4–8 m width and 8–40 cm depth that receives the WWTP effluent from a nearby town. Along the study reach, we defined three study sites: one located 200 m upstream of the WWTP input (hereafter referred to as UP site) and two sites located 25 m and 950 m downstream the WWTP input (hereafter referred to as DW1 and DW2 site, respectively; Figure 5.1). Previous studies in this reach have shown that the WWTP effluent increases stream water DIN concentration and ^{15}N-DIN signature and changes the relative proportion of NO_3 and NH_4 (Merseburger et al. 2005; Ribot et al. 2012). In particular, the input of the WWTP effluent increases concentrations (Figure 5.1A) and ^{15}N signatures (Figure 5.1B) of NH_4 of the receiving stream. While DW1 and DW2 can be similar in concentrations and ^{15}N signatures of NH_4 and NO_3 (Figure 5.1A & B; Merbt et al. 2011) , DW1 has more dense canopy cover, and thus lower light availability than DW2.

5.3.2 Translocation experiment

Our experimental approach consisted in the simultaneous translocation of different autotrophs (i.e. biofilm, filamentous algae, bryophytes, submerged and emergent macrophytes) from La Tordera headwaters to each one of the three study sites (UP, DW1 and DW2; Figure 5.1). We collected the organisms from La Tordera headwaters because both concentrations and ^{15}N signatures of the two DIN species ($^{15}N\text{-}NH_4$ = *below detection limit*; $^{15}N\text{-}NO_3$ = 4.2 ‰) and of autotrophs are known to be lower than at any of the sites in the study reach (Table 5.1; Pastor et al. 2013). Therefore, after their translocation, all autotrophs were exposed to higher ^{15}N-DIN signatures; and thus, we could monitor how their ^{15}N signatures achieved a new equilibrium at each study site. For the biofilm, we used unglazed ceramic tiles (2.5 x 5 cm) as artificial substrata for microbial colonization. Tiles were glued onto 15 bricks (30 tiles per brick) and deployed in La Tordera headwaters one month prior the beginning of the experiment to allow biofilm colonization. Stream cobbles naturally colonized by filamentous algae (*Ulothrix sp.*) and bryophytes (*Rhynchostegium riparioides*), as well as emergent macrophytes (*Apium nodiflourm*) were manually collected in the headwaters and transported to each study site into recipients filled with stream water. Submerged macrophytes (*Callitriche stagnalis*) were collected in a near low-order stream and sorted by groups of 5-10 individuals to be set into 10 x 20 cm mesh bags of 1 cm^2 pore size that were previously tested to reduce light availability less than 5%.

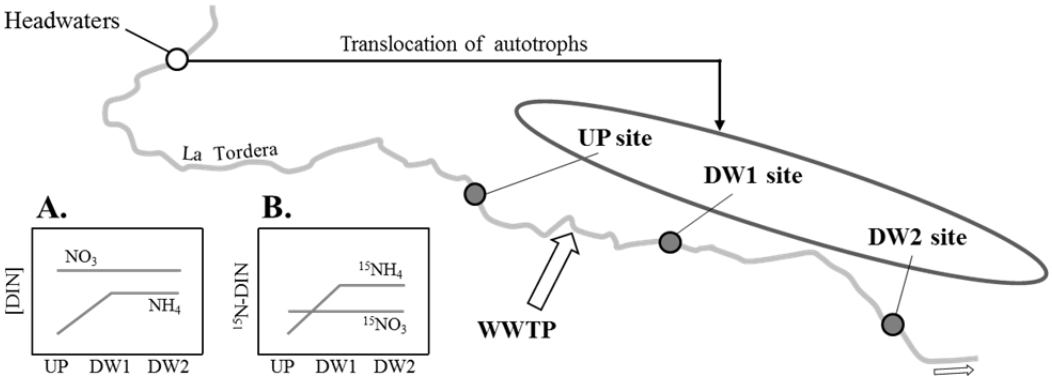


Figure 5.1 Illustration of the sampling sites along La Tordera stream and of the experimental set up. The thin arrow indicates he direction of water flow and the thick arrow indicates the site where the effluent from the wastewater treatment plant (WWTP) enters the river. Bottom panels show the *a priori* expected patterns of variation in DIN concentration (A) and in ^{15}N-DIN values (B) among study sites, which were based on results from previous studies in this reach (Merseburger et al. 2005, Ribot et al. 2012).

At each study site (UP, DW1 and DW2), we translocated 5 biofilm-colonized bricks, 10 cobbles colonized by filamentous algae, 10 cobbles colonized by bryophytes, 10 mesh bags with submerged macrophytes and 10 individuals of emergent macrophytes. Autotrophs were deployed in their natural stream habitat; riffles-run for biofilm, filamentous algae and bryophytes, pools for submerged macrophytes and stream margins for emergent macrophytes. All organisms were sampled at each study site before and 2, 4, 8, 16, 24, and 36 days after the translocation.

5.3.3 Field measurements and sample collection

Synchronically to the organisms sampling, several environmental variables were recorded throughout the experiment. Discharge was estimated every sampling day at each study site by conducting a cross-sectional profile (every

20 cm) of water depth and velocity using a tape and a velocity meter (Schiltknecht Messtechnik). Water temperature and PAR were recorded at 20–min intervals over the study period at all sites using a waterproof temperature and light data loggers (HOBO Pendant® UA-002-64). DIN concentration and ^{15}N-DIN signatures for both NO_3 and NH_4 from stream and groundwater in the stream-riparian edge, were analyzed in triplicated samples at each sampling date and site, from 4 L water samples. Samples for surface water were collected from the right, the left and the center sides of the stream channel. Groundwater samples were collected from three pre-installed wells using a Masterflex (Vernon Hills, Illinois, USA) L/S battery-powered peristaltic pump. Sampling wells consisted of 3.5 cm PVC cylinders with a bored portion of 30–cm long at the bottom of the well, inserted 50 cm beneath the riparian soil at 1 m from one of the stream margins. Water samples were stored on ice for less than 4 h and afterwards filtered through ashed glass fiber filters (GFF; 0.7-mm pore size, Albet) in the laboratory. Filtered samples for $^{15}NH_4$ were immediately processed in the laboratory (see details in *laboratory analyses*), whereas $^{15}NO_3$ and nutrient samples were frozen until further processing and analysis. Samples for the analysis of autotrophs ^{15}N signatures (five replicates per site and autotroph) were randomly collected from translocated organisms on each sampling day. Only tips were collected for bryophytes and leaves for macrophytes. In addition, we also collected samples for autotrophs naturally growing at each study site (five replicates per site, autotroph, and sampling day) as our reference samples.

Biomass of biofilm, bryophytes and emergent macrophytes was estimated at 0, 16, and 36 days after the translocation. Biomass of submerged macrophytes was estimated at the beginning and at the end of the experiment,

and biomass of filamentous algae was only estimated at the beginning of the translocation because it was unexpectedly degraded in all sites four days after the translocation began. Reference and translocated biofilm was sampled by scraping five cobbles and four tiles per brick, respectively, and filtered onto ashed pre-weighted glass fiber filters (GFF; 0.7-mm pore size, Albet). Bryophytes and filamentous algae (n = 5) were sampled by collecting all material found within a known area (6.25 cm^2). Biomass of submerged macrophytes (n = 5) was estimated by measuring the total wet weight at each 10 x 20 cm mesh bag after shaking the sample during 20 s in a salad spinner, and then converted to dry weight by assuming plant wet weight is 10% dry weight. Individual aerial biomass of emergent macrophytes was estimated from ten different plants randomly collected at each study site.

5.3.4 Laboratory analyses

Stream water samples were analyzed for $N\text{-}NH_4$, $N\text{-}NO_3$ and soluble reactive phosphate (SRP) concentrations following standard colorimetric methods (APHA 1995) on an Automatic Continuous Flow FUTURA–ALLIANCE Analyzer at the CEAB-CSIC Biogeochemistry laboratory. The ^{15}N natural abundance of NH_4 and NO_3 was determined following procedures adapted from Holmes et al. (1998) and Sigman et al. (1997) respectively. Details for this method are described in von Schiller et al. (2009).

Biomass samples of all autotrophs were oven-dried at 60ºC during at least 72 hours, weighted to estimate the dry mass (mg) on a Sartorius (Goettingen, Germany) MC1 analytical balance, and combusted at 500ºC for 5-6 h to estimate ash free dry mass (AFDM). Dry mass samples for the ^{15}N analysis of autotrophs were ground, thoroughly homogenized and weighted to the nearest 0.001 mg on a Mettler- Toledo (Greifensee, Switzerland) MX5

microbalance. Samples were encapsulated in tins and stored until isotope analysis. All ^{15}N samples ($^{15}NH_4$, $^{15}NO_3$ and $^{15}N_{autotrophs}$) were analyzed at the UC Davis Stable Isotope Facility (California, USA). The carbon (C) and N content (as a percentage of total dry mass) and the stable isotope signatures of N either for the water samples or autotrophs were determined using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). All ^{15}N signatures are expressed in terms of delta values ($\delta^{15}N$), which are parts per thousand (‰) differences from a standard (i.e., N_2).

5.3.5 Data analysis

Comparison of physical and chemical variables among the study sites was done using a Kruskal-Wallis nonparametric ANOVA (site as a factor) followed by a single-step multiple comparison procedure (Siegel and Castellan 1988). Comparisons of biomass, N content, and C:N ratios for each autotroph among sites (UP, DW1 and DW2) and treatments (reference and translocated samples) were evaluated by two-way ANOVA tests (site and treatment as factors) with repeated measures (sampling day as the within effect). The ANOVA tests were done with ln-transformed values to fit requirements of normality and homogeneity. Results were considered significant for $p < 0.05$.

Incorporation of stream water ^{15}N into autotrophs was evaluated by following the rate of change of their ^{15}N natural abundance over the incubation period after the translocation. Calculations assumed first-order ^{15}N kinetics. Changes in ^{15}N signature of autotrophs over time (t) at each study site were expressed as follows:

$$\delta^{15}N_{(t)} = \delta^{15}N_{assymptote} \times (1 - e^{-kt}) \quad (1)$$

where $\delta^{15}N_{(t)}$ are the ^{15}N signatures of autotrophs corrected by background values ($\delta^{15}N_{t=0}$) over time after the translocation, $\delta^{15}N_{asymptote}$ is the asymptote value reached by $^{15}N_{autotrophs}$ signatures at equilibrium, and *k* is the isotopic incorporation rate (in units of d^{-1}), which can be considered a surrogate of the N turnover of each particular autotroph. Based on these empirical parameters, we estimated the DIN uptake rate of each autotroph (in units of mgN m^{-2} d^{-1}) following the equation:

$$\text{DIN uptake rate } (U_{DIN}) = k \times \text{TN} \times (\delta^{15}\text{N}_{assymptote} \,/\, \delta^{15}\text{N}_{\text{DIN}}) \qquad (2)$$

where TN is the N standing stock of each autotroph (mgN m^{-2}) and the $\delta^{15}N_{DIN}$ is the background-corrected ^{15}N signal of DIN calculated as the concentration–dependent sum of $\delta^{15}N\text{-}NH_4$ and $\delta^{15}N\text{-}NO_3$ values. General relationships between N turnover and uptake rates were examined using Pearson correlations. While comparison of N uptake rates and turnover among autotrophs and sites was assessed using two-way ANOVA with organism and site as factors. Because of the small and relatively unbalanced data set available to compare how variation in *k* values, $\delta^{15}N_{asymptote}$, and DIN uptake rates differ among autotrophs (n = 5 organism; 2-3 observations per organism) and among sites (n = 3 sites; 3-5 observations per site) we employed two-way ANOVA of *z* scores:

$$z \text{ score} = \frac{(y_i - \bar{y})}{SD} \qquad (3)$$

where y_i, $\overline{y}$, and SD are observed values, mean values, and standard deviations, respectively, for each of those three measured parameters. This generates a standardized normal distribution (mean = 0 and SD = 1) for each parameter and normalizes measures for organism and site variation, respectively.

Significant ANOVAs differences among factor levels (organism or site) were assessed using post hoc Tukey Honestly Significant Difference tests with $p < 0.05$. All statistical tests were done using R 3.0.0 (R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/).

5.4 Results

5.4.1 Physical and chemical parameters

Stream discharge was relatively high (0.2 – 0.4 $m^3 s^{-1}$) at all study sites and rather constant during the first 4 weeks of the experiment. However, on day 29, intense rainfalls lasting for 3 days increased discharge to approximately 1$m^3 s^{-1}$ until the end of the experiment. On average, discharge below the WWTP input (DW1 and DW2) was 25% higher than at the UP site due to the WWTP effluent input (Table 5.1). Water temperature did not vary significantly among the study sites or over the study period (Table 5.1). Daily PAR at DW2 site was on average three and four times higher than at UP and DW1 sites, respectively (Table 5.1).

Surface water NH_4 concentration at the UP site was similar to the headwaters and over an order of magnitude lower than at both DW sites, although this difference was reduced over the study period as discharge increased (Table 5.1). There was not any significant difference in surface water NO_3 concentration among sites, but NO_3 concentration was on average 10 times higher than in the headwaters (Table 5.1). Concentrations of NH_4 and NO_3 in surface water were not significantly different between the two DW sites (Table 5.1). Surface water SRP concentration increased significantly from the UP to both DW sites (Table 5.1). DIN:P ratio in surface water at the UP site (66.1 ± 3.2) was high compared to DIN:P values below the WWTP input (average: 33 ± 1.8). Concentration of NH_4 in groundwater decreased from DW1 to DW2, while NO_3

concentration was highest at DW2, lowest at DW1, and intermediate at the UP site (Table 5.1).

Table 5.1 Physical and chemical characteristics of La Tordera stream at the headwaters, and at the upstream (UP) and downstream (DW1 and DW2) sites of the input from the wastewater treatment plant during the study period. Nutrient data are means and SE, in brackets, from 7 sampling dates, and *p*-values are for Kruskal-Wallis tests (site as a factor). Means in a row with identical superscripts are not significantly different ($p > 0.05$).

Parameter	Headwaters	UP	DW1	DW2	*p*
Physical					
Average discharge ($m^3\ s^{-1}$)	0.1	0.3 (0.1-0.9)	0.4 (0.2-1.1)	0.4 (0.2-1.2)	0.281
Daily mean temperature (ºC)	14.4	16^{a} (0.1)	16.4^{ab} (0.2)	16.8^{b} (0.2)	0.008
Daily PAR (mol quanta $d^{-1}\ m^{-2}$	2.9	19.8^{ab} (2.2)	11.7^{a} (1.3)	35.6^{b} (3.9)	<0.001
Surface water chemistry					
NH_4 concentration (mg N L^{-1})	0.009	0.01^{a} (0.001)	0.7^{b} (0.1)	0.6^{b} (0.1)	<0.001
NO_3 concentration (mg N L^{-1})	0.229	2.2 (0.2)	2 (0.1)	2.3 (0.1)	0.091
SRP concentration (mg P L^{-1})	0.006	0.03^{a} (0.001)	0.1^{b} (0.007)	0.1^{b} (0.009)	<0.001
Groundwater chemistry					
NH_4 concentration (mg N L^{-1})		0.01^{b} (0.02)	0.2^{a} (0.02)	0.01^{b} (0.005)	<0.001
NO_3 concentration (mg N L^{-1})		1.2^{a} (0.1)	0.6^{b} (0.1)	2.6^{c} (0.4)	<0.001
SRP concentration (mg P L^{-1})		0.1^{a} (0.004)	0.1^{a} (0.02)	0.2^{b} (0.02)	<0.001

Values between parentheses for discharge are minimum and maximum values

PAR, Photosynthetically active radiation; NH_4, ammonium; NO_3, nitrate; SRP, soluble reactive phosphorous

Variation both among sites and over time of $\delta^{15}N\text{-}NH_4$ was greater than that of $\delta^{15}N\text{-}NO_3$ (Figure 5.2). $\delta^{15}N\text{-}NH_4$ in surface water shifted from negative values at the UP site (–4.7 ± 0.6; Figure 5.2A) to highly ^{15}N-enriched values at the DW1 (23 ± 1.7; Figure 5.2B) and DW2 sites (24 ± 0.7; Figure 5.2C). $\delta^{15}N\text{-}NH_4$ values in groundwater differed among sites in agreement to the patterns observed in surface water (Figure 5.2D, E and F). In general, temporal variation in $\delta^{15}N\text{-}NH_4$ values of both surface and groundwater was greater at DW sites than at the UP site (Figure 5.2). Surface water $\delta^{15}N\text{-}NH_4$ values were lower and more variable under the elevated stream flow conditions of the last week of the study (Table

5.1; Figure 5.2). Conversely, the δ^{15}N-NO_3 values did not varied neither among sites nor over time (Figure 5.2A, B and C) being around 10‰, except for groundwater at DW1 that was highly ^{15}N-enriched during all the study period (Figure 5.2E).

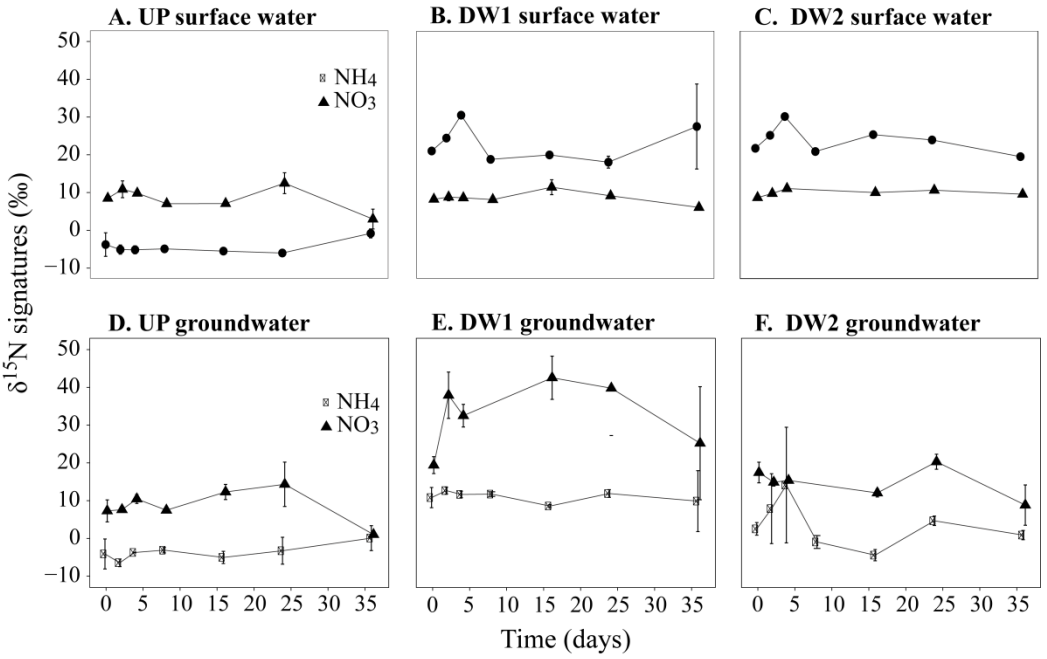


Figure 5.2 Variation of $\delta^{15}NH_4$ and $\delta^{15}NO_3$ values during the translocation experiment at the different study sites (UP, DW1, and DW2). For each sampling date, symbols are the means of 3 replicates and vertical lines across the symbols indicate the SE.

5.4.2 Nitrogen standing stocks of autotrophs

Biomass of autotrophs did not vary significantly among the study sites (Table 5.2), except for submerged macrophytes which showed on average two times higher biomass at both DW sites (11.3±2.8 g m^{-2}) compared with the UP site (5.3±0.6 g m^{-2}). When comparing among treatments, we found that

reference plants of emergent macrophytes had on average lower biomass compared to translocated plants at all the study sites (Table 5.2). Contrastingly, biomass of reference biofilm (average: 15.9 ± 5.1 g m^{-2}) was consistently higher than biomass of translocated biofilm (average: 10.8 ± 5.1 g m^{-2}).

Site and treatment had significant effects on the N content of most autotrophs (Table 5.2). We observed similar patterns for biofilm and filamentous algae when comparing among sites and treatments. Biofilm and filamentous algae showed higher N content at both DW1 (biofilm: 3.2 ± 0.3%, filamentous algae: 5.7±1.1%) and DW2 sites (biofilm: 2.4 ± 0 .3%, filamentous algae: 2.2 ± 0.4%) than at the UP site for reference and translocated samples (Table 5.2). We also found significantly higher N content for submerged macrophytes at both DW sites (average: 4.4 ± 0.1%) than at the UP site (average: 3.7 ± 0.1%; Table 5.2). Overall, N content tended to be lower in biofilm and filamentous algae and higher in bryophytes and macrophytes, regardless of the stream site.

Biofilm, filamentous algae, and emergent macrophytes did not showed significant differences in C:N ratios among the study sites and averaged 4.7 ± 0.3, 7.7 ± 0.4, and 8.9 ± 0.3, respectively. Conversely, we observed significant differences in C:N ratios for bryophytes and submerged macrophytes (Table 5.2), which showed relatively high C:N at the UP site (bryophytes: 14.7 ± 0.9, s. macrophytes: 10.1 ± 0.7) than at both DW sites (bryophytes: 11 ± 0.4, s. macrophytes: 8.9 ± 0.5). These two autotrophs did also showed higher C:N ratios in translocated than in reference samples, especially in the case of bryophytes (Table 5.2).

Table 5.2 Results from two-way ANOVA, using stream site (UP, DW1, and DW2) and treatment (reference and translocated) as factors and sampling date as repeated measures, for biomass (that is, ash free dry mass for biofilm and dry mass for the rest of autotrophs), N content (as percentage of dry weight) and C:N (mass ratio). Tests were separately conducted for each of the primary uptake autotroph considered. Values highlighted in bold indicate significant effects ($p < 0.05$).

Organisms	Biomass		N percentage		C:N	
	F	*p*	*F*	*p*	*F*	*p*
Biofilm						
Treatment	113.7	**<0.01**	8.4	**<0.01**	4.1	0.06
Site	1.6	0.23	5.6	**0.01**	1.3	0.28
Site × Treatment	2.2	0.15	13.6	**<0.01**	11.4	**<0.01**
Sampling day	18.9	**<0.01**	1.9	0.08	1.7	0.18
Filamentous algae						
Treatment			15.7	**<0.01**	2.7	0.12
Site			16.9	**<0.01**	0.3	0.71
Site × Treatment			4.8	**0.02**	2.4	0.12
Sampling day			4.1	**<0.05**	12.6	**<0.01**
Bryophytes						
Treatment	1.8	0.21	0.03	0.84	252.4	**<0.01**
Site	0.1	0.89	2.7	0.09	6.9	**<0.01**
Site × Treatment	0.1	0.77	0.5	0.49	0.5	0.47
Sampling day	9.4	**<0.01**	16.5	**<0.01**	42.6	**<0.01**
Submerged macrophytes						
Treatment						
Site	0.1	**<0.01**	22.1	**<0.01**	15.1	**<0.01**
Site × Treatment						
Sampling day	0.1	0.78	14.8	**<0.01**	30.7	**<0.01**
Emergent macrophytes						
Treatment	4.7	**0.04**	9.9	**<0.01**	11.7	**<0.01**
Site	1.1	0.34	0.6	0.54	2.4	**0.11**
Site × Treatment	1.4	0.26	3.5	**0.05**	3.9	**0.03**
Sampling day	4.1	**<0.05**	0.9	0.41	0.2	0.81

5.4.3 ^{15}N transfer from stream water to autotrophs

At all study sites, ^{15}N signatures of the translocated autotrophs followed an asymptotical increase after the translocation, except those of the emergent macrophytes (Figure 5.3). Most of the autotrophs, and particularly biofilm and

filamentous algae, rapidly achieved a $\delta^{15}N_{asymptote}$ and therefore equilibrium with the isotopic conditions in the stream water (Figure 5.3). At equilibrium, $\delta^{15}N$ values of translocated autotrophs were similar to those of reference autotrophs, regardless of the study site (Figure 5.3). Consistently for all autotrophs, values of $\delta^{15}N_{asymptote}$ were considerably higher in both DW sites than in the UP site in agreement with differences in $^{15}N\text{-}NH_4$ signatures among sites (Table 5.3). In general, there were larger differences in $\delta^{15}N_{asymptote}$ (Figure 5.4A & B) among sites than among the different autotrophs. However, submerged macrophytes showed higher ^{15}N signatures; and thus, higher values of $\delta^{15}N_{asymptote}$ than the rest of organisms (Figure 5.4A).

5.4.4 Uptake rates and turnover of N in autotrophs

Differences in N turnovers (*k*) were larger among the different autotrophs than among the study sites (Figure 5.4C & D). Variability of N turnover among sites was different for each autotroph (Table 5.3). Variation and mean values for N turnover of submerged macrophytes were much lower compared to the other autotrophs, regardless of the sampling site (Figure 5.4C). Biofilm and bryophytes had intermediate and similar turnover at the UP and DW1 site but not at DW2, where biofilm showed a faster N turnover than bryophytes (Table 5.3). Filamentous algae exhibited the fastest and more variable N turnover at the two sites where they were present (DW 1 and DW2), with a maximum for this study (0.72 d^{-1}). This result however, must be carefully interpreted because the time series of exposure was shorter (4 days) than for the other autotrophs (Figure 5.3).

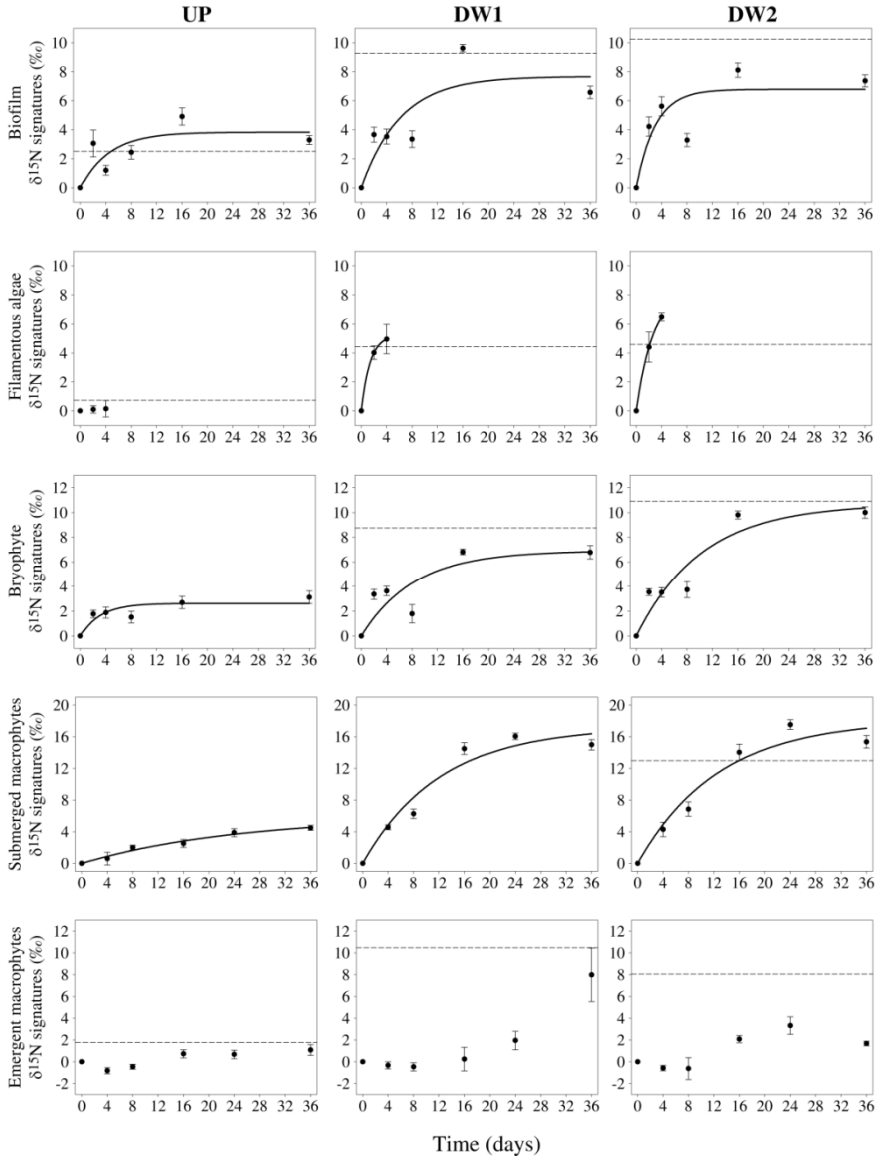


Figure 5.3 Variation in natural abundance of $\delta^{15}N$ values (corrected by $\delta^{15}N_{t=0}$ values) of translocated autotrophs over the study period at each different site. Solid black lines represent the expected results from fitting the data into *equation 1* (see Table 5.3 for details on equation parameters for each case). Dashed lines represent the corrected-mean $\delta^{15}N$ value of the reference samples for each autotroph and site. For each sampling date, symbols are the means of 5 replicates and vertical lines across the symbols indicate the SE.

For all autotrophs, DIN uptake rates (U_{DIN}) were almost two times higher at the DW sites than at the UP site (Table 5.3). Submerged macrophytes showed on average higher U_{DIN} rates than any other autotroph, but similar variation than biofilm (Figure 5.4E). Average U_{DIN} rates at DW2 were higher than at the other two study sites although variation within each site was similar (Figure 5.4F). Variation in U_{DIN} rates among autotrophs was slightly lower than variation observed among the study sites (Figure 5.4E & F). Overall, U_{DIN} rates were not significantly correlated neither with N turnover ($r = 0.1$, $p = 0.778$) nor with $\delta^{15}N_{asymptote}$ values ($r = 0.5$, $p = 0.149$), while the correlation between N turnover and $\delta^{15}N_{asymptote}$ values was also not significant ($r = 0.2$, $p = 0.529$).

Table 5.3 $\delta^{15}N_{assymptote}$ ± SD, N turnover ± SD (*k*), N standing stocks ($N_{biomass}$), and DIN uptake rates ± SD (U_{DIN}) for each autotroph at each study site (UP, DW1, and DW2), which was calculated using the results from the rate of change in ^{15}N natural abundance of the autotrophs during the translocation experiment (*Equation 1*). R^2 shows the goodness of fit for the rate of change in ^{15}N signatures. Only results from significant regressions between ^{15}N signatures and time were included in the table ($p < 0.05$).

Site	Organisms	$\delta^{15}N_{assymptote}$ (‰)	k (d^{-1})	R^2	$N_{biomass}$ (mgN m^{-2})	U_{DIN} (mgN $m^{-2}d^{-1}$)
UP	Biofilm	3.8 ± 0.4	0.22 ± 0.09	0.40	187.9	30.1 ± 12.3
	Bryophytes	2.6 ± 0.2	0.32 ± 0.12	0.45	348.3	55.5 ± 20.8
	Submerged macrophytes	5.9 ± 1.7	0.04 ± 0.02	0.73	502.4	23.9 ± 12.5
DW1	Biofilm	7.7 ± 0.6	0.16 ± 0.04	0.67	117.7	14.8 ± 3.7
	Filamentous algae	5.3 ± 1.2	0.72 ± 0.55	0.71	83.5	45.4 ± 34.7
	Bryophytes	6.9 ± 0.7	0.12 ± 0.03	0.60	357.2	30.3 ± 7.6
	Submerged macrophytes	17.2 ± 1.1	0.08 ± 0.01	0.92	449.8	63.5 ± 7.9
DW2	Biofilm	6.8 ± 0.4	0.33 ± 0.09	0.63	329.6	72.6 ± 19.8
	Filamentous algae	8.3 ± 3.6	0.38 ± 0.3	0.81	90.1	37.7 ± 29.7
	Bryophytes	10.7 ± 0.7	0.10 ± 0.02	0.84	386	40.5 ± 8.1
	Submerged macrophytes	18.1 ± 1.3	0.08 ± 0.01	0.90	532.3	75.9 ± 9.5

5.5 Discussion

In this study we used a translocation experiment to evaluate how metabolically and structurally distinct stream autotrophs cycle N by following their changes in the ^{15}N natural abundance over time under different nutrient and light conditions. The translocation experiment was based on the exposure of autotrophs from headwaters to stream sites with increased concentration and ^{15}N signature of NO_3 (UP site) and of both NO_3 and NH_4 (DW sites). In addition, autotrophs translocated to the DW2 site were exposed to higher light availability than those at the DW1 site. We assumed that the evolution of ^{15}N labelling in autotrophs over time would indicate incorporation of stream water DIN. Therefore, the comparison of this evolution among autotrophs and sites would allow examining the relevance of intrinsic (i.e., structural and functional characteristics of the autotrophs) and extrinsic (i.e., DIN concentration and light availability) factors controlling the variation in N cycling rates of autotrophs. To our knowledge, this is a novel experimental approach to examine N cycling of benthic autotrophic compartments in streams. However, it has previously been used for stream consumers to examine the carbon (C) transfer from the resources into their tissues based on changes in ^{13}C (Fry and Arnold 1982, del Rio and Carleton 2012). The present translocation experiment demonstrated to be sensitive enough to trace the asymptotical increase of ^{15}N natural abundance of autotrophs, even though 15N increases at equilibrium (that is, $\delta^{15}N_{assymptote}$) were not particularly high (e.g., < 3‰ at the UP site). The similarity between $\delta^{15}N$ of reference autotrophs and $\delta^{15}N_{assymptote}$ of translocated autotrophs indicated that the time frame of the experiment was enough for autotrophs to reach equilibrium with stream water ^{15}N-DIN. Nonetheless, the sampling interval was probably too wide for biofilm and filamentous algae,

which incorporated the stream water ^{15}N-DIN very fast. Overall, this translocation experiment has proved to be efficient to estimate N uptake and turnover rates of several autotrophs that play an important role on the N cycling at whole-reach scale (Mulholland et al. 2006, von Schiller et al. 2009, Riis et al. 2012). Nevertheless, by comparing rates among differing sites we found that the robustness and accuracy of the translocation experiment seem to be contingent upon the initial conditions and the magnitude of the changes in ^{15}N-DIN at which organisms are exposed.

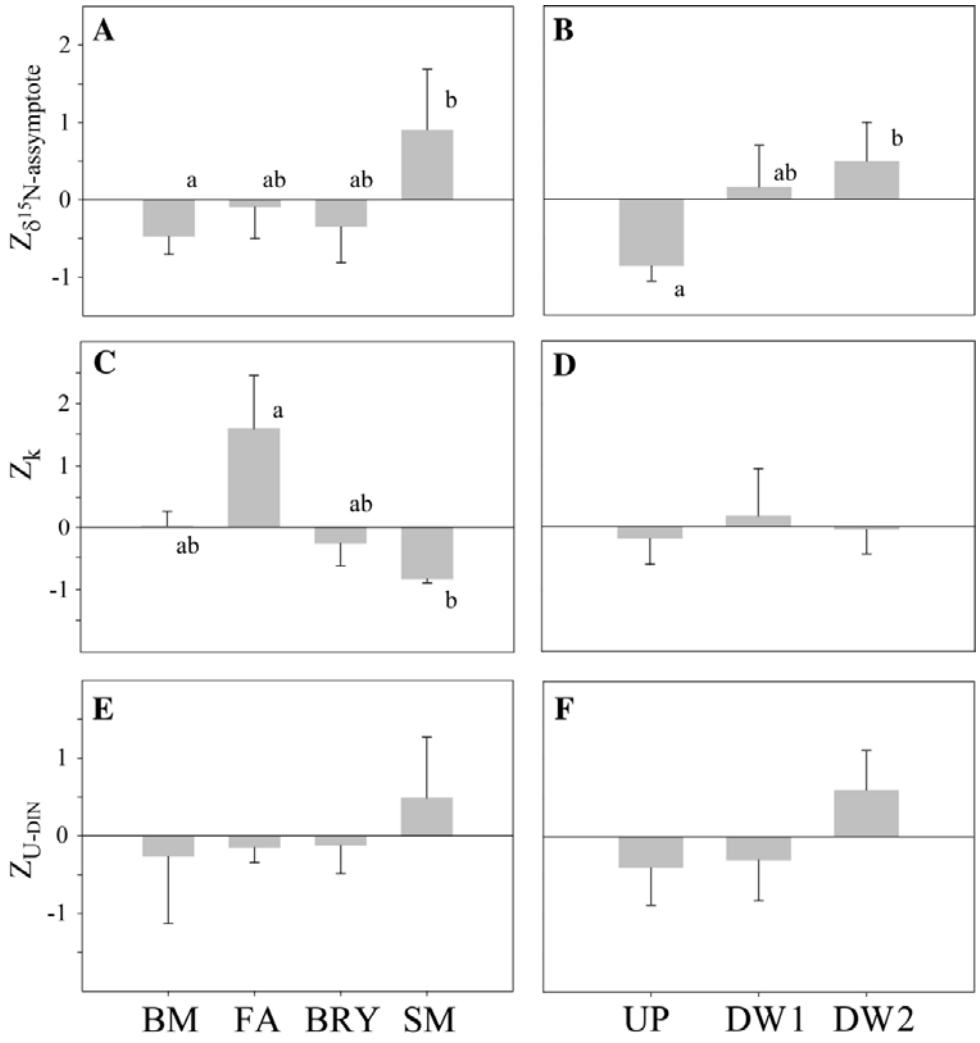


Figure 5.4 Variation in autotrophs (A, C, and E) and study sites (B, D, and F) of the parameters estimated from the rate of change in ^{15}N natural abundance of autotrophs during the translocation experiment. Columns represent the mean ± SE of the *z* scores calculated for $\delta^{15}N_{assymptote\text{-}corrected}$, N turnover (*k*), and DIN uptake rates (U_{DIN}). Error bars for each column indicate variation within an autotroph or a site, while differences between columns indicate variation among autotrophs or among sites. Uppercase letters indicate significant differences between the columns of each panel ($p < 0.05$ from Tukey's HSD after two-way ANOVA). Abbreviations correspond to biofilm (BM), filamentous algae (FA), bryophytes (BRY), and submerged macrophytes (SM).

5.5.1 Intrinsic vs. extrinsic control on the ^{15}N natural abundance of autotrophs

Nitrogen isotopic signatures of autotrophs are expected to be influenced by the ^{15}N signatures of bioreactive N (NH_4 and NO_3) and the extent to which these two DIN pools are utilized. The former directly affects the $\delta^{15}N$ that autotrophs will reach at equilibrium by determining the proportion of ^{15}N atoms present in each DIN pool (Robinson 2001, Fry 2008), while the concentration of NH_4 and NO_3 influences the uptake rates at which these two N forms are assimilated (Earl et al. 2006, O'Brien et al. 2007, Ribot et al. 2013) and the degree of isotopic fractionation (Fry 2008). In addition, light availability is known to enhance N uptake by autotrophs (Mulholland et al. 2006, von Schiller et al. 2007); and thus, this factor may indirectly influence $\delta^{15}N$ of autotrophs. Therefore, at equilibrium, we expected clear differences in the $\delta^{15}N$ of autotrophs among the study sites due to the substantial differences in $\delta^{15}N$ and concentration of DIN and in light availability (i.e., extrinsic factors). Consistent differences in $\delta^{15}N_{asymptote}$ observed among sites for all autotrophs considered supported this expectation. Values of $\delta^{15}N_{asymptote}$ were lowest at the UP site, intermediate at DW1, and highest at DW2, coinciding with higher DIN concentration and ^{15}N signatures at the DW sites and with higher light availability at the DW2 site. This finding suggests that ^{15}N signatures of autotrophs at equilibrium (i.e., natural abundance) are strongly influenced by the local environmental conditions where they develop. This is in agreement with other studies showing the influence of these extrinsic factors on the variability of ^{15}N natural abundance for different primary uptake compartments at larger scales (Peipoch et al. 2012, Pastor et al. 2013). In contrast, we found that differences among autotrophs were more remarkable for the velocity at which ^{15}N

signatures reached equilibrium. The time to reach isotopic equilibrium was shorter for biofilm and filamentous algae than for bryophytes and macrophytes, regardless of stream site, probably as a result of the larger N standing stocks and structural complexity of bryophytes and macrophytes compared to those of biofilms and filamentous algae. Therefore, ^{15}N natural abundance of biofilm and filamentous algae seems to be a good integrator of short-term ^{15}N-DIN variability in the stream water (e.g., ~ daily variations), while ^{15}N natural abundance of bryophytes and, particularly, macrophytes may be a better integrator of medium to long-term ^{15}N-DIN variability (e.g., several days or weeks).

5.5.2 Intrinsic vs. extrinsic control on N uptake by autotrophs

Macrophytes clearly showed the highest DIN uptake rates (U_{DIN}). The rest of autotrophs showed on average similar U_{DIN} rates, despite previous studies have shown large differences in NH_4 uptake rates among them within a stream (*see* Mulholland et al. 2000b, Tank et al. 2000). Nevertheless, in this study, U_{DIN} differed more across the study sites than among the different autotrophs, suggesting that nutrient and light availability provide the basis for variation in N uptake of the stream autotrophic community. In particular, U_{DIN} rates were greater when these two extrinsic factors operated together. The importance of the extrinsic controls on DIN uptake is also observed for each autotroph type separately, although the effects of these controls seem to slightly vary among autotrophs. The high variation in biofilm U_{DIN} rates encountered among the study sites (from 14.8 to 72.6 mgN m^{-2} d^{-1}) follows a similar pattern to that observed by Simon et al. (2007) for biofilm NH_4 uptake in seven open-canopy streams along a gradient of NH_4 and NO_3 concentrations (from 8.5 to 80.5 mgN m^{-2} d^{-1}). Higher biofilm N uptake observed at DW2 may additionally respond to

higher metabolic activity and N demand due to higher light availability (von Schiller et al. 2007). Contrasting patterns between bryophytes and submerged macrophytes in variation of N uptake rates among sites may respond to the negative effects of high nutrient loads on bryophytes (Vanderpoorten et al. 2000) and to the positive effects on biomass production of macrophytes (Pedersen and Borum 1997), which is in agreement with patterns observed in this study. Overall, differences in spatial patterns of DIN uptake among autotrophs suggests that extrinsic factors contribute to the variation found in autotrophic N uptake, but that their effects vary for each autotroph.

5.5.3 Intrinsic vs. extrinsic control on N turnover of autotrophs

Nitrogen turnover calculated in this study (*k* values) can be considered as a measure of the internal N cycling of autotrophs, because they were empirically quantified along a time period large enough (~30 days) to reach equilibrium, which indicates that both uptake and release processes were occurring. Previous studies using ^{15}N-tracer additions at constant rate during similar time periods have shown that turnover of assimilated DIN is occurring at the observed time-scale either for specific biotic compartments (Dodds et al. 2004) or for whole-stream reaches (Peterson et al. 2001). Our results confirmed that N turnover differed greatly among autotrophs as expected by their conspicuous differences in metabolic rates, biomass, C:N ratios, and structural complexity (Brown et al. 2004, Dodds et al. 2004). We found that N turnover was faster for biofilm and filamentous algae than for macrophytes, which is in agreement with previous studies (Simon et al. 2007, Riis et al. 2012). Strong differences among autotrophs regardless of the site location suggest that the intrinsic metabolic character of each autotroph provide the basis for the variation in autotrophic N turnover. Low variation in autotrophic N turnover among the

study sites contrasted with the remarkable among-site differences observed for autotrophic N uptake. N uptake is an enzyme-mediated process; therefore, changes in NH_4 and NO_3 concentration may certainly produce faster changes in uptake than in turnover. In addition, N turnover was calculated based on the time span for ^{15}N of autotrophs to reach an asymptotic value; and thus, autotrophic N turnover may represent the estimated time at which uptake and mineralization processes were compensated. Thus, our results suggest that increases in N uptake may lead to higher N mineralization (or vice versa) resulting in a certain balance between uptake and mineralization processes reducing the variation in autotrophic N turnover compared to autotrophic DIN uptake. The lack of a general correlation between uptake rates and turnover seems to support this assumption. Uptake and mineralization compensation for stream autotrophs suggested by the results of this study is in agreement with results observed at larger scales. Previous studies have shown that in-stream net N uptake (i.e., gross uptake – mineralization) at whole-reach scale is predominantly nil during seasons with high autotrophic activity (Brookshire et al. 2009, Bernal et al. 2012). Overall, these results seem to indicate that autotrophic N turnover have a more homeostatic character than DIN uptake probably due to physiological constraints such as elemental stoichiometry (Dodds et al. 2004); and thus, it does not seem to be particularly influenced by changes in nutrient or light availability, or at least to a lesser extent than DIN uptake.

5.5.4 Summary

The translocation experiment demonstrated to be an efficient and accurate approach to understand the factors influencing the ^{15}N natural abundances of autotrophs as well as to estimate the N uptake and turnover rates of different

autotrophs under different nutrient and light availability. Results from this study allowed evaluating the relative influence of intrinsic and extrinsic factors on the role of autotrophs in stream nutrient retention. Variation in DIN uptake of stream autotrophs was principally driven by nutrient concentration and light incidence, although other extrinsic factors may also be important. Intrinsic control on the autotrophic N uptake seems to be overcome by the extrinsic control that N and light availability exert on uptake rates, except when comparing macrophytes to the rest of autotrophs. Contrastingly, variation in turnover was more evident among autotrophs than among study reaches, suggesting an intrinsic control on N turnover relatively independent of environmental influences. Therefore, despite the rapid and different responses in uptake rates by each autotroph to changes in nutrient and light availability, the role of autotrophic community on the net N retention within the stream channel may be more determined by the intrinsic control on N turnover.

Acknowledgments

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6

Whole-reach implications of spatial heterogeneity in microbial N uptake at the microhabitat scale in stream ecosystems

6.1 Abstract

Ecologists have historically examined stream nitrogen (N) cycling by addressing patterns at the reach scale, from which we have learned that a great deal of the among-streams variation in whole-reach N uptake is influenced by factors operating at larger scales such as the hydrology or the amount of resource subsidies. Nonetheless, the large and not always predictable variation in microbial contribution to whole-reach N uptake points out the need to further understand factors operating at smaller spatial scales, which may dictate the mechanisms driving in-stream N uptake and ultimately explain observed variation in N cycling at the whole-reach scale. Here, we investigated the spatial variability of microbial N uptake at microhabitat scale, the biophysical factors controlling it, and how this variability contributes to explain N cycling at the whole-reach scale. In general, spatial heterogeneity of microbial N uptake at the microhabitat scale was characterized by a mosaic of patches dominated by low N uptake microhabitats and by a low abundance of highly active microhabitats (i.e., hot spots). However, factors influencing microbial N uptake at fine spatial scales differed between microbial assemblages developed on cobbles (epilithon) or in fine benthic organic matter (FBOM) accumulations. Spatial variation at microhabitat scale of epilithon N uptake was mostly driven by flow velocity, which probably influenced the N transfer from the water column to the epilithon layers. Differently, spatial variation at microhabitat scale of FBOM N uptake was controlled by a combination of biophysical factors, which influenced the relevance of stream water N as a nutrient source for microbial assemblages in FBOM. Results from this study demonstrate that the degree of spatial variation in microbial N uptake at fine scales may differ among streams with different

stream-riparian linkages, and how microbial N cycling is strongly related to factors operating at fine spatial scales. Finally, this study highlights that losses of spatial heterogeneity in stream ecosystems can derive in a decreased capacity of streams to process nitrogen.

6.2 Introduction

Streams are highly dynamic and complex ecosystems due to the high degree of spatial heterogeneity and the hydrological interactions operating at different spatial scales (Ward 1989, Ward and Stanford 1995). These physical properties generate multiple niches for benthic aquatic organisms, which ultimately govern the functioning of streams (Jones et al. 1995, Lewis et al. 2007, Battin et al. 2008). Moreover, spatial heterogeneity in streams is hierarchically organized by a set of subsystems, which interact from large (e.g., segment or reach) to small spatial scales (e.g., habitat or microhabitat; Frissell 1986, O'Neill et al. 1986). Within this context, some studies have investigated the relationships between patterns and processes at different spatial scales to explore how spatial heterogeneity at one scale influences the patterns observed at larger scales (Fisher et al. 1998, Dent and Grimm 1999, Poole 2002). Feedbacks between patterns and processes across different scales are based on the principle that patterns observed at a given scale are constrained by factors operating at larger scales and driven by mechanisms acting at smaller scales (sensu Levin 1992).

Nutrient cycling is one of the key ecological processes with relevance at the ecosystem scale. Research addressing nutrient dynamics in streams has been hitherto focused at the whole-reach scale (~100 m), which is the spatial unit where nutrient spiraling metrics are commonly quantified (Webster and Patten

1979, Newbold et al. 1983). Estimates of nutrient uptake, transformation, and retention derived from nutrient spiraling metrics at this scale have been used as an integrative measure to assess and compare nutrient cycling among different streams or over time within a stream. Results have demonstrated that the magnitude and variation among streams of nutrient uptake at the whole-reach scale are strongly constrained by factors operating at larger scales such as stream-riparian interactions (Sabater et al. 2000, Webster et al. 2003, Valett et al. 2008) or resource subsidies from the adjacent catchment (Mulholland et al. 2008, von Schiller et al. 2009). Much less is known, however, about the factors operating at smaller scales, which may affect mechanisms driving in-stream nutrient retention and ultimately explain observed variation in nutrient retention at the whole-reach scale among streams. In this study, we investigate the spatial variability of microbial nitrogen (N) uptake at microhabitat scale (i.e., <1m), the different biophysical factors controlling it, and how this variability contributes to explain N cycling at the whole-reach scale (Figure 6.1). Ultimately, this study aims to provide understanding on mechanisms that may help explaining variation in N uptake among streams.

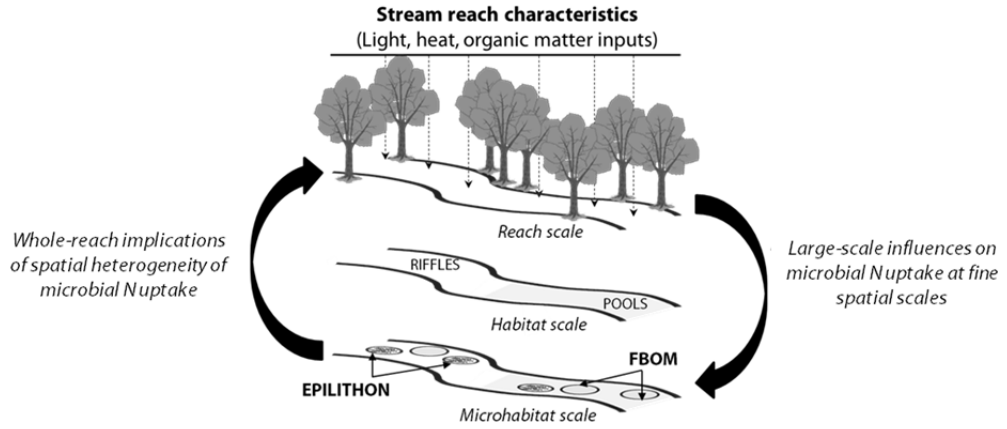


Figure 6.1 General scheme of the main goal of this study illustrating the three spatial scales considered (microhabitat, habitat, and whole-reach); the possible implications of factors driving microbial N uptake at fine scales to whole-reach N uptake (arrow on the left); and how well-known constrains on stream N uptake by factors operating at the reach scale, such as the type of stream-riparian interaction, downscales to influence microbial N uptake (arrow on the right).

6.2.1 Role of microbial assemblages in stream nitrogen uptake

Uptake, transformation, and retention of N in stream ecosystems are mostly driven by microbial assemblages living in the stream benthos, although in some streams other benthic compartments, such as filamentous algae, bryophytes, or macrophytes can substantially contribute to stream N cycling (see Grimm 1987, Mulholland et al. 2000, Riis et al. 2012). Stream microbial assemblages are ubiquitous in stream benthos, but they can exhibit a large variation in their biotic composition, habitat distribution, and the dominant energy and matter sources they require. For instance, microbial assemblages develop on mineral substrata (e.g., rocks, cobbles, gravel and sand) form complex biofilm structures (here referred to as epilithon), which are composed by a mixture of algae, normally dominated by diatoms (Sheath and Cole 1992,

Hamilton et al. 2001), and bacteria (Lock et al. 1984, Lock 1993) embedded in a mucopolisacharide matrix. On the other hand, fine benthic organic matter (here referred to as FBOM) accumulated in depositional zones of the stream channel hosts microbial assemblages mostly dominated by bacteria (Findlay et al. 2002). Despite differences in the nature of epilithon and FBOM, several studies have suggested that both algae and bacteria in these biotic stream compartments play an active role in taking up dissolved nutrients from the stream water column (Ward and Aumen 1986, Hall and Meyer 1998, Romani and Sabater 1999).

Epilithon and FBOM are commonly present in streams; however, the relative abundance of FBOM (i.e., in terms of N standing stocks) is generally larger than that of epilithon (Tank et al. 2000). In contrast, the proportion of biologically active N tends to be lower in FBOM (3–7% in Sanzone et al. 2001) due to the high content of detrital matter in this compartment; and thus, epilithon has normally a high N uptake efficiency (i.e., N uptake per unit of N mass) compared to FBOM (Ashkenas et al. 2004, Dodds et al. 2004, von Schiller et al. 2009). The relative abundance and uptake efficiency of epilithon and FBOM are important factors to determine their contributions to whole-reach N uptake. In this regard, it has been demonstrated that factors operating at large-scale, such as the density of the riparian canopy, can strongly influence the relative abundance of algae in epilithon (Sabater et al. 2000, Simon et al. 2005) and can ultimately determine nutrient uptake at the whole-reach scale (Marti and Sabater 1996, Roberts and Mulholland 2007). Despite recognizing the influences of large-scale factors on the relative importance of microbial N uptake to whole-reach N uptake, there is still a lack of understanding about the large variation in N uptake of epilithon and FBOM observed among streams. In

part, this variation could be driven by differences in riparian canopy cover among streams because it can influence the relative abundance and the particular nature of these two primary uptake compartments. Studies have shown that in open-canopied streams, N uptake of epilithon can be either much higher (Simon et al. 2004, Simon et al. 2007) or slightly lower (Dodds et al. 2000) than N uptake of FBOM. While studies in streams covered by a dense canopy have shown that N uptake of epilithon is lower than N uptake of FBOM either before (Mulholland et al. 2000) or after (Tank et al. 2000) leaf emergence, although similar N uptake for epilithon and FBOM have been also observed (Merriam et al. 2002). Overall, the lack of consistent patterns in the role of epilithon and FBOM to whole-reach patterns of N uptake among streams with different canopy cover suggests that factors operating at smaller scales may be also relevant to further understand the spatial variation in microbial N uptake and how this can influence observed patterns at whole-reach N uptake.

6.2.2 Influence of spatial heterogeneity on stream nitrogen cycling

Large variation in N cycling among different streams can be partly explained by the exceptional spatial heterogeneity of stream ecosystems at either large or fine scales. However, at whole-reach scale, variation in N cycling has been more consistently explained by the effects of riparian canopy (Sabater et al. 2000, Valett et al. 2008), nutrient availability (Mulholland et al. 2008, Hall et al. 2009) and discharge (Peterson et al. 2001, Wollheim et al. 2001) than by factors related to hydraulic and geomorphologic characteristics of the stream channel (but see Martí and Sabater 1996, Valett et al. 1996). At finer spatial scales, Simon et al. (2004) showed differences in N uptake of benthic compartments among habitats, which were principally associated with a higher demand of N by primary producers in riffles, even though their relative abundance was

generally greater in pool habitats. These finding suggests that the spatial configuration of stream reaches associated with the presence and distribution of riffles and pools may also contribute to explain among-stream variation in N uptake because factors operating at habitat scale are relevant for microbial N uptake. However, considering the key role of microbial assemblages on stream N cycling, it is reasonable to expect that factors operating at even finer scales (i.e., microhabitat < 1m) can help explaining observed variation in N uptake at larger scales. In fluvial ecosystems, the influence of spatial heterogeneity at the microhabitat scale has been examined on contrasting processes such as fish foraging (Grossman and Freeman 1987), stream hydrodynamics (Mulholland et al. 1994, Battin et al. 2003), or the composition of invertebrate communities (Ward et al. 1998), but not on biogeochemical aspects, such as N cycling processes. However, recent studies on the variation of the microbial structure at microhabitat scale have indicated its relevance for hydrodynamic retention and biogeochemical processes at the reach scale (Battin et al. 2003).

To our knowledge, despite some theoretical contributions describing feedbacks between structure and function across hierarchical scales in stream ecosystems (e.g., Pringle 1988, Poole 2002, Fisher et al. 1998), process-related studies quantifying spatial heterogeneity and examining controlling factors of N cycling at microhabitat scale are rather scarce. Nevertheless, this information can be relevant to understand the mechanisms that drive observed patterns of N cycling at larger scales (i.e., whole-reach scale).

Here, we quantified the contribution of epilithon and FBOM microbial compartments to whole-reach N uptake in two reaches differing in the degree of canopy cover based on the estimation of patch-specific uptake rates and the relative abundance of the two compartments. We also evaluated the spatial

heterogeneity of N uptake at the microhabitat scale of these two compartments and the main biophysical factors influencing it. Finally, we assessed how spatial heterogeneity in N uptake at microhabitat scale can contribute to explain N uptake patterns observed at the whole-reach scale. This experimental design aimed to contribute to a mechanistic understanding of whole-reach N cycling based on the examination of controlling factors on microbial N uptake operating at different spatial scales.

6.2.3 Study site

Fuirosos is a third order stream draining a catchment of 16.2 km^2, which is located in the Montnegre-Corredor Natural Park (50 km north of Barcelona, northeast Spain), a forested mountain range parallel to the Mediterranean Sea coastline. The climate of this region is typically Mediterranean, with hot, dry summers and cool, wet winters. Typical basal flow discharge ranges between 0 and 25 L/s, but during storms discharge can increase up to 500 L/s (Butturini et al. 2008, Vazquez et al. 2013).

Two reaches differing in the density of riparian vegetation were defined in Fuirosos: a shaded reach (SR) of 315 m, with a dense canopy cover mainly made up of sycamore (Platanus x hispanica), alder (Alnus glutinosa), and hazel (Corylus avellana); and an open reach (OR) of 360 m, where selective harvesting of riparian tree species have considerably reduced the canopy cover. Consequently, leaf area index was on average more than two times lower in OR than in SR, and daily photosynthetic active radiation (PAR) reaching the stream channel and water temperature in OR were higher than in SR (Table 6.1). The streambed morphology of the two reaches was very similar and dominated by cobbles and sand. The OR and SR reaches had similar stream gradient and average wetted width, as well as similar percentages of riffle/pool areal

coverage (OR=65%/35% and SR=58%/42%). Stream hydrology during the study was also very similar in the two reaches, and was characterized by low discharge and shallow water depths (Table 6.1). Dissolved inorganic N (DIN; both as NH_4 and NO_3) concentration was relatively low, especially at the OR (Table 6.1). Soluble reactive phosphorus (SRP) concentration was two times higher in the OR than in the SR (Table 6.1), resulting in a DIN:P ratio of 5.5 and 26.8 at the OR and SR, respectively.

Table 6.1 Geographical, physical and chemical characteristics of the open and shaded stream reaches during the study. Geographical coordinates correspond to the addition point at each study reach. Data correspond to mean values (SD). Means in a row with different superscripts are significantly different (Kruskal-wallis, $p > 0.05$).

Characteristic	Open reach	Shaded reach
Geographical parameters		
Latitude	41º 42' 35.4'' N	41º 41' 24.6'' N
Longitude	2º 34' 33.4'' E	2º 34' 28.1'' E
Reach elevation (m)	93	145
Physical parameters		
Canopy cover (LAI)	1.7 (0.7)^{A}	4.2 (1.1)^{B}
Daily PAR (molquanta/m^2d)	25.9 (14.3)^{A}	7.6 (3.6)^{B}
Daily mean temperature (ºC)	16.1 (0.3)^{A}	15.6 (0.3)^{B}
Stream gradient (%)	1.4	2.5
Discharge (L/s)	7.4	6.5
Wetted width (m)	2.9 (0.6)^{A}	3.1 (0.5)^{A}
Depth (cm)	12.1 (6.6)^{A}	11.5 (6.3)^{A}
Water velocity (m/s)	0.03(0.005)^{A}	0.03 (0.005)^{A}
Chemical parameters		
NH_4 concentration (µg N/L)	16.9 (5.1)^{A}	15.7 (4.9)^{A}
NO_3 concentration (µg N/L)	22.9 (6.6)^{A}	46 (9.3)^{B}
SRP concentration (µg P/L)	7.2 (0.7)^{A}	2.3 (1.2)^{B}

6.3 Methods

6.3.1 Experimental design

At each study reach, we defined a total of 8-9 sampling stations along the entire reach, a sub-reach located between stations 1-5 and characterized by consecutive sequence of three riffles and three pools habitats included a total of 30 sampling points at microhabitat scale (15 within riffles and 15 within pools). In order to estimate the N cycling in microbial assemblages across these three different spatial scales (whole-reach, habitat, and microhabitat), we conducted a ^{15}N addition at the OR on May 5th 2012 and another one two days later at the SR, in which an identical sampling design was performed. Samples of water and organisms collected at each reach station were used to estimate whole-reach N uptake and the relative contribution of different benthic compartments to it following procedures from Mulholland et al. (2000) and von Schiller et al. (2009). While, samples collected at each microhabitat station were used to characterize the spatial heterogeneity in N cycling of epilithon and FBOM at the microhabitat scale.

6.3.2 ^{15}N additions

At each study reach, we conducted a 24-h constant rate addition of ^{15}N-NH_4 (as 99% enriched $^{15}NH_4Cl$). The $^{15}NH_4Cl$ additions were designed to produce a 10,000-fold increase in the ^{15}N:^{14}N ratio of stream water N-NH_4, which elevated ambient N-NH_4 concentration by 6 and 5% at the OR and SR, respectively. Additionally, before (i.e., background sampling), after ~24h of the beginning of the $^{15}NH_4$ addition (i.e., plateau sampling), and ~14h after the end of the $^{15}NH_4$ addition (i.e., post-addition sampling) we collected water samples for nutrient chemistry and isotopic ^{15}N signal at each station along the reach. All water

samples were immediately filtered through ashed Albet (Barcelona, Spain) FVF glass fiber filters (0.7µm pore size). Filtered samples for nutrient chemistry (15 mL, two replicates per station) were stored on ice in the field, and then stored at -20ºC in the laboratory until analysis. Samples for $^{15}NH_4$ (4 L, one replicate per station) were immediately processed as explained in the laboratory methods section, while samples for $^{15}N\text{-}NO_3$ (1 L, one replicate per station) were stored on ice in the field and then kept frozen until further analysis. Two days after the $^{15}NH_4$ additions were performed, a constant rate addition of NaCl (used as a conservative tracer) was conducted to characterize each reach in terms of hydraulics. Changes in the concentration of the conservative tracer over time were assessed by the measurement of conductivity as a surrogate of chloride concentration. During this addition, conductivity was automatically recorded at the first and the last sampling stations along the reaches every 10s using WTW (Weilheim, Germany) 3110 conductivity meters. Additionally, at plateau conditions we measured conductivity at all sampling stations to assess the dilution from lateral inflow along the reach.

6.3.3 Characterization of canopy cover and benthic compartments at the whole-reach scale

Differences in canopy cover between the two reaches were assessed by comparing leaf area index (LAI) measurements in and out of the stream channel during the study period using a Sunfleck PAR ceptometer (ICT international, Australia). Water temperature and PAR was recorded at each station, as well as at each riffle and pool for the microhabitat survey, at 20–min intervals from one week before to one week after each ^{15}N addition using a waterproof temperature and light data loggers (HOBO Pendant® UA-002-64). Two days prior the $^{15}N\text{-}NH_4$ release, we determined the wetted width, the

percent reach cover of riffle and pool habitats, and the percent reach coverage of different substrates (i.e., sand, pebbles, cobbles, boulders, bedrocks). One day prior the ^{15}N addition, we visually estimated the percent reach coverage of different benthic compartments (i.e., epilithon, FBOM, and leaf litter) at 5 m intervals along the reach. The percent coverage of filamentous algae, bryophytes, and macrophytes was estimated by determining their presence-absence at 10cm intervals at stream transects done at 5 m intervals along the reach. Standing stocks of the different benthic compartments at reach scale were calculated using average values from patch-specific measures of dry mass and ash free dry mass (AFDM) at each sampling station. For these measures, epilithon was sampled by scraping the surface of each selected cobble and filtering it onto ashed pre-weighted FVF glass fiber filters. The sampled surface of each cobble was estimated by covering it with aluminum foil and using a weight-to-area relationship. FBOM was collected with a syringe from the material suspended by manual agitation of the surface (1-3 cm) sediment in a known volume within a 104cm^2 plastic corer placed into the sediments (~15cm). The collected aliquot of FBOM was then filtered through ashed pre-weighted FVF glass fiber filters. Coarse benthic organic matter (CBOM) was sampled by collecting all organic matter found within a 104cm^2 area. Suspended particulate organic matter (SPOM) was sampled by filtering 2-6L of stream water through ashed pre-weighted FVF glass fiber filters. Filamentous algae, bryophytes, and macrophytes were sampled by scraping all material from a known area covered by these compartments. All samples were dried at 60 ^oC in the laboratory and stored until analysis for AFDM, elemental content (C and N), and ^{15}N and ^{13}C analysis (see *Laboratory methods*). Reach-weighted N standing stock of each benthic compartment was calculated using their

percent reach coverage and patch-specific biomass. To estimate the relative contribution of each compartment to whole-reach N uptake, at the ^{15}N addition plateau we collected composite samples of each compartment for the ^{15}N analysis at each station (3-5 replicates) following the methods described for biomass samples.

6.3.4 Characterization of hydraulic properties and microbial assemblages at the microhabitat scale

Prior each ^{15}N-NH_4 addition, a total of 30 samples at microhabitat scale for both epilithon and FBOM compartments were randomly collected following the stratified sampling design of ~5 samples at each of the 3 riffles and pools selected within each reach. The location of each microhabitat sample was recorded based on the nearest sampling station. Before the collection of each epilithon and FBOM sample, we measured local water depth and velocity using a velocity meter (Schiltknecht, Messtechnik), and quantified the relative abundance of green algae, cyanobacteria, and diatoms of these compartments using a BenthoTorch (bbe, Germany) portable fluorometer probe. Once these measurements were done, samples of epilithon and FBOM were collected for ash free dry mass (AFDM), C and N elemental content, and ^{15}N and ^{13}C analysis following the same procedures described for the whole-reach scale sampling. At each microhabitat, we also collected two subsamples of epilithon and FBOM for analysis of extracellular activities (4mL) and one subsample of epilithon for analysis of extracellular polymeric substances (EPS, 10mL). These samples were stored on ice in the field, and then refrigerated at 4 ^oC and stored at -20^oC in the laboratory until further processing and analysis (see *Laboratory methods*). At the plateau sampling, we collected samples of epilithon and FBOM for the ^{15}N analysis (30 per compartment and reach) at the same locations where

background samples had been collected following the methods described for biomass samples.

6.3.5 Laboratory methods

Stream water samples were analyzed for ammonium ($N\text{-}NH_4$), nitrate ($N\text{-}NO_3$) and SRP concentrations following standard colorimetric methods (APHA 1995) on an Automatic Continuous Flow FUTURA–ALLIANCE Analyzer at the CEAB-CSIC Biogeochemistry laboratory. The ^{15}N content of stream water NH_4 and NO_3 was determined following the ammonia diffusing procedure adapted from Holmes et al. (1998) and Sigman et al. (1997), respectively. For the analysis of $^{15}N\text{-}NH_4$ signatures, water samples were amended with 3 g L^{-1} of MgO and 50 g L^{-1} of NaCl and a Teflon filter packet. The Teflon filter packets consisted of a pre-combusted Whatman GF/D glass fiber filter (1 cm diameter), which was previously acidified by adding 25 µl of K_2HPO_4 to trap the diffusing NH_3, sealed within a packet formed by two pieces of Teflon tape. Immediately after adding the packets, the samples were tightly capped and incubated at 40 ºC in a shaker table during three weeks. For $^{15}N\text{-}NO_3$ analysis, NH_4 was initially removed by boiling the samples with 3.0 g of MgO and 5.0 g of NaCl. The resulting samples (0.1 L) were then transferred into acid-washed HDPE plastic bottles that were amended with 0.5 g MgO, 0.5 g Devarda's alloy and a Teflon filter packet. Immediately after adding the Teflon filter packets, samples were tightly capped and incubated at 60ºC for 48 h to reduce NO_3 to NH_4, and then incubated in a shaker table for seven days at room temperature. At the end of the incubations, filter packets from $^{15}N\text{-}NH_4$ and $^{15}N\text{-}NO_3$ samples were removed from the bottles, placed in scintillation vials, and dried in a desiccator until further processing. Further details on these methods can be found in von Schiller et al. (2009).

Biomass samples of all benthic compartments were oven-dried at 60ºC for 72 hours, weighted to the nearest 0.1 mg on a Sartorius (Goettingen, Germany) MC1 analytical balance, and combusted at 500ºC for 5-6 h to estimate AFDM. Dry mass samples for elemental and isotopic analyses of carbon (C) and N were ground, thoroughly homogenized, weighted to the nearest 0.001 mg on a Mettler- Toledo (Greifensee, Switzerland) MX5 microbalance, encapsulated in tins, and stored until analysis. These samples were analyzed at the UC Davis Stable Isotope Facility (California, USA). The C and N content (as a percentage of total dry mass) and their stable isotope signatures for all samples were determined using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). All ^{15}N and ^{13}C signatures are expressed in terms of delta values ($\delta^{15}N$ and $\delta^{13}C$), which are parts per thousand (‰) differences from a standard.

The extracellular activities of the enzymes leucine-aminopeptidase (EC 3.4.11.1) and β-D-1, 4-glucosidase (EC 3.2.1.21) in the epilithon and FBOM were measured spectrofluorometrically (UV-2401PC, ultraviolet–visible spectrometer, Shimadzu) 24 hours after collection, by using the fluorescent-linked substrates L-leucine-4-methyl-7-coumarinylamide (Leu-AMC, Sigma–Aldrich) and 4-methylumbelliferyl β-D-glucopyranoside (4-MUF β -D-glucoside, Sigma–Aldrich), as described by Proia et al. (2012). We used substrate concentrations of 0.3 mM based on saturation curves performed previously for both epilithon and FBOM samples from the study stream.

The extraction of EPS from the epilithon was performed using a cation exchange resin (Dowex Marathon C, Na-form, srongly acid, Sigma-Aldrich) following the procedure described in Romaní et al. (2008) and Ylla et al. (2010). The polysaccharide content in the EPS fraction was measured by the phenol–

sulfuric acid assay (Dubois et al., 1956). Standards of glucose (0–200 µg/mL) were also run with the set of samples. Results for EPS are given as glucose equivalents per cm^2 of epilithon surface area.

6.3.6 Parameter calculations

Calculations of N uptake rates at both whole-reach and compartment-specific scales were done following methods described by Mulholland et al. (2000). In brief, whole-reach ammonium uptake length (NH_4-S_w; m) was calculated as the inverse of the slope of the regression between natural logarithm of the ^{15}N-NH_4 flux and downstream distance with data from plateau sampling corrected by data from background sampling (Newbold et al. 1981). The ^{15}N-NH_4 flux (mg ^{15}N/s) at each station was calculated using the following equation:

$$\delta^{15}NH_4\ flux = (\delta^{15}NH_4/1000) \times 0.003663 \times Q \times [NH_4] \quad (1)$$

where $\delta^{15}NH_4$ are background-corrected values, Q is stream discharge at each station estimated from a dilution factor based on the conservative tracer data, and [NH_4] is NH_4 concentration. NH_4-S_w was then used to estimate uptake velocity (NH_4-V_f; m/min) and areal NH4 uptake rate (NH_4-U; mgN/m^2d) following equations described in (Webster and Valett 2006). Whole-reach rates of ^{15}N-NH_4 nitrification and regeneration of assimilated ^{15}N-NH_4 were estimated from the longitudinal profiles in the reaches of ^{15}N-NH_4 and ^{15}N-NO_3 fluxes from water samples collected 14h after the end of the ^{15}N addition by using the Microsoft Excel 2010 Solver and following model procedures described by Mulholland et al. (2000).

The amount of added ^{15}N mass retained in each benthic compartment ($^{15}N_{biomass}$; mg$^{15}N/m^2$) at the end of the ^{15}N addition and at each sampling station

was determined using the compartment-specific background-corrected $\delta^{15}N$ values at plateau and the total reach-weighted N standing stock (TN) of each compartment following the equation:

$$^{15}N_{biomass} = (\delta^{15}N_{biomass}/1000) \times 0.003663 \times (TN) \quad (2)$$

The total ^{15}N retained in the reach and the relative contribution of each compartment to this retention was estimated from a mass balance between added ^{15}N mass (concentration in the solution, addition flow rate, and addition duration) and the sum of ^{15}N biomass in each compartment measured at the end of the addition along the reach. Total ^{15}N biomass in each compartment along the reach was estimated by integrating values obtained at each station using the best-fit exponential decline model. Total ^{15}N retained in the reach was calculated as the sum of integrated ^{15}N mass values for each compartment. The relative contribution of each compartment to total ^{15}N retained was estimated as the fraction of ^{15}N retained by the compartment relative to the total ^{15}N retained.

Compartment-specific NH_4 uptake rates (mgN/m^{2}d) for each benthic compartment were calculated based on ^{15}N data from the plateau sampling using the following equation:

$$NH_4\ uptake\ rate = ((\delta^{15}N/1000) \times 0.003663 \times N_{biomass}/\delta^{15}NH_4)/T \quad (3)$$

where $\delta^{15}N$ are the plateau values of each compartment, $N_{biomass}$ is N standing stock of each compartment (mgN/m^2), $\delta^{15}NH_4$ are stream water $\delta^{15}N$-NH_4 plateau values, and T represents the elapsed time since the start of the ^{15}N-NH_4 addition (24 hours). Compartment-specific NH_4 uptake rates for epilithon

and FBOM were calculated at both whole-reach and microhabitat scales. To calculate compartment-specific NH_4 uptake rates at whole-reach scale (i.e., data from each station) we used the reach-weighted $N_{biomass}$ of each compartment. The sum of whole-reach NH_4 uptake rates for each compartment represents the total assimilatory NH_4 uptake rate at whole-reach scale. To calculate compartment-specific NH_4 uptake rates of epilithon and FBOM at the microhabitat scale (i.e., data from microhabitat samples) we used the patch-specific $N_{biomass}$ of each microhabitat sample

Turnover rate (d^{-1}) for each benthic compartment at the whole-reach scale was calculated using the slope of the regression between ln-transformed $\delta^{15}N$ values from post-addition samples collected at the stations 1-3. Turnover rates of epilithon and FBOM at the microhabitat scale were estimated by dividing compartment-specific NH_4 uptake rates (mg N/m^2d) by the patch-specific $N_{biomass}$ of each sample.

6.3.7 Statistical analysis

Comparisons of biotic, physical, and chemical parameters between the two study reaches and between epilithon and FBOM at the microhabitat scale were done using separate Kruskal-Wallis nonparametric ANOVA analyses (reach and compartment as factors) followed by a single-step multiple comparison procedure (Siegel and Castellan 1988). To examine how biotic and physical variables at microhabitat scale contributed to the observed spatial variation of the two microbial compartments we conducted separate principal component analyses (PCA) for each compartment (epilithon and FBOM) considering data from the two reaches together. Variables were standardized and a correlation matrix was used for the PCA.

Variability in uptake and turnover rates of microbial assemblages at the microhabitat scale was examined by calculating the distribution and the inequality of the observed data over each study reach. Because original data for uptake and turnover rates did not meet normality requirements, we used kernel density estimations (KDE, n=30 for each assemblage and reach, bandwidth=0.1) to estimate the probability density function of uptake and turnover rates of epilithon and FBOM. Comparisons of probability distributions among open and shaded reaches for uptake and turnover rates were done using Kolmogorov-Smirnov tests. The spatial inequality in uptake and turnover rates of epilithon and FBOM at the microhabitat scale was calculated using Lorenz curves and Gini coefficients. The Lorenz curve is a plot of the cumulative percent of variable response (here, uptake rates) versus the cumulative percent of observations ranked from lowest to highest. From this plot, we calculated Gini coefficients (G), which range from 0 to 1, and measure the inequality within a distribution. For G=0, all observations make an equal contribution to uptake and turnover, as G increases towards 1 the inequality increases being G=1 a situation where a single observation account for the 100% of the measured uptake rates.

The influence of biotic and physical factors on the variation in uptake and turnover rates of epilithon and FBOM at microhabitat scale was examined by performing multiple lineal regression analysis with pooled data from the two reaches (i.e., considering all variation). LAI, daily PAR, and water temperature were not included in the model since these variables were not measured at the same spatial resolution than the rest of the explanatory variables. Prior to regression analysis, variables were normalized by log transformation when they did not meet the requirements for normality. Variables included in the

multiple regression models were selected by using a forward stepwise approach. We used Bayesian methods to check if the observations were consistent with the fitted model for each compartment and reach. The Bayesian method is based on the use of the posterior predictive distribution. Briefly, this method allows comparisons between probability distributions of response values calculated from the multiple regression models and each observed data. If many observed response values fall far from the center of the posterior predictive distribution, then the adequacy and predictive power of the model are poor. Spatial heterogeneity at microhabitat scale in NH_4 uptake rates of epilithon and FBOM was mapped using significant regressions between water depth and NH_4 uptake rates observed at each study reach, and cross-sectional profiles (every 20cm) of water depth every 20m along the study reach. Regression estimations of NH_4 uptake rates by each reach were mapped in a three-dimensional plot (x=reach length, y=reach width, z=NH_4 uptake rate).

Finally, we scaled up the results observed for epilithon and FBOM at the microhabitat scale to the whole-reach scale. We examined how variation in microbial NH_4 uptake is filtered across three different scales (reach, habitat, and microhabitat) by calculating the total NH_4 uptake variance observed at each scale. We also calculated the standard error of the mean (SEM) of microbial NH_4 uptake as a proxy of the variability of the mean estimation for microbial NH_4 uptake at each scale. In addition, we also examined the relevance of highly active microhabitats of microbial NH_4 uptake at whole-reach scale by subtracting different percentages of the spatial variation in microbial NH_4 uptake at the microhabitat scale, starting from the highest to the lowest uptake rates observed in each reach, and then recalculating the whole-reach NH_4

uptake after each different subtraction. All statistical tests were done using R 3.0.0 (R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/).

6.4 Results

6.4.1 Whole-reach ^{15}N uptake rates

Values of NH_4-Sw were relatively short and similar in the two reaches and averaged 58 m, while NH_4-Vf and total NH_4-U were slightly higher at the OR than at the SR (Table 6.2). Total regeneration of N assimilated by stream biotic compartments was larger at the SR than at OR (Table 6.2). Assimilatory NH_4 uptake calculated from the sum of compartment-specific NH_4 uptake rates accounted for 24% and 9% of the total NH_4 uptake at the OR and SR, respectively (Table 6.2). However, nitrification rates were around two orders of magnitude lower than assimilatory NH_4 uptake in each study reach (i.e., ~0.4 mgN/m^2d), indicating that assimilatory NH_4 uptake accounted for a large proportion of NH_4-U estimated from the uptake length. Both assimilatory NH_4-Vf and NH_4-U were much higher at the OR (Table 6.2), where the number of different benthic compartments contributing to assimilatory NH_4 uptake was greater than at the SR.

Table 6.2 Summary of total and assimilatory NH_4 uptake rates in each study reach. Parameters of total NH_4 uptake were calculated from the decay in tracer ^{15}N-NH_4 flux along the reach, while parameters for assimilatory NH_4 uptake were calculated as the sum of compartment-specific NH_4 uptake rates at whole-reach scale for each measured compartment. Total regeneration is estimated from the tracer ^{15}N-NH_4 flux in the water column after 14h of the end of the addition.

Parameter	Open	Shaded
TOTAL UPTAKE		
NH_4-Sw (m)	55.73	60.45
NH_4-Vf (m/min)	$3.40x10^{-3}$	$2.15x10^{-3}$
NH_4-U (mgN/m^2d)	73.38	57.03
Total N regeneration (d^{-1})	0.16	0.52
ASSIMILATORY UPTAKE		
NH_4-Sw (m)	233.95	667.20
NH_4-Vf (m/min)	$8.04x10^{-4}$	$1.95x10^{-4}$
NH_4-U (mgN/m^2d)	17.37	5.18
Percentage of total NH_4 Uptake	23.6%	9.1%

6.4.2 Contribution of benthic compartments to whole-reach NH_4 uptake rates

Nitrogen biomass ($N_{biomass}$) in the reaches was dominated by epilithon and FBOM, even though FBOM covered ~25% of the stream bottom at each reach (Table 6.3). N biomass in autotrophic compartments was higher at the OR than at SR, with large N standing stocks in filamentous algae (*Cladophora glomerata* and *Lemanea sp*), bryophytes, and macrophytes (Table 6.3). In addition, the sum of N standing stocks in detrital compartments (FBOM and CBOM) was also much higher at the OR than at the SR because FBOM showed almost ten times larger $N_{biomass}$ than any other compartment at this reach (Table 6.3).

Cyanobacteria (*Oedogonium sp.*) was the compartment showing lowest $N_{biomass}$, especially at the OR; whereas CBOM was almost two times higher at the SR than at the OR but represented less than 10% of the total $N_{biomass}$ at the two reaches (Table 6.3).

Microbial NH_4 uptake (epilithon and FBOM) had a high and similar contribution to whole-reach NH_4 uptake at the two reaches (57% at the OR, 60% at the SR). The sum of compartment-specific NH_4 uptake rate at the OR was strongly dominated by FBOM, followed by *Cladophora glomerata*, epilithon, and bryophytes (Table 6.3). The contribution of the rest of studied compartments to sum of compartment-specific NH_4 uptake rate was mostly irrelevant (Table 6.3). At the SR, compartment-specific NH_4 uptake rates were similar to the ones at the OR except for the FBOM, which was much lower at the SR (Table 6.3). In this reach, epilithon was the compartment with higher contribution to sum of compartment-specific NH_4 uptake rate at reach scale (Table 6.3).

Table 6.3 Reach-weighted N standing stocks, compartment-specific NH_4 uptake, and turnover rates for the different benthic compartments at the two reaches.

Biomass compartment	N standing stock (mgN/m^2)		NH_4 uptake rate (mgN/m^2d)		Contribution to whole-reach NH_4 uptake (%)		N turnover (d^{-1})	
	Open	Shaded	Open	Shaded	Open	Shaded	Open	Shaded
Epilithon	16.09	32.84	2.80 (0.65)	2.23 (0.29)	16.1 (3.7)	42.8 (5.6)	1.244	0.374
FBOM	164.63	45.89	7.23 (2.25)	0.94 (0.30)	41.6 (12.9)	17.9 (5.7)	0.261	0.029
Filamentous algae								
Cladophora glomerata	10.68		5.13 (0.99)		29.5 (5.7)		0.440	
Lemanea sp.	4.30	12.56	0.72 (0.28)	0.58 (0.22)	4.2 (1.6)	11.1 (4.2)	0.440	0.494
Cyanobacteria								
Oedogonium sp.	0.91	5.16	0.22 (0.03)	0.67 (0.27)	1.2 (0.2)	12.9 (5.2)	0.183	0.155
Bryophytes	12.06	8.89	1.12 (0.37)	0.68 (0.30)	6.4 (2.1)	13.1 (5.7)	0.090	0.042
Macrophytes	5.49		0.13 (0.03)		0.7 (0.2)		0.472	
CBOM	4.37	8.14	0.02 (0.01)	0.12 (0.13)	0.1 (0.0)	2.3 (2.5)	0.152	0.450
TOTAL	218.53	113.48	17.36 (4.61)	5.21 (1.51)				

Nitrogen turnover rates of epilithon was one order of magnitude faster than that of any other compartment at the OR, but within the same range of other compartments at the SR (Table 6.3). FBOM and bryophytes were the compartments showing lower N turnover rates at both study reaches (Table 6.3). Low turnover rates of FBOM combined with its large contribution to assimilatory NH_4 uptake at the OR are in agreement with the lower N regeneration observed for this reach (Table 6.2 and 6.3).

6.4.3 Contribution of benthic compartments to whole-reach N retention

The total ^{15}N tracer retained by benthic compartments at the end of the addition accounted for 17.1 and 6.1% of added ^{15}N at the OR and SR, respectively. Retention in each study reach was dominated by microbial compartments, which accounted for ~60% of the total N retained by the benthic compartments (Figure 6.2). However, microbial N retention was dominated by FBOM at the OR and by epilithon at the SR (Figure 6.2). The remaining ~40% of total ^{15}N retention was mostly attributed to *Cladophora glomerata* (28.5%) at the OR, and equally divided among *Lemanea sp.*, *Oedogonium sp.*, and bryophytes at the SR (Figure 6.2).

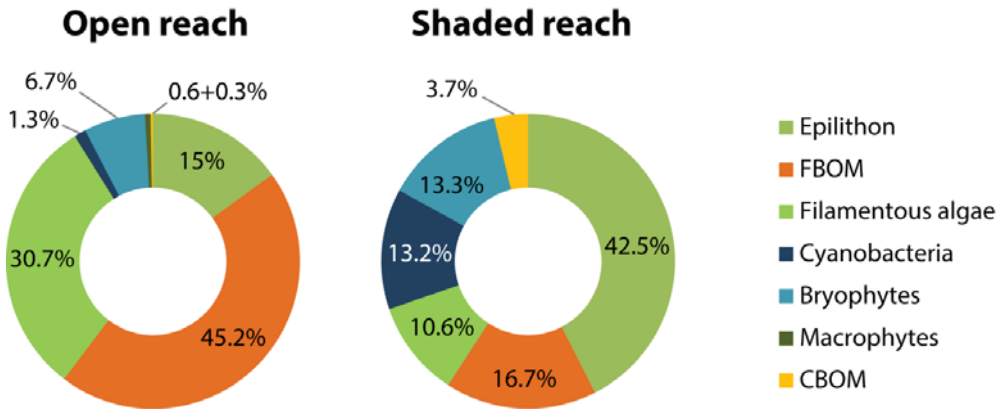


Figure 6.2 Results from the mass balance of tracer ^{15}N mass retained in the benthic stream compartments after 24h of ^{15}N addition showing the percentage contribution of each compartment to total ^{15}N retention at each study reach.

6.4.4 Characterization of hydraulics and microbial assemblages at the microhabitat scale

Variation in water depth measured above microhabitat samples was similar among the two reaches and microbial assemblages, but water velocity measured over epilithon samples was significantly higher at the OR (Table 6.4). Epilithon showed greater percentages of organic matter content and much lower microhabitat variation than FBOM, but no clear differences between the two study reaches were observed for any of the two microbial compartments (Table 6.4). Similarly, N content in epilithon was higher than in FBOM, and significantly lower at the OR compared with the SR in the case of epilithon (Table 6.4). N biomass was on average one order of magnitude higher in FBOM (44.4 ± 22.6 μg/cm^2) than in epilithon (4.4 ± 2.5 μg/cm^2), and significantly lower at the OR for epilithon (Table 6.4). The C:N ratio was higher in FBOM than in epilithon and showed no significant differences between the two reaches for any of the two compartments (Table 6.4). Algal biomass (measured as chlorophyll *a* content) was generally higher and less variable in epilithon than

in FBOM. Chl-*a* showed significant differences between the two reaches only for epilithon, with higher values at the SR (Table 6.4). Content of green algae was higher in epilithon than in FBOM, and in epilithon it was significantly higher at the OR than at the SR (Table 6.4). Content of cyanobacteria and diatoms was within the same range in epilithon and FBOM. In epilithon, content of these two groups was higher at the SR, while in FBOM it was higher at the OR (Table 6.4). Ratios of chl-*a*:AFDM showed lower mean values and higher spatial variation in FBOM than in epilithon (Table 6.4).

Extracellular enzymatic activities (EEA) were generally higher and more variable in epilithon than in FBOM (Table 6.4). EEA at the OR were on average higher than at the SR in epilithon, whereas the reverse pattern was found for EEA in FBOM (Table 6.4). Content of extracellular polymeric substances (EPS) in epilithon did not differ between the two reaches (Table 6.4). For both epilithon and FBOM, natural abundance of $\delta^{13}C$ showed no significant differences between the study reaches; whereas $\delta^{15}N$ signatures were similar between the two microbial compartments, but higher in the OR and the SR for epilithon and FBOM, respectively (Table 6.4).

Table 6.4 Microhabitat variation in hydraulics and characteristics of epilithon and fine benthic organic matter within each study reach. Data correspond to mean values (coefficient of variation: CV; n=30 for each compartment and in each reach) and *p*-values are for Kruskal-Wallis tests (reach as a factor).

Parameter	Eplitihon			Fine benthic organic matter		
	Open	Shaded	*p* -value	Open	Shaded	*p* -value
Water depth (cm)	10.1 (63.4)	11.2 (62.5)	0.681	13.9 (45.3)	11.9 (46.2)	0.379
Water velocity (m/s)	0.2 (108)	0.06 (166.7)	**<0.05**	0.04 (75)	0.05 (120)	0.344
Organic matter content (%)	60.3 (88.5)	53.4 (24.5)	0.268	81.9 (4.1)	78.6 (7.4)	0.198
Nitrogent content (%)	0.4 (25)	0.5 (40)	**<0.001**	0.3 (33.3)	0.3 (33.3)	0.314
Nitrogen biomass (µgN/cm^2)	3.4 (32.3)	5.4 (72.2)	**<0.001**	63.3 (59.6)	25.4 (29.5)	0.174
Molar C:N	11.7 (12.8)	9.86 (10.1)	0.061	12.6 (9.5)	13.2 (9.1)	0.151
Green algae biomass (µg/cm^2)	0.5 (100)	0.3 (200)	**<0.001**	0.001 (900)	0.01 (300)	0.156
Cyanobacteria biomass (µg/cm2)	0.3 (66.6)	0.7 (71.4)	**<0.001**	0.3 (66.6)	0.2 (150)	**<0.05**
Diatoms biomass (µg/cm^2)	1.1 (45.4)	2.6 (53.8)	**<0.05**	1.3 (61.5)	1.1 (72)	0.426
Total chl-*a* (µg/cm^2)	1.8 (44.4)	3.6 (47.2)	**<0.001**	1.6 (62.5)	1.3 (76.9)	0.267
Chl *a* : AFDM	0.6 (50)	1.1 (72.7)	0.124	0.06 (116.7)	0.09 (100)	0.158
Aminopeptidase activity (µmol AMC/gAFDM h)	76.9 (42.5)	29.1 (33.6)	**<0.001**	6.9 (20.3)	9.6 (48.9)	**<0.001**
β-glucosidase activity (µmol MUF/gAFDM h)	3.4 (50)	1.6 (93.7)	**<0.05**	1.1 (36.4)	2.5 (24)	**<0.001**
Extracellular polymeric substances (µg glucose-eq/cm^2)	18.3 (38.8)	21.3 (26.3)	0.037			
δ^{13}C signature (‰)	-27.6 (11.9)	-26.5 (9.1)	0.171	-28.2 (2.8)	-28.5 (2.4)	0.162
δ^{15}N signature (‰)	2.7 (22.2)	1.9 (31.6)	**<0.05**	2.3 (65.2)	3.2 (0.9)	**<0.05**

Spatial variability in epilithon and FBOM characteristics at microhabitat scale was similar and mostly attributed to algae abundance and EEA, although it showed contrasting patterns in differences between reaches depending on each compartment. When all variables for epilithon were considered together in a PCA, epilithon from OR and SR were clearly separated along the first component of the PCA, which explained 35.2% of the variance (Figure 6.3A). Nitrogen content (%N, $\delta^{15}N$, and C:N ratio) and EEA were the most significant variables explaining differences in epilithon between reaches, with higher leucine-aminopeptidase activity and C:N ratios at the OR (Figure 6.3A). The second component accounted for 16.7% of the variance and mostly showed spatial variation at microhabitat scale within epilithon of each particular reach, especially for the OR (Figure 6.3A). Variation along the second PCA component was mostly attributed to differences in the relative abundances of different algal groups (green algae, diatoms, and cyanobacteria) and to $\delta^{13}C$ natural abundances of epilithon (Figure 6.3A). In contrast, results from the PCA with data for FBOM assemblages did not clearly separated FBOM from the two reaches either for the first (24.3% of explained variance) or the second (18.6% of explained variance) components of the PCA (Figure 6.3B). Nevertheless, variation between and within reaches in FBOM characteristics was principally attributed to differences in %N, EEA activities, and algae abundances as with epilithon, despite this did not coincided with clear differences between reaches (Figure 6.3B). Unlike epilithon, FBOM at the SR showed higher EEA activities and lower cyanobacteria and diatoms abundances than at the OR (Figure 6.3B).

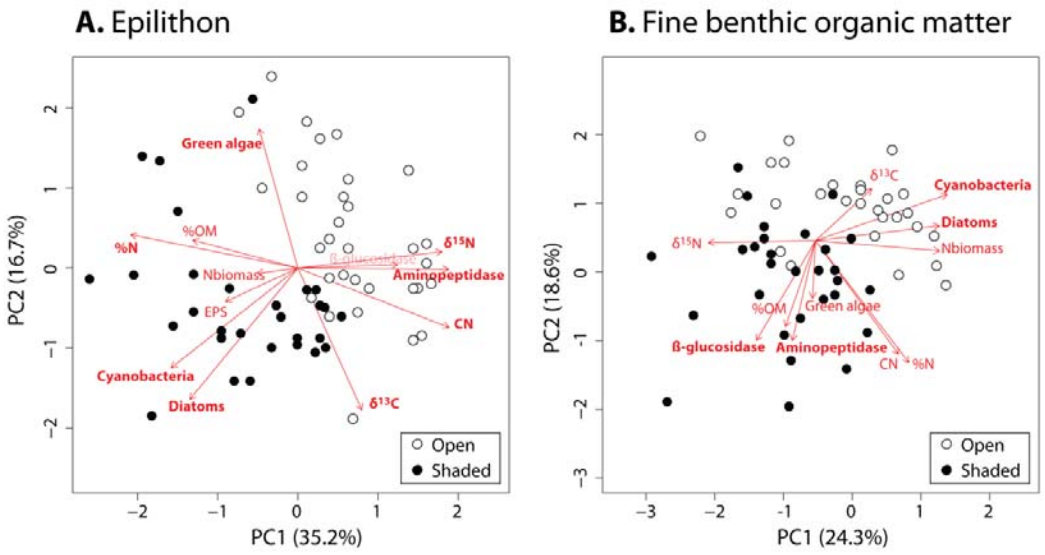


Figure 6.3 Principal components analysis (PCA) of variables associated with epilithic (a) and FBOM (b) microbial assemblages at the microhabitat scale. The percentage of variability explained by each PCA component is indicated in brackets on each axis.

6.4.5 Spatial heterogeneity in N cycling rates of microbial assemblages at the microhabitat scale

Spatial variation of NH_4 uptake rate at microhabitat scale was slightly higher for epilithon than for FBOM, regardless of the study reach (APPENDIX 6.1). NH_4 uptake rate of epilithon showed higher coefficient of variation (CV) at OR than at SR (CV_{OR}=94%, CV_{SR}=79%), while no differences between reaches in CV were observed for NH_4 uptake rate of FBOM (CV_{OR}=62%, CV_{SR}=67%; APPENDIX 6.1). Nevertheless, differences in average NH_4 uptake rate between the two reaches were much larger for FBOM than for epilithon, with rates being 3 times higher at the OR (APPENDIX 6.1). Probability distribution for uptake and turnover rates of epilithon were characterized by a dominance of lower values and relatively few high rates at both stream reaches (Figure 6.4A and C). In contrast, the

distribution of uptake and turnover rates of FBOM was significantly different between the two reaches (Figure 6.4B and D). NH_4 uptake rates of FBOM at the OR showed a more normal distribution compared to those at the SR (Figure 6.4B). Dominance of low NH_4 uptake rates, especially for epilithon, resulted in large inequality of microbial NH_4 uptake at both reaches. Lorenz curves showed that only 20% of the total observations corresponding to the highest patch-specific NH_4 uptake rates observed for epilithon accounted for more than 60% of the sum of NH_4 uptake rates of epilithon (Figure 6.5A). Similarly, the highest patch-specific NH_4 uptake rates observed for FBOM accounted for ~20% of the total observations but for ~40% of the NH_4 uptake rates (Figure 6.5B). These results indicate that patch-specific NH_4 uptake rates of epilithon were more unequally distributed over the stream reach (i.e., higher Gini coefficients) than NH_4 uptake rates of FBOM, regardless of the study reach (Figure 6.5A and B).

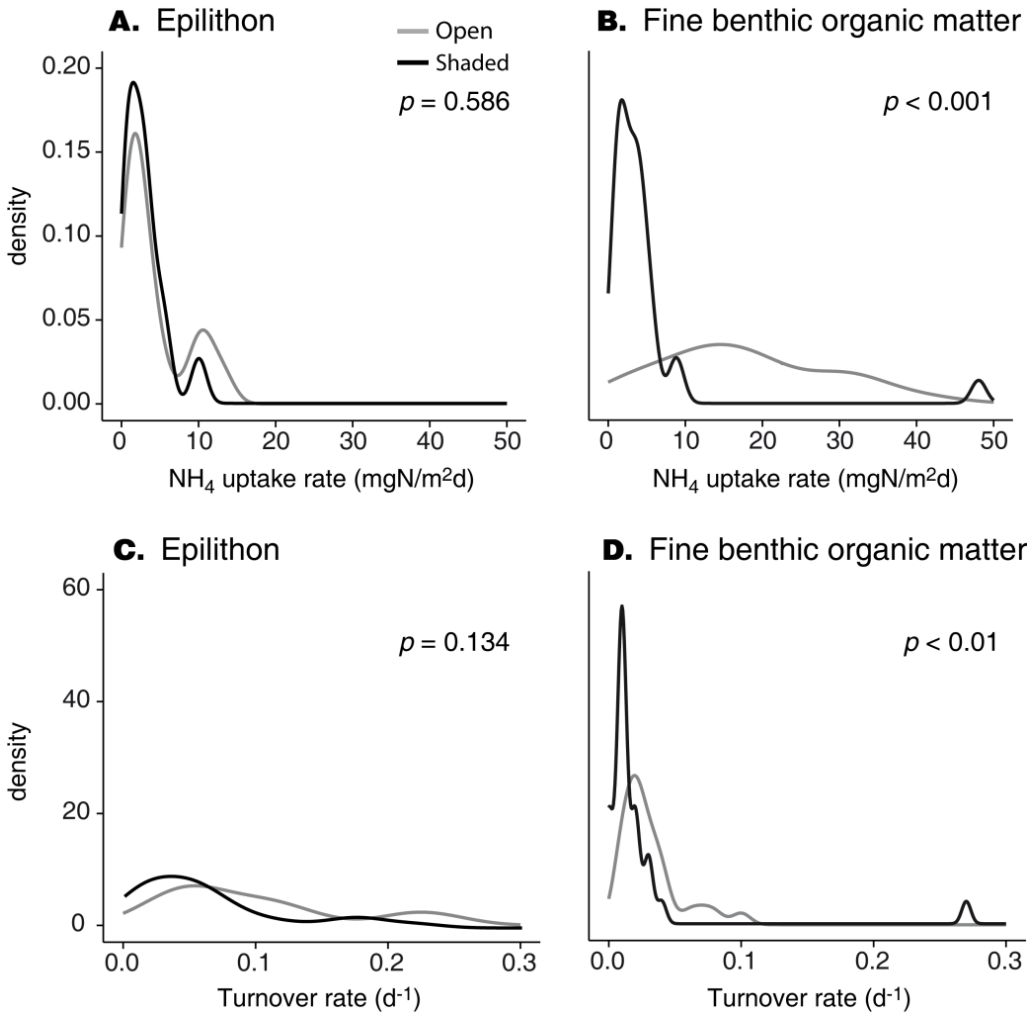


Figure 6.4 Kernel density estimations (KDE) for N uptake (A and B) and turnover (C and D) rates at the open (grey lines) and shaded (dark lines) reaches. The bandwidth was adjusted to 0.1, and n=60 for each compartment and random variable.

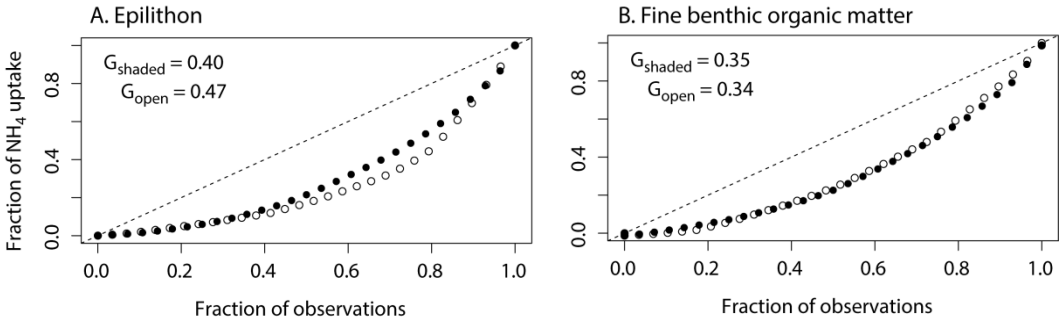


Figure 6.5. Lorenz curves for uptake rates of epilithon (A) and FBOM (B) at the open (empty dots) and shaded (filled dots) reaches. Lorenz curve relates the cumulative percentage of the 30 observations per compartment and reach (*x* axis) to the cumulative percentage of the sum of all NH_4 uptake rates per compartment and reach (*y* axis). Gini coefficients (G) are shown at each panel, for G = 0 all observations make equal contribution to uptake or turnover, whereas as G increases towards 1 the inequality increases.

6.4.6 Biophysical factors influencing N cycling of stream microbial assemblages at the microhabitat scale

In general, models for microhabitat variation in uptake and turnover rates showed sufficient predictive power for both compartments and study reaches, even though uptake and turnover rates varied across 3 orders of magnitude (APPENDIX 6.2). Biotic and physical variables explained a higher percentage of the variation in uptake and turnover rates of epilithon than of FBOM (on average, r^2=0.6 for epilithon and r^2=0.5 for FBOM). Outliers (observed response values falling outside the 90th interval band) were less than 4% of total observed values (n=60) at any of the fitted models (APPENDIX 6.2). Variation in epilithon uptake and turnover rates was mostly explained by water velocity and depth, which together accounted for 60% and 80% of the total variance explained by uptake and turnover models, respectively (Table 6.5). Biotic factors associated with epilithon characteristics such as C:N, green algal

content, and % OM did also significantly contribute to explain the variation in uptake and turnover rates observed for this compartment (Table 6.5). However, a single lineal relationship between water velocity and epilithon uptake rates in a log-log scaled plot showed almost the same predictive power than the multiple regression model, indicating that water velocity was by far the strongest predictor of the spatial variation in epilithon uptake rates (Figure 6.6A). In contrast, the most important factor explaining spatial variation in FBOM uptake rate was water depth (Table 6.5). However, in this case, a single exponential relationship between water depth and FBOM uptake showed much lower predictive power than the multiple regression model indicating the contribution of other factors explaining the spatial variation in FBOM uptake (Figure 6.6B). Specifically, organic matter content and EEA of FBOM contributed to explain 30 and 35% of the variation in FBOM uptake, respectively (Table 6.5). Variation in turnover rates of FBOM was mostly explained by water velocity and %N (Table 6.5).

Table 6.5 Results of stepwise multiple regression analysis between biophysical factors and that influenced ther ates of nitrogen uptake and turnover of epilithon and FBOM. Values within brackets refer to the total variability explained by each model (i.e., adjusted-R^2). For each selected variable the table provides data on estimated regression coefficients (β), standard error for β (SE), relative contribution of each factor (relative-R^2) to the total variance explained by each model, and level of singificance (*p*-value).

Response variable	Compartment	Selected variables	β	SE	relative-R^2	*p* -value
NH_4 Uptake rate*	Epilithon (0.64)	Water velocity*	0.39	0.05	0.61	**<0.001**
		C:N	-0.12	0.30	0.18	**<0.001**
		Green algae*	0.08	0.03	0.12	**<0.001**
		%OM*	-1.46	0.32	0.08	**<0.05**
	FBOM (0.51)	Water depth	0.04	0.01	0.31	**<0.001**
		%OM*	-1.91	0.50	0.3	**<0.005**
		β-glucosidase*	-0.25	0.10	0.25	**<0.05**
		Aminopeptidase*	-0.48	0.19	0.12	**<0.05**
Turnover rate*	Epilithon (0.62)	Water velocity*	0.39	0.07	0.55	**<0.001**
		Water depth*	-0.35	0.15	0.25	**<0.05**
		Green algae*	0.08	0.04	0.17	**<0.05**
		%N*	-0.54	0.30	0.02	0.081
	FBOM (0.4)	Water velocity*	0.50	0.12	0.52	**<0.001**
		%N*	-1.89	0.55	0.38	**<0.005**
		Water depth	0.02	0.01	0.09	**<0.05**

* Variables that were log-transformed

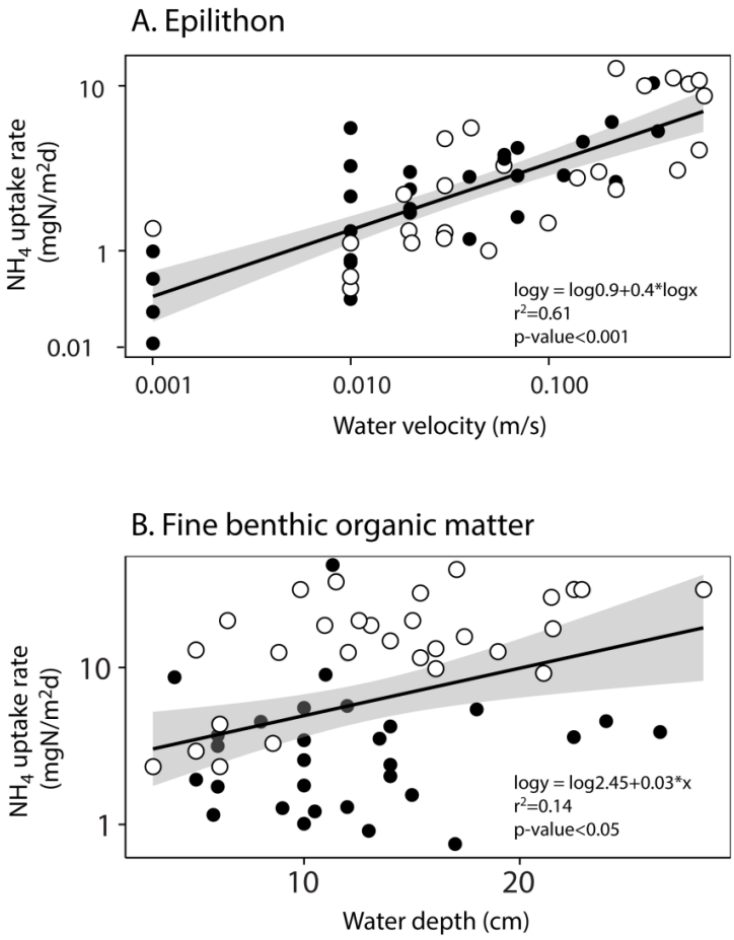


Figure 6.6 Linear regression between water velocity and NH_4 uptake rates of epilithon (a), and between water depth and NH_4 uptake rates of FBOM (b). Note the axis expressed in a logartihmic scale. Shaded bands represent 95% confidence regions. Empty and filled dots represent data from open and shaded reach, respectively.

Reach-specific regressions between water depth and NH_4 uptake rates of epilithon (OR: uptake=18.6*depth$^{-0.87}$, r^2=0.66; SR: uptake=12.6*depth$^{-0.96}$, r^2=0.53) were used to map epilithon NH_4 uptake within each study reach (Figure 6.7). Results showed large spatial heterogeneity in epilithon NH_4 uptake rates at the microhabitat scale with hot spots of NH_4 uptake rates (i.e., red zones) concentrated in a small percentage of the total streambed area, being generally located in the riffle habitats of the two reaches (Figure 6.7). In the case of NH_4 uptake rates of FBOM, we only found a significant but positive regression with water depth at the OR (uptake=3.4exp$^{0.09*depth}$, r^2=0.53; Figure 6.7), where zones of high NH_4 uptake by FBOM represented a large percentage of the total streambed compared to epilithon (Figure 6.7).

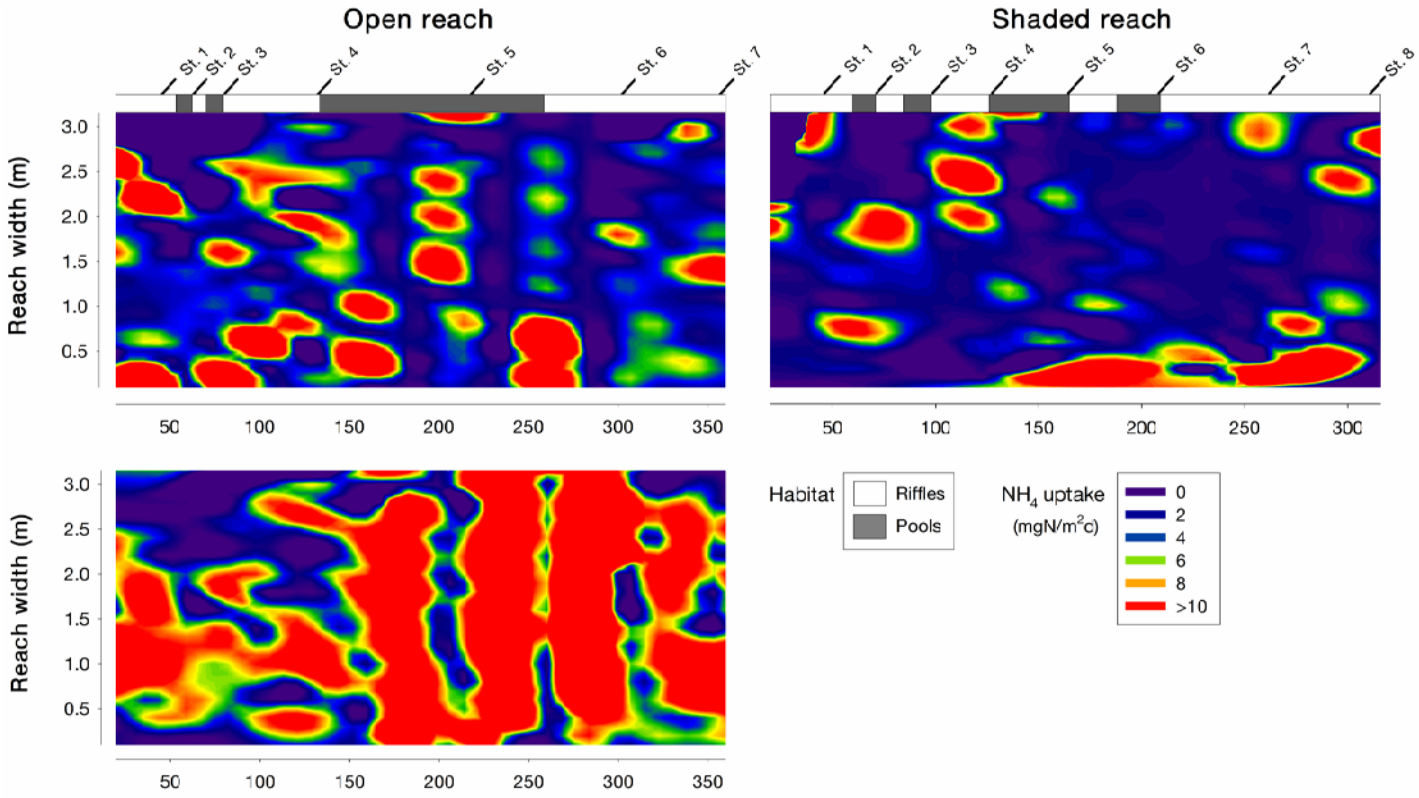


Figure 6.7 Spatial variatiability of patch-specific NH_4 uptake rates for epilithic (upper panels) and FBOM (bottom panels) microbial assemblages in the open and shaded reaches. Data is derived from the hydromorphological configuration of each stream reach and the significant relationship between NH_4 uptake rates of epilithon and FBOM and water depth at the two reaches. Bands at the top of each panel indicate the location of sampling stations and the distribution and longitudinal extend of riffle and pool habitats in each reach.

6.4.7 Relevance of microhabitat variation in microbial NH_4 uptake at the whole-reach scale

Averaging the observed microhabitat variation in microbial NH_4 uptake to habitat (riffle/pool) and to whole-reach (sampling stations) scale had different effects depending on the study reach and the microbial assemblage considered (Figure 6.8). However, greater SEM observed at larger scales indicated that averaging variation of NH_4 uptake from fine to large spatial scales increased uncertainty on the mean estimation of NH_4 uptake, especially for epilithon (Figure 6.8A). Spatial variation in epilithon NH_4 uptake at the OR showed its major decrease when averaging from habitat to whole-reach scale (~75% reduction), and from microhabitat to habitat scale at the SR (~50% reduction; Figure 6.8A). In contrast, for FBOM, spatial variation in NH_4 uptake at the OR showed a similar decrease across the three spatial scales, and almost a 70% decrease from microhabitat to habitat scale at the SR (Figure 6.8B).

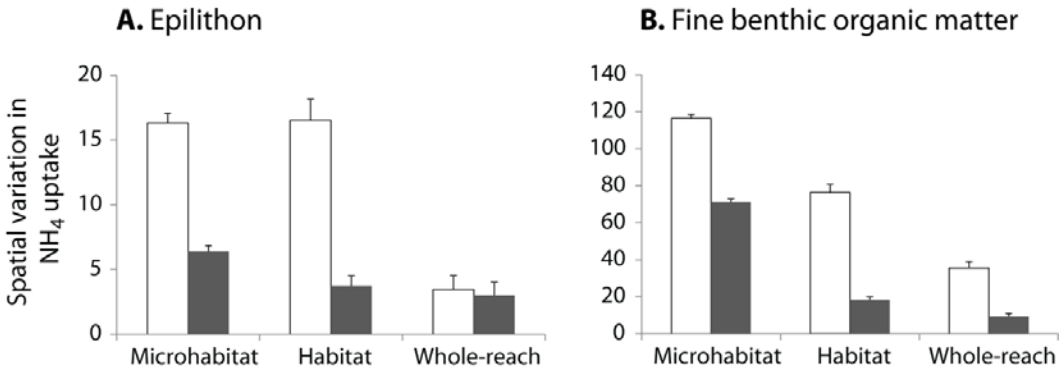


Figure 6.8 Variation in microbial NH_4 uptake filtering it through different spatial scales. Columns represent the measured variance for patch-specific NH_4 uptake of epilithon and FBOM in the OR (empty columns) and SR (filled columns) at three different scales: microhabitat (n=30), habitat (n=6; 3 riffles + 3 pools), and whole-reach (n=3-4 stations). Errors bars correspond to the standard error of the mean (SEM) of NH_4 uptake rates and are used as a sampling size-corrected measure of the variation in mean uptake estimation at each scale.

Simulated reductions in spatial variation of microbial NH_4 uptake at the microhabitat scale generated large decreases of NH_4 uptake at the whole-reach scale (Figure 6.9). In particular, when microhabitats with the highest microbial NH_4 uptake were extracted, which represented ~20% of the total observations, whole-reach NH_4 uptake decreased also around 20% at each study reach (Figure 6.9A and B). These results indicate that hot spots of NH_4 uptake represent an important part of the microbial contribution to whole-reach NH_4 uptake. Greater reductions in spatial variation of microbial NH_4 uptake showed that whole-reach NH_4 uptake could be reduced up to 50% if only microhabitats with lowest microbial NH_4 uptake were extracted (Figure 6.9A and B). This is particularly relevant because microhabitats with low NH_4 uptake rates were the most abundant in both study reaches (Figure 6.3). Similar patterns observed at both study reaches are consistent with the similar contribution of microbial NH_4 uptake to whole-reach NH_4 uptake (Figure 6.9; Table 6.3).

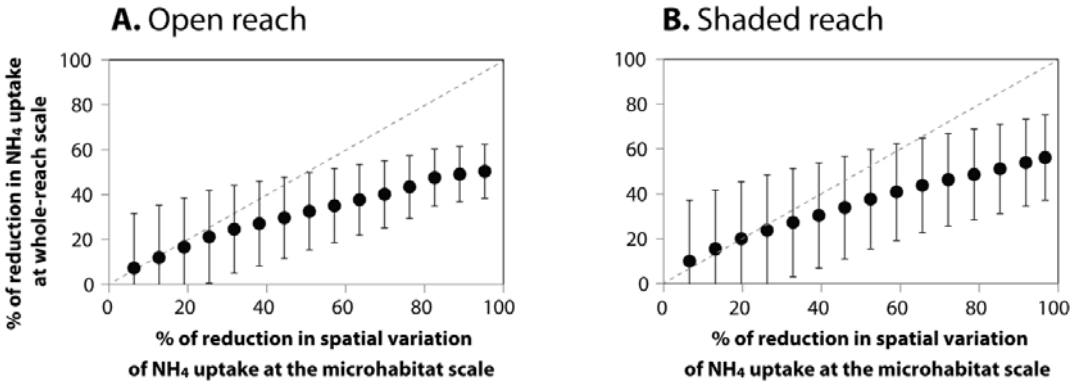


Figure 6.9 Relationships between the percentage of reduction in spatial variation of microbial NH_4 uptake at microhabitat scale (*horizontal axis*) and the percentage of reduction in whole-reach assimilatory NH_4 uptake (*left vertical axis*). Error bars correspond to SD. Dashed line represents the 1:1 relationship between *horizontal* and *left vertical axis*.

6.5 Discussion

6.5.1 Contribution of microbial assemblages to reach-scale N uptake

Total NH_4 uptake did not vary between the study reaches despite their different degree of canopy cover and PAR availability, and showed NH_4 uptake rates very similar to values observed in other open (Dodds et al. 2000, Simon et al. 2007) and shaded (Mulholland et al. 2000, Tank et al. 2000, Merriam et al. 2002) stream reaches. Uptake velocity (i.e., NH_4-Vf), which is a parameter commonly used to compare N uptake among different streams, was also similar to the average NH_4-Vf reported for 12 headwater streams from different biomes (Peterson et al. 2001). In contrast, the low nitrification rates measured in our study contrasted with previous studies that showed a higher contribution of nitrification to total NH_4 uptake (Mulholland et al. 2000, Merriam et al. 2002). These results indicated that total NH_4 uptake in our reaches was dominated by assimilatory NH_4 uptake. In this regard, microbial N uptake accounted for >50% of the assimilatory NH_4 uptake in both reaches,

confirming microbial assemblages as key players in stream N cycling. However, the particular contribution of N uptake by epilithon and FBOM to whole-reach N uptake differed between reaches, with FBOM being more relevant at the open reach and epilithon being more relevant at the shaded reach, despite N standing stock of FBOM was larger than that of epilithon at the two study reaches. These results point at the need to consider not only the relative abundance of the different microbial assemblages, but also the particular nature of them at smaller scales to understand the underlying mechanisms driving N uptake at the reach scale.

Differences between reaches, mostly associated with light availability, also had an effect on the spatial heterogeneity of microbial N uptake at finer scales. Spatial variation in microbial N uptake at the habitat and microhabitat scales was lower at the shaded reach; and thus, differences between compartments at the reach scale were more driven by the relative presence of each compartment along the reach than by their differences in N uptake at finer scales (Figure 6.10). Contrastingly, higher light availability in the open reach maximized the differences in N uptake between epilithon and FBOM at both habitat and microhabitat scales (Figure 6.10). Overall, these results indicate that light may enhance the influences of controlling factors operating at fine scales on the microbial N cycling, by increasing for instance the autotrophic N demand.

The present study also provides valuable information on the remarkable spatial variation of microbial N uptake rates within the reaches and the factors controlling it. Results show that rates of N uptake by microbial assemblages are not homogeneously distributed within the stream channel and that their spatial variability is mostly associated with the hydraulic characteristics of the

microhabitats. Microbial NH_4 uptake rates ranged by three orders of magnitude within each reach. This range is comparable to that observed at the reach scale for NH_4 uptake rates from 12 headwater streams across different biomes (Peterson et al. 2001). In particular, spatial heterogeneity of microbial N uptake was mostly attributed to the presence of hot spots of N cycling (McClain et al. 2003, Harms and Grimm 2008) that lead to a non-uniform spatial variation (i.e., high inequality) in N uptake within the stream channel, especially in the case of epilithon.

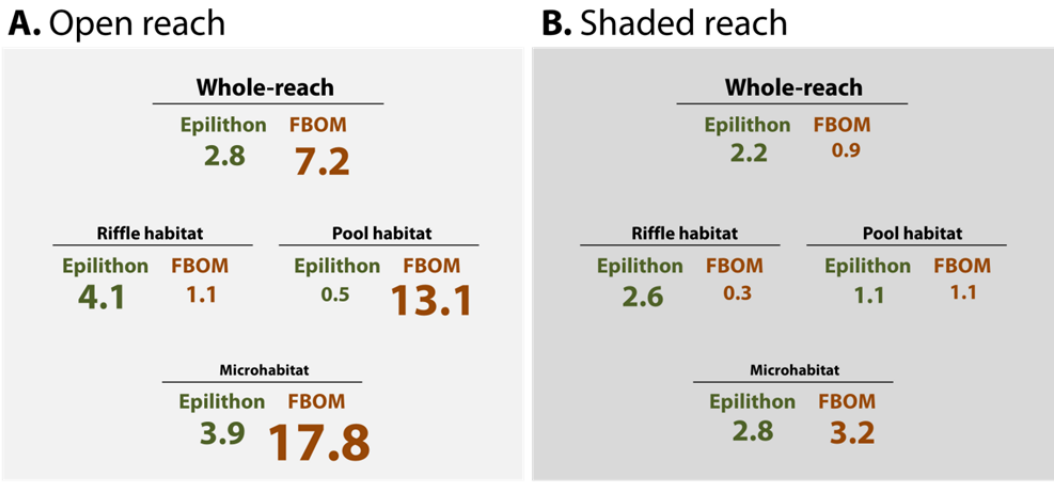


Figure 6.10 NH_4 uptake rates (mg N/m^{2}d) of epilithon and FBOM measured across the different spatial scales at the open (A) and shaded (B) reaches. Values at whole-reach and habitat scales are compartment-specific NH_4 uptake rates based on the reach-weighted $N_{biomass}$ at each scale. Values at microhabitat scale correspond to patch-specific NH_4 uptake rates.

6.5.2 Spatial heterogeneity in epilithic microbial assemblages at the microhabitat scale

Characteristics of the epilithic microbial assemblages considered in this study revealed a considerable variability at microhabitat scale, although some general patterns emerged from them. Interestingly, comparison between reaches showed lower algal abundance (in terms of Chl a content) and higher heterotrophic activities (i.e., EEA) in the epilithon at the open reach, even though lower canopy cover was expected to enhance photoautotrophic activity of epilithon. In contrast to these between-reach differences, epilithon N uptake did not differed between reaches and its variability at microhabitat scale was mostly influenced by external factors, such as flow velocity in the stream channel. At the time of our study, despite the large spatial heterogeneity in the characteristics of the epilithon within each reach, EPS values indicate that epilithic microbial assemblages were mature with a well-developed structure (Romani et al. 2008). Under these conditions, positive feedbacks between algae and bacteria has been previously described (Romani and Sabater 1999, Rier and Stevenson 2002, Proia et al. 2012), which may explain lack of a clear response in N uptake between the distinct epilithic microbial assemblages from the two reaches. .

The strong relation between flow velocity and epilithon N uptake at the microhabitat scale followed a nonlinear positive relation, which was common for epilithon developed on the two reaches. The influence of water velocity on N uptake at microhabitat scale could be explained by a facilitation of mass transfer of substrates from the stream water into the epilithon as water velocity increases, resulting in increases in N uptake rates. This is supported by previous results showing that slow velocities can lead to lower mass transfer into the

epilithon matrix due to a higher effect of boundary layer constrains, which is commonly associated with thicker epilithic biofilms (Gantzer et al. 1988, Battin et al. 2003, Singer et al. 2010). This hypothesis was further confirmed by the negative correlation observed in our study between water velocity and δ13C natural abundance of epilithon ($r=0.6$, $p<0.001$). Values of δ13C in epilithon are known to be higher under low water velocities because of the lower isotopic fractionating caused by constrains in diffusion rates through the epilithon boundary layer (Trudeau and Rasmussen 2003). An alternative explanation for the N uptake-water velocity relationship could be that epilithon in microhabitats with higher water velocities are exposed to higher N fluxes which could support a higher N demand by epilithon. This N load effect could be particularly relevant in streams with low N concentrations as it is the case in this study; however, this deserves further experiments under controlled conditions.

6.5.3 Spatial heterogeneity in FBOM microbial assemblages at the microhabitat scale

Microbial assemblages in FBOM are embedded in a high pool of fine particles mostly from detrital organic matter from autochthonous and allochthonous origin (Bonin et al. 2000, Findlay et al. 2002, Sakamaki and Richardson 2013), which contrasts with the biofilm structure of epilithic microbial assemblages. Riparian canopy cover can be a critical factor for the composition of FBOM because it regulates light incidence and inputs of organic matter from terrestrial vegetation, thus presence or absence of canopy cover may influence the proportion of photoautotrophs and heterotrophs in FBOM (Gessner and Chauvet 1994, Findlay et al. 2002). In particular, Artigas et al. (2008) showed that FBOM in Fuirosos generally contains greater algal than

bacterial biomass during periods of high light availability such as before riparian tree leaf-out in spring. Our results are in agreement with these previous studies, showing a higher algal content in FBOM at the OR and a higher heterotrophic activity (i.e., EEA) at the SR. In addition, several parameters measured in our study, such as N biomass, algal abundance, or chl-a:AFDM ratios, showed a larger microhabitat variation in FBOM compared to epilithon. However, the spatial variation of N uptake by FBOM within the reaches was more equally distributed than that of epilithon, especially at the open reach.

Spatial variation in N uptake rates of FBOM at the microhabitat scale was positively controlled by water depth, and negatively controlled by organic matter content and EEA. The combination of positive and negative effects from these factors may explain the more homogeneous distribution of N uptake rates of FBOM within the reach compared to that of epilithon because they can counterbalance each other. The intrinsic nature of FBOM dictates the distribution of it in the reaches, with higher accumulation on the stream edges or in deeper zones of the stream channel where water velocity is slower. These depositional zones are known to be hot spots of organic matter decomposition (Hedin 1990, Fuss and Smock 1996). Our results indicate that these zones may also be hot spots of N uptake by FBOM within the reach. This finding suggests that previous results showing a relationship between organic matter decomposition and N cycling at reach scale (Tank and Webster 1998, Sanzone et al. 2001, Meyer et al. 2005) are also consistent at finer spatial scales. However, these results further indicate that N uptake by FBOM is not only dictated by the location where it accumulates, but also by its composition. FBOM is normally dominated by heterotrophic bacteria, which are responsible for the decomposition of the accumulated organic matter by using it as

substrate sources (Findlay et al. 2002). We found that FBOM with higher organic matter content showed lower N uptake rates, suggesting this FBOM is more independent from the water column as a N source. This is further supported by the negative relationship between FBOM uptake and EEA, which suggests that microbial organisms in FBOM can obtain nutrients from the detrital organic matter in FBOM. In addition, the higher N uptake by FBOM at the open reach, coinciding with a higher content of Chl a, seems to indicate that the use of water column N as a relevant nutrient source for microbial assemblages in FBOM is related to the relative abundance of algae in FBOM.

6.5.4 Implications of spatial heterogeneity in microbial N uptake at the microhabitat scale for N cycling at the reach scale

Previous research conducted in the context of the hierarchical organization of streams has frequently highlighted the high spatial heterogeneity of these ecosystems at different scales (Dent et al. 1997, Poole 2002, Lewis et al. 2007). Notwithstanding, the use of a hierarchical framework to examine highly dynamic and complex systems, such as fluvial ecosystems, is based not only on quantifying the spatial heterogeneity per se at each hierarchical level, but also on understanding the influence of it on ecologically relevant attributes and the linkages across different scales (see Dent et al. 2007). Our results show that upscaling the observed spatial heterogeneity in N uptake by microbial assemblages from microhabitat to reach scales demands an understanding of how spatial variation is filtered through different scales (e.g., microhabitat, habitat, and reach), and also of how factors controlling N uptake at small spatial scales vary at larger scales.

Across-scales filtering of spatial variation in microbial N uptake differed between the two microbial assemblages examined in this study, and also

between the two reaches. At the shaded reach, spatial heterogeneity in N uptake by the two microbial assemblages exhibited a major drop when filtering from microhabitat to habitat scales, a pattern that was not observed at the open reach. This may be explained by the fact that light infiltration through the foliage of a dense riparian canopy cover generates illuminated patches along the stream reach, the size of which is probably more similar to the microhabitats size (~1m) than to the habitats size (~10m). Light can be a limiting factor for N uptake by photoautotrophic communities (Mulholland et al. 2006, von Schiller et al. 2007) and it was a major distinction between the study reaches. Therefore, light availability may have contributed to the large loss in spatial variation of microbial N uptake when averaging it by habitats at the shaded reach. On the other hand, in the open reach, across-scales filtering of spatial variation in microbial N uptake showed very distinct patterns depending on each microbial assemblage. The similar variation found between microhabitat and habitat for epilithon N uptake is consistent with the strong control of N uptake by water velocity, which is also a major distinction between riffle and pool habitats. In fact, previous studies have also observed consistent differences in N uptake rates of epilithon between riffle and pool habitats (Simon et al. 2004). On the contrary, N uptake by FBOM at the open reach showed a gradual decrease of its spatial variation from microhabitat to reach scale, which is in agreement with the more equally distribution of FBOM uptake rates at the microhabitat level observed at open reach.

Large inequality in the distribution of microbial N uptake in the stream channel, with hot spots of N uptake representing only around 20% of the total microhabitat variation can also have important consequences on the contribution of microbial assemblages to reach-scale N uptake. Epilithon and

FBOM were clearly the most important benthic compartments contributing to N uptake in the two reaches, but we found that their contribution can be particularly vulnerable to natural and human disturbances that alter the stream channel morphology. Our results showed that a decrease in the spatial heterogeneity of the stream channel could drastically affect N uptake at the reach scale, especially if changes alter microhabitats associated with hot spots of microbial N uptake. It is therefore reasonable to argue that alteration of hydromorphologic characteristics of stream and river channels (i.e., channelization, sediment clogging, etc.) can have severe effects on the niche partitioning at the microhabitat level and ultimately on the microbial contribution to N uptake at reach scale. Several studies have shown how N enrichments at the reach scale derived from human activities reduce the stream capacity to uptake and retain nutrients (Marti et al. 2004, Gucker et al. 2006, von Schiller et al. 2009). However, in most cases N enrichments are combined with a simplification of the stream channel. Under these conditions, the reduced capacity of in-stream nutrient uptake may be also partly explained by the lost in spatial heterogeneity. Together, these findings also provide a basis for stream restoration projects from a functional perspective.

6.5.5 Synthesis

Spatial heterogeneity of stream ecosystems generates a mosaic of microhabitats that result in a large spatial variation of microbial assemblages. This study shows that spatial variation of microbial assemblages is associated with a remarkable variation in N cycling within the stream channel, and provides evidence of hot spots of microbial N cycling at microhabitat scale. Results also indicate key factors influencing variation in microbial N uptake at fine spatial scales, showing that they differ depending on the nature of the

microbial assemblages studied. N uptake by epilithon was mostly driven by a non-lineal effect of flow velocity, which seems to favor the N transfer from the water column into the epilithon. On the other hand, N uptake by FBOM was influenced by a combination of biophysical factors, which may determine microbial demand of N from the water column. Therefore, understanding patterns of microbial N uptake among different streams may need to consider not only how the streams differ at the reach scale, but also how biophysical factors driving microbial N uptake at fine spatial scales can vary among different streams.

Our results demonstrated that the degree of spatial variation in microbial N uptake at both habitat and microhabitat scale may differ among streams with different stream-riparian linkages. Moreover, the extent to which spatial variation in microbial N uptake at fine spatial scales is integrated at the reach scale is potentially affected by factors operating at the ecosystem level, as shown by distinct patterns between the open and shaded reaches. For instance, changes in the degree of spatial variation in microbial N uptake when upscaling from microhabitat to habitat scales differed between reaches. These results suggest that inconsistent patterns in the microbial contribution to N uptake at reach scale among streams can be in part caused by how spatial variation at fine scales is captured when averaging N uptake at the reach scale. Moreover, the large inequality in spatial variation of microbial N uptake rates in the reach hinder even more the predictions of how spatial variation at fine scales is filtered to larger scales. Overall, results from this study provides a mechanistic understanding of how the loss of spatial heterogeneity in stream ecosystems can ultimately result in a decreased capacity of streams to uptake and retain N.

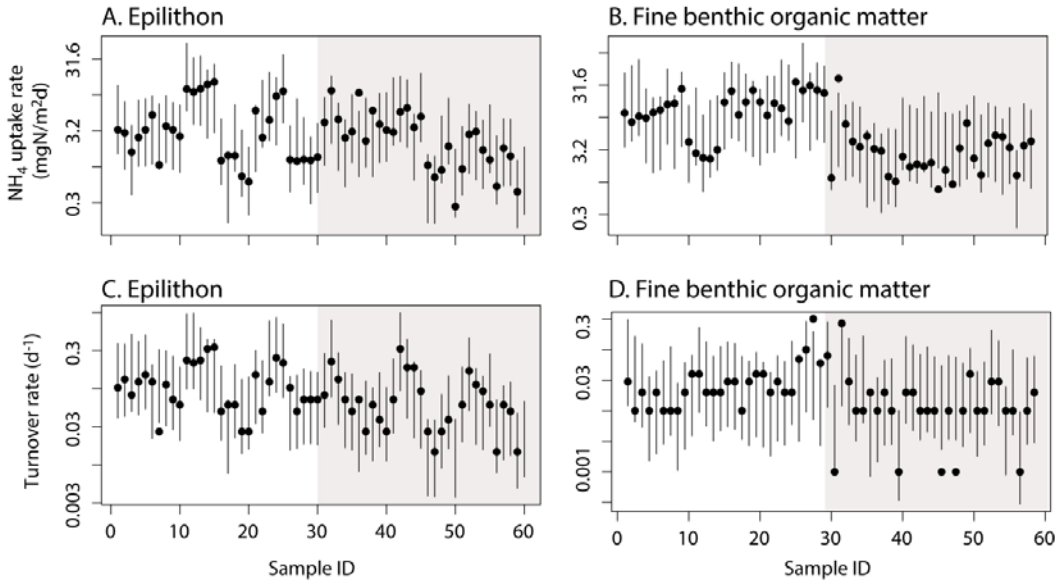
Acknowledgements

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Appendixes

Appendix 6.1 Patch-specific NH_4 uptake (mgN/m^2d) and turnover rates (d^{-1}) for epilithon and FBOM at each study reach. Data correspond to mean values (SD) and *p*-values are for Kruskal-Wallis tests (reach as a factor).

	Eplitihon			Fine benthic organic matter		
Parameter	Open	Shaded	*p* -value	Open	Shaded	*p* -value
NH_4 uptake rate	3.93 (3.69)	2.77 (2.20)	0.666	17.77 (10.96)	3.23 (2.18)	**<0.001**
Turnover rate	0.11 (0.08)	0.08 (0.08)	0.092	0.04 (0.06)	0.01 (0.01)	**<0.05**



Appendix 6.2 Comparison between 90th interval band of the predictive distribution for uptake and turnover rates from the model (solid lines) and each observed response value (solid points). Data is organized from upstream to downstream for open (white background) and shaded (grey background) reaches,respectively.

7

General discussion:
learning from ^{15}N signatures to examine the role of PUCs on stream N cycling

The overall goal of this PhD thesis was to understand the role of primary uptake compartments on stream nitrogen (N) uptake and retention, as well as the potential factors controlling it. This knowledge is especially relevant in the current context where fluvial ecosystems are facing important changes derived from human activities. The stable isotopes of N (^{15}N), both as natural abundances and as a tracer, for dissolved inorganic N (DIN) and primary uptake compartments (PUCs) were used as the main empirical tool to infer how variation in DIN availability, intrinsic characteristics of PUCs, or variation across spatial scales may ultimately affect the N uptake by PUCs. Notwithstanding, the extensive quantification of the variation in ^{15}N natural abundances has allowed not only examining the role of different PUCs on stream N uptake, but it has also revealed the principal sources of variation in their ^{15}N signatures across different spatial and temporal scales. This second aspect is not less relevant because N isotope signatures are used to approach a wide range of aspects in ecological studies such as ecosystem biogeochemistry (Angradi 1993, Chen et al. 2009), trophic web interactions (Coat et al. 2009, Boecklen et al. 2011), and physiological (Ehleringer et al. 1986, Adams and Sterner 2000), taxonomic (Vanderklift and Ponsard 2003) or biogeographic questions (Schlacher et al. 2005, Peuke et al. 2006). Therefore, the detailed examination of the patterns of variability in ^{15}N natural abundances of PUCs provided in this Thesis, and how different intrinsic and environmental factors may influence them, is itself valuable information, which may be of interest for other ecological studies using ^{15}N signatures. For instance, variation in ^{15}N-PUC signatures should be particularly relevant for food web studies examining consumer's trophic position since ^{15}N variation can be transferred from basal to upper trophic levels (Post 2002).

Results of this Thesis also point at some of the limitations in the use of ^{15}N natural abundances to address the role of PUCs on stream N cycling. Although many interesting linkages between ^{15}N signatures and the N cycling in PUCs were observed under natural conditions, the narrow spatial variation in ^{15}N natural abundances in relatively pristine streams was particularly problematic when trying to evaluate the N transfer from DIN to PUCs in these ecosystems. The restraints observed under natural conditions evidenced which types of questions can be solved by examining ^{15}N natural abundances and which ones require the use of ^{15}N-tracers (i.e., ^{15}N additions). Contrastingly, in human-altered streams, ^{15}N natural abundances of DIN and PUCs demonstrated that anthropogenic N inputs can sometimes be harnessed as an opportunity to examine the N transfer from the stream water to the different PUCs using ^{15}N signatures at natural abundance levels.

Considering data from each particular study conducted together, in this chapter we aim to provide a synthesis overview on 1) global and local factors influencing variation in the ^{15}N-PUC signatures examined in the context of global change alterations on stream ecosystems; 2) the extent to which ^{15}N natural abundances of PUCs can help understanding the contribution of each PUC to stream N retention; and 3) the N cycling of different PUCs across spatial scales and their relevance at the ecosystem level.

7.1 Factors influencing variation in ^{15}N signatures of primary uptake compartments in stream ecosystems: from local to global scales

Nitrogen isotopes in the form of DIN are constantly circulating through fluvial networks since the moment they reach stream waters from natural or anthropogenic N sources within the catchment until they are locally retained by organisms at the base of aquatic food webs (i.e., primary uptake compartments; PUCs). Therefore, ^{15}N natural abundance of PUCs reflects to some extent the ^{15}N signatures of their DIN sources in the stream water because of the ^{15}N transfer during N uptake (Cifuentes et al. 1988, Handley and Raven 1992, Pastor et al. 2013). However, DIN inputs into the streams may show a substantial variation in their ^{15}N signatures given the several and distinct sources that often differ in their ^{15}N signatures depending on their natural or anthropogenic origin (Kendall 1998, Mayer et al. 2002, Panno et al. 2006). Moreover, prior to local processes of DIN uptake by PUCs, ^{15}N signatures of DIN (as both ammonium and nitrate) have likely experienced several changes through the multiple in-stream N transformations affecting ^{15}N-DIN signatures such as nitrification (Mayer et al. 2002), denitrification (Diebel and Vander Zanden 2009, Houlton and Bai 2009), or atmospheric deposition (Paerl and Fogel 1994). As a result, ^{15}N-DIN signatures in the stream water can be seen as the net result of an unknown number of mixing and transformation processes taking place along the stream network, which will be sooner or later transferred into the PUCs through N uptake processes. Ultimately, this means that ^{15}N signatures of PUCs bear to some extent the imprints of both local processes of

DIN uptake and regional mixing and transformations of DIN sources within the catchment and along the streams.

Both local and global effects on the ^{15}N-PUC signatures are suggested by the results from this Thesis, which show that spatial variability in ^{15}N signatures of all PUCs is generally lowest at habitat scales, intermediate at reach scales, and highest at the world-wide scale (Figure 7.1). The increase in ^{15}N-PUCs variation from local to global scales is not surprising given the increased ^{15}N-DIN variation at larger scales probably due to a greater number of distinct N sources and processes affecting ^{15}N-DIN signatures. However, differences in the ^{15}N signatures among PUCs are similar either when comparing habitats within one stream reach or when comparing streams across contrasting biomes (Figure 7.1); which suggests that even though ^{15}N-PUC variation increases from small to larger scales via increasing ^{15}N-DIN variation, local influences, such as the intrinsic characteristics of each PUC, may be also relevant to explain spatial variation in ^{15}N signatures among the different PUCs of stream ecosystems. As an example, systematic lower ^{15}N signatures of coarse particulate organic matter (CPOM) relative to values of other PUCs are probably related to strong local effects on its ^{15}N variation (*Chapter 1*). Microbial assemblages colonizing CPOM are known to rely more on N sources from this terrestrially-derived organic matter than on DIN from the stream water (Cheever et al. 2013), which are normally ^{15}N depleted compared to ^{15}N from ammonium and nitrate in the water column (Fry 1991). In contrast, large variation in ^{15}N of filamentous algae observed at the habitat scale is probably related to a highest sensitivity to ^{15}N-DIN variation because of its reliance on this source and its rapid N turnover (*Chapter 3*). Overall, variation across spatial scales seems to indicate that total variability in ^{15}N-PUC signatures may be mostly explained by large-scale factors

such as the existence of human-derived N inputs into stream ecosystems, while the ^{15}N variation among the different PUCs would be mostly attributed to local factors influencing N uptake processes such as the intrinsic characteristics of the different PUCs.

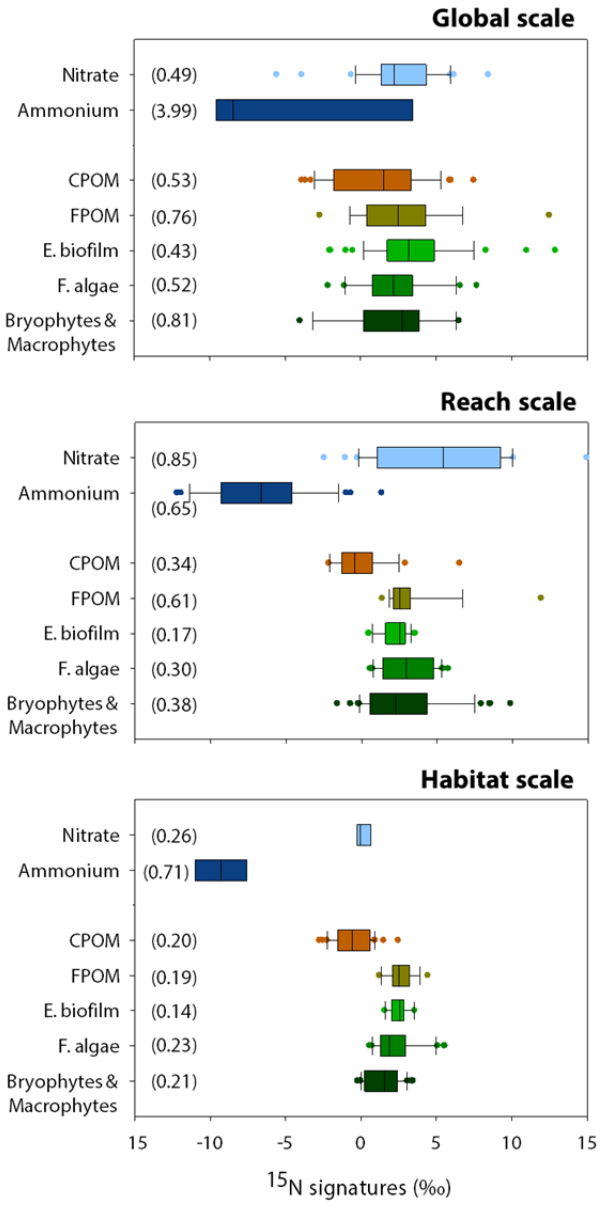


Figure 7.1: Spatial variation in ^{15}N signatures of DIN (ammonium and nitrate) and PUCs in relatively pristine streams. Habitat scale represents variation among riffles and pools in Fuirosos stream; reach scale corresponds to ^{15}N data along four stream reaches (two in Fuirosos stream, and two in La Tordera river); and global scale represents ^{15}N data from reference streams worldwide. Values in brackets correspond to standard error of the mean.

7.1.1 Effects of C:N stoichiometry on the ^{15}N signatures of primary uptake compartments

Ecological stoichiometry states that primary producers have the ability to change their elemental composition (i.e., C:N ratios; Elser et al. 2000) according to the balance of resources supplied to the ecosystems, either if these resources refer to two different nutrients (Sterner and Elser 2002) or to energy (light) and nutrients (Sterner et al. 1997, Chrzanowski and Grover 2001). Therefore, C:N stoichiometry of primary producers will be influenced by external variation in nutrients supply, which in turn will also influence the N cycling trough primary producers. Specifically, C:N ratios of both photoautotrophic and heterotrophic PUCs in streams are negatively related to their N-specific uptake rates suggesting that compartments with low C:N ratios (e.g., microbial compartments) have higher N turnover rates (Dodds et al. 2000). Additionally, Dodds et al. (2004) showed that C:N ratios of epilithon from different streams were positively related to its N demand, as a proxy of the degree of N limitation.

At present, there are no clear descriptions of how ecological stoichiometry can be related to the ^{15}N natural abundance of PUCs. In fact, it is unlikely that ^{15}N and C:N ratios of PUCs could be related when considering ^{15}N data from different streams because variation in the ^{15}N signatures of DIN sources would probably mask the ^{15}N-C:N relationships. However, within reaches (i.e., habitat and microhabitat scales), linkages between C:N stoichiometry and the N cycling of each PUC may lead to some characteristic imprints on the ^{15}N-PUC signatures. For instance, results from La Tordera headwaters (*Chapter 3*) showed a positive relationship between ^{15}N and C:N ratios for epilithon (r^2=0.4, p<0.05), and negative ^{15}N-C:N relationships for filamentous algae (r^2=0.6; p-

value<0.01), submersed macrophytes (r^2=0.5; *p*-value<0.05), and emergent macrophytes (r^2=0.5; *p*-value<0.05). Results at the microhabitat scale in Fuirosos stream (*Chapter 4*) also showed that ^{15}N signatures of epilithon were positively related to their C:N ratios, and that ^{15}N signatures of FBOM were negatively related to their C:N ratios (Figure 7.2). The mechanisms behind these significant relationships between ^{15}N and C:N ratios of PUCs are not straightforward, but they seem to be consistent and different between epilithon and the rest of PUCs. In the case of epilithon, the positive relationship between ^{15}N and C:N ratios may indicate that higher N demand, related to high C:N ratios (Dodds et al. 2004), results in more enriched ^{15}N signatures due to higher internal N cycling. The greater C:N ratios and N turnover rates of epilithon observed at the open reach seem to confirm this hypothesis (*Chapter 4*). In the case of FBOM, the negative relation between ^{15}N and C:N ratios should be principally driven by the variation in the detrital contribution to N bulk of FBOM. ^{15}N signatures of FBOM may be mostly representative of the N from the detrital pool rather than from the active N pool from microbes colonizing it (Tank et al. 2000, Sanzone et al. 2001, Findlay et al. 2002). Therefore, higher C:N ratios may correspond to a larger contribution of the detritus bulk, most of it being from terrestrial origin which is known to have higher C:N ratios (Dodds et al. 2004) and lower ^{15}N signatures (Fry 1991, Seifert and Scheu 2012). On the other hand, the negative relationships between ^{15}N and C:N ratios of PUCs with higher structural complexity, such as filamentous algae and macrophytes, cannot be explained by their variation in N turnover rates as it could be for the epilithon. In this case, a possible explanation may be that the variety of sources from which aquatic plants can use N and their contrasting uses for growth or storage usually have distinct ^{15}N signatures (Robinson et al. 1998). Therefore,

higher C:N ratios of filamentous algae and macrophytes, which are indicative of greater N demand, may lead to lower N storage, ultimately affecting their ^{15}N signatures. However, although results from this Thesis on the relationships between ^{15}N and C:N ratios of the different PUCs are potentially interesting, they do not allow disentangling the mechanisms behind them, and further controlled experiments should be conducted to understand them. In this sense, most recent stable isotope techniques linking high-resolution microscopy with isotopic analysis (NanoSIMS; Herrmann et al. 2007) or using isotope-labelling on RNA (Neufeld et al. 2007) may help disentangling the influence of elemental stoichiometry on microbial ^{15}N signatures in the upcoming future.

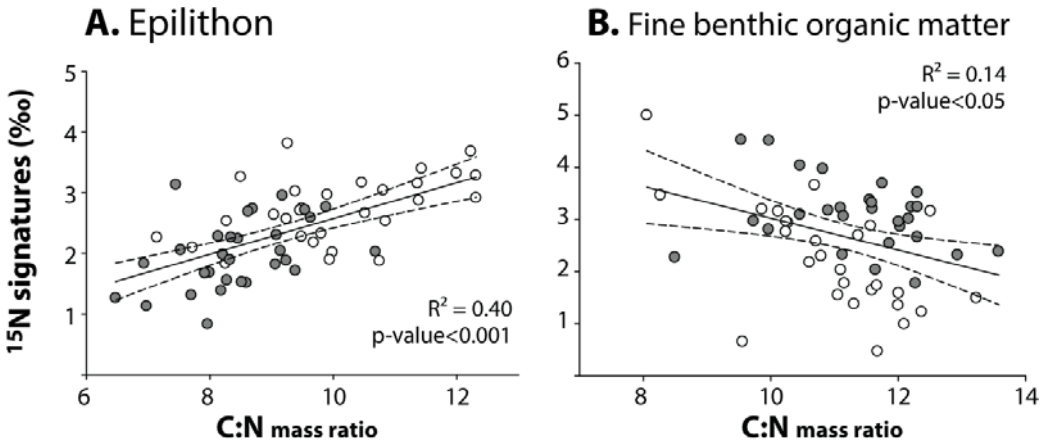


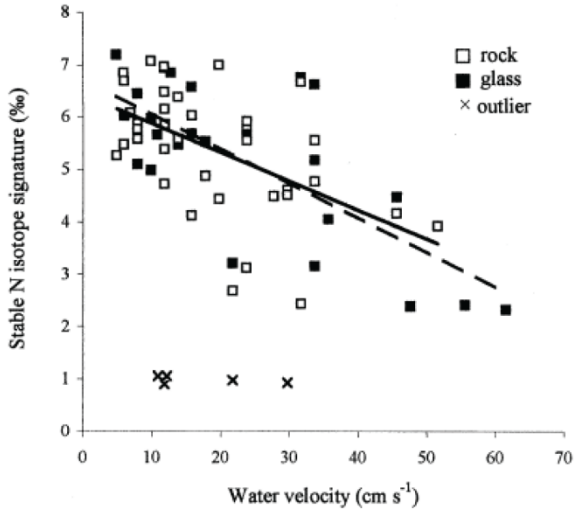
Figure 7.2: Relationships between C:N mass ratios and ^{15}N signatures of epilithon (A) and FBOM (B) at the microhabitat scale in Fuirosos stream. Empty dots represent ^{15}N data from the open-canopied reach (higher light availability) and filled dots represent ^{15}N data from the shaded reach (lower light availability). Coefficients of determination (r^2) and *p*-values are indicated in each panel. Dashed lines indicate the 95% confidence intervals for the regression line (solid line).

7.1.2 Effects of water depth and velocity on the ^{15}N signatures of primary uptake compartments

At the microhabitat scale, we found that hydraulic factors have critical effects on the variation in N cycling of in-stream microbial compartments (*Chapter 4*). This suggests that ^{15}N natural abundance of microbial compartments should bear some imprints of this fine-scale spatial variation in N cycling. Trudeau and Rasmussen (2003) conducted a controlled experiment from which they proposed that thicker boundary layers in epilithic biofilms exposed to low water velocities result in reduced isotopic discrimination compared to their nutrient sources and thus in higher ^{13}C and ^{15}N signatures (Figure 7.3 A). This mechanism was also observed under field conditions in Fuirosos stream for the epilithon ^{13}C signatures, but not for ^{15}N signatures. Although the ^{15}N range was considerably narrower than the one observed for Trudeau and Rasmussen (2003), boundary layer effects on epilithon ^{15}N did not seem to be appreciated under field conditions (Figure 7.3 B). A field study examining the water velocity effect on epilithon ^{15}N, with a similar range of 15N values than in Fuirosos, stream did not find any significant relationship between velocity and epilithon ^{15}N either (Rasmussen and Trudeau 2007). Two plausible explanations for the non-significant relation between water velocity and epilithon ^{15}N are that 1) velocity measurements did not captured the required microscale resolution or 2) other important factors affecting epilithon ^{15}N signatures at fine scales such as light availability or their metabolic activity (MacLeod and Barton 1998) masked the effects of water velocity. However, ^{15}N natural abundances of epilithon were positively related to water depth in Fuirosos stream (r^2=0.3; p-value<0.05), which was also an important predictor for epilithon N uptake. Water depth can also lead to thicker epilithic biofilms

and the subsequent reduced isotopic discrimination (Trudeau and Rasmussen 2003). Therefore, it seems that the lack of significant relationships between water velocity and epilithon ^{15}N signatures under field conditions could be caused by a non-sufficient resolution of the water velocity measurements. In this case, water depth may be a more powerful predictor of epilithon ^{15}N variation than water velocity under field conditions due to its easier measurement. On the other hand, microhabitat variation in hydraulic conditions did not influence ^{15}N natural abundance of FBOM even though water depth and velocity were significant predictors for both FBOM uptake and turnover. The lack of significant relationships between ^{15}N natural abundance of FBOM and the important drivers for its N cycling may again indicate that ^{15}N bulk signatures of FBOM are mostly representative of the detrital pool rather than of the active microbial organisms.

A. Laboratory experiment: *artificial stream*



B. Field experiment: *Fuirosos stream*

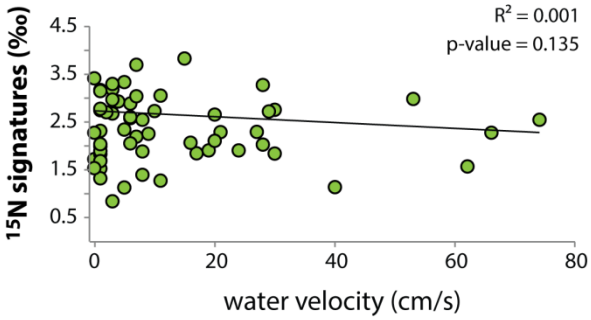


Figure 7.3: Relationship between water velocity and ^{15}N natural abundance of epilithon in A) a controlled experiment conducted by (Trudeau and Rasmussen 2003), and B) in a field experiment conducted in Fuirosos stream.

At the habitat scale, neither epilithon nor FBOM showed any significant variation in their ^{15}N signatures between riffle and pool habitats, despite their clear hydraulic differences, as have been observed in other studies (Finlay et al. 2002). Nonetheless, filamentous algae and bryophytes showed greater ^{15}N signatures at pools habitats, which are characterized by slower velocities and deeper waters, than at riffle habitats (ANOVA, *p*-value<0.05). Although these two photoautotrophic PUCs have much higher structural complexity than

microbial assemblages, variation between habitats in their ^{15}N signatures seems to be consistent with the lower isotopic discrimination generated by the effect of a thicker boundary layer in the two compartments (MacLeod and Barton 1998, Trudeau and Rasmussen 2003).

7.1.3 Effects of urban N sources on the ^{15}N-PUC signatures

In general, imprints of local N processing on the ^{15}N signatures of PUCs are difficult to be discerned in relatively pristine streams. A good example of this is that clear relationships between C:N ratios and ^{15}N-PUC signatures observed in La Tordera headwaters were not necessary observed in Fuirosos stream, and vice versa. The main reason for that is the generally low ^{15}N labeling of DIN sources in pristine streams. In particular, ^{15}N-PUC signatures are affected by almost every step during their internal N cycling because of the isotope fractionation associated with uptake (Evans 2001, Robinson 2001, Bedard-Haughn et al. 2003), assimilation (Waser et al. 1998, Casciotti et al. 2003, Cohen and Bradham 2010), mineralization (Mariotti et al. 1981, Macko et al. 1987, Hogberg 1998) or NH_4/NH_3 state changes (Handley and Raven 1992, Hogberg 1998). As a result, the effects that elemental stoichiometry, N turnover, or preferential DIN sources can have on DIN uptake by PUCs and ultimately influence their ^{15}N signatures would be more easily mask by internal isotope fractionation when the ^{15}N signal of the DIN input (i.e., ^{15}N-DIN content) is low, as normally happens under pristine conditions. Contrastingly, when stream and groundwater DIN sources (ammonium and nitrate) exhibit a more enriched and heterogeneous ^{15}N signatures, the intrinsic and extrinsic processes controlling N cycling through PUCs are more easily traced by their ^{15}N signatures. This is in fact the conceptual idea of the ^{15}N tracer additions, in which a DIN source (nitrate or ammonium) is ^{15}N-enriched to trace the fates

and processes of DIN within the stream channel (*see* Mulholland et al. 2000). However, the paradox relies on some urban streams, where the highly enriched ^{15}N-DIN signatures contribute to amplify the imprints of N processing on the ^{15}N-PUC signatures. Examples of this effect are found in streams receiving inputs from urban wastewater treatment plants (WWTP) as shown in *chapters 2 and 3*.

Stream ecosystems influenced by WWTP inputs show a remarkable discontinuity in stream water ^{15}N-DIN signatures between reaches located upstream and downstream of the inputs (Ribot et al. 2012, Pastor et al. 2013). Compilation of data from this Thesis shows that the variation in ^{15}N-PUC natural abundance increases from low- to high-order streams (Figure 7.4), reflecting the greater variation in ^{15}N-DIN as stream order increases probably because their increase in DIN sources with differential ^{15}N signatures and also the increase in the relevance of the in-stream processes affecting ^{15}N signatures of DIN sources. In addition, the data show that natural abundance of ^{15}N-PUCs in WWTP-influenced streams are clearly higher than values observed across stream orders, indicating that urban activity has a strong effect on the ^{15}N isotopic signals of DIN sources reaching the stream, which are further transferred to stream water PUCs. Higher ^{15}N-DIN signatures in WWTP effluents are due to the intense isotope fractionation associated with nitrification-denitrification processes undergoing in the WWTP operation. As a result, downstream the WWTP inputs, stream DIN loads are higher (Marti et al. 2004, Merseburger et al. 2005) and have higher ^{15}N-DIN signatures. Nevertheless, ammonium and nitrate can have distinct ^{15}N signatures in the receiving streams depending on the relative proportion of these two DIN species in the WWTP effluent (Ribot et al. 2012, Pastor et al. 2013). In addition, spatial

variability in ^{15}N-DIN signatures along the receiving streams is remarkable (Lofton et al. 2007, Gammons et al. 2010, Merbt et al. 2011) due to intense in-stream N cycling.

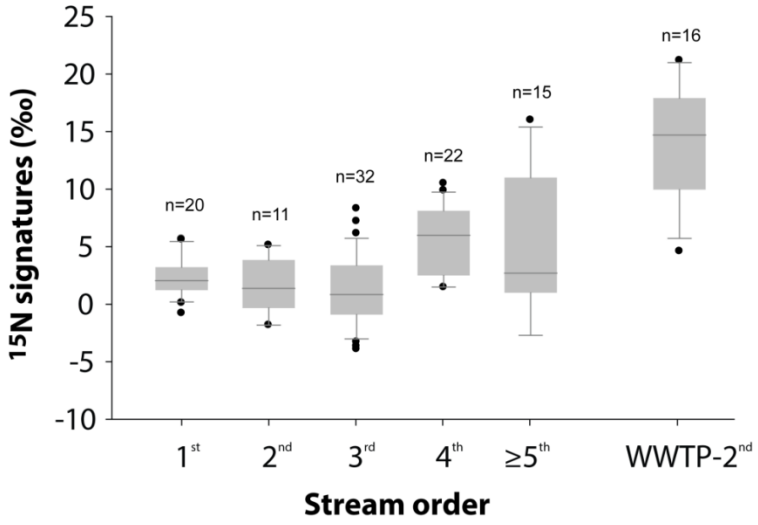


Figure 7.4: Variability in ^{15}N-PUC signatures among streams of different stream orders. Data from literature-compilation in *Chapter 1*. Variation in ^{15}N-PUC signatures of WWTP-influenced 2nd order streams is also displayed at the right part of the graph. The number of data (averaged per compartment and stream) for each stream order is showed over each box plot. Box plots display 10th, 25th, 50th, 75th and 90th percentiles, and individual outliers outside the 10th and 90th percentiles are shown as dots.

In this Thesis it has been demonstrated that the ^{15}N-DIN enrichment and its longitudinal variability along WWTP-receiving streams constitute an opportunity to examine N cycling by PUCs in highly loaded streams based only on natural abundances instead of ^{15}N tracer additions, which are economically-constrained in these types of streams. Once in the stream water, WWTP-derived DIN is subjected to similar processes governing N dynamics in relatively pristine streams, but the footprint that these processes leave on the ^{15}N-PUC

natural abundances is magnified because of the higher ^{15}N signal in the DIN sources, which leads to more clear relationships at natural abundance levels than those observed in pristine streams. A good example of this effect is observed when comparing the relationships between N content and ^{15}N signatures of PUCs in a reference and an urban-influenced stream (Figure 7. 5). Percentage of N in PUCs, which apparently has no influence on their ^{15}N signatures at the reference stream, is a powerful predictor of ^{15}N-PUC signatures in the urban stream. Although the range of %N in PUCs is almost the same in both types of streams, the range of ^{15}N-PUC is much wider in the urban stream, which allows determining the fate of the urban-derived N in the stream biota (Figure 7.5). Contrastingly, the larger ^{15}N range in the urban stream does not particularly magnify the relation between ^{15}N and C:N ratios of PUCs, probably because C:N stoichiometry is more physiologically constrained; and thus; it is less influenced by the effects of urban-derived DIN (Figure 7.5).

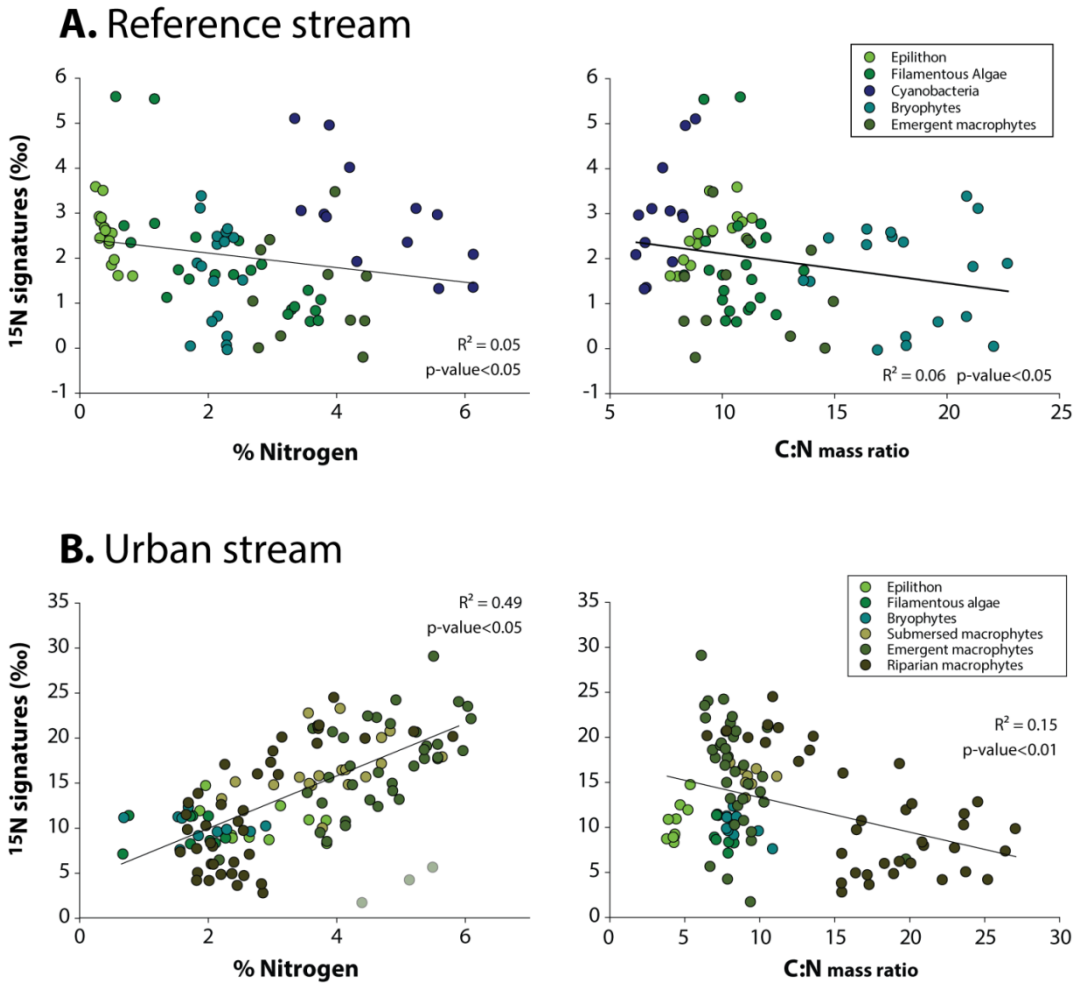


Figure 7.5: Relationships between %N or C:N ratios and ^{15}N signatures of PUCs in Fuirosos stream (A) and in a WWTP-influenced reach in La Tordera river (B). Coefficient of determinations (r^2) and *p*-values are indicated in each panel. Graphs consider values for all different PUCs together.

The contrasted findings between pristine and urban streams indicate that tracing the N transfer from DIN to PUCs by using ^{15}N signatures at natural abundance levels is more easily observed under stream conditions of high variation in ^{15}N-DIN signatures and isotopically-enriched values. This is

illustrated in Figure 7.6, in which the inclusion of ^{15}N data from WWTP-influenced streams expands the ^{15}N-DIN and ^{15}N-PUC ranges leading to a significant relationship between the two variables, although we observe a clear segregation of values between pristine and urban streams, as previously discussed. Interestingly, this relationship is better explained by a log-log regression (Figure 7.6), indicating that ^{15}N-PUC signatures tend to reach a steady state regardless of increases in ^{15}N-DIN. This is further supported by results from the translocation experiment (*Chapter 3*). A possible explanation for this pattern is that ^{15}N-PUC signatures are controlled by the N uptake rate of each compartment (*Chapter 3*). Alternatively, because usually increased ^{15}N-DIN signatures are associated with higher DIN concentrations, the steady state in ^{15}N-PUC signatures may reflect the uptake saturation at higher DIN concentrations (O'Brien et al. 2007, Ribot et al. 2013). Overall, these findings are in agreement with previous studies showing that high and usually distinct ^{15}N signatures of NH_4 and NO_3 are well correlated with the ^{15}N-PUC signatures (Kohzu et al. 2008, Ribot et al. 2012, Pastor et al. 2013). However, our results additionally indicated that this relation is particularly significant in PUCs with a high dependence on stream water DIN (*Chapter 2*) and/or with fast N turnover (*Chapter 3*).

An interesting result comes from the comparison of the relationship between ^{15}N-PUC and the ^{15}N of the two DIN species. Existing literature has mostly concentrated in the measurement of ^{15}N signatures of NO_3 as the dominant DIN species. In this Thesis we particularly considered the ^{15}N signatures of the two major DIN species and found that their ^{15}N isotopic signatures can be clearly distinct. This isotopic difference has allowed a further examination on the preferential use of the two DIN species by PUCs. At the

reference streams, ^{15}N-PUC signatures seem to be generally higher than ^{15}N-DIN signatures, especially for ^{15}N-NH_4 (Figure 7.6), which suggests that given the low ^{15}N-DIN variation in the stream water, intrinsic factors controlling the ^{15}N variation of PUCs may be more relevant to explain patterns of ^{15}N-PUC signatures. In contrast, at urban streams, ^{15}N-PUC signatures are in between the ^{15}N signatures of NH_4 and NO_3, although generally towards ^{15}N-NO_3 (Figure 7.6); which suggests that both DIN species are important N sources for the different PUCs. The close similarity with ^{15}N-NO_3 signatures indicates that, despite NH_4 seems to be the preferred N source by most PUCs (Naldi and Wheeler 2002, Ribot et al. 2013), the commonly greater concentrations of NO_3 may favor uptake of N-NO_3 because its higher concentration compensates for the higher energetic costs during NO_3 assimilation (Dortch 1990). The concentration effect is further supported by results from urban streams where both concentration of NH_4 and NO_3 are high. In this case, ^{15}N-PUC signatures seem to reflect a balanced isotopic mixing between ^{15}N-NH_4 and ^{15}N-NO_3 signatures, indicating a similar use of the two DIN species.

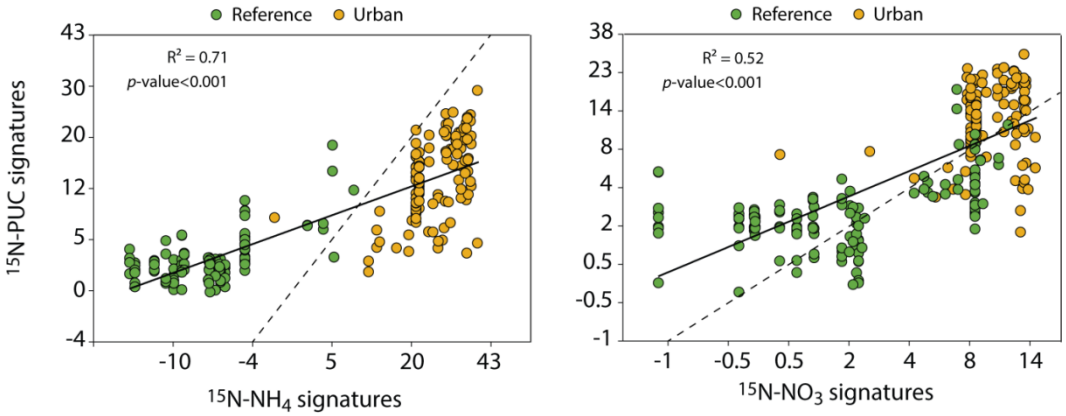


Figure 7.6: Log-log relationships between ^{15}N-NH$_4$ signatures (left panel) and ^{15}N-NO$_3$ signatures (right panel) and the ^{15}N signatures of PUCs. Data comes from samplings conducted in several reference and urban streams, and includes the variation at different spatial scales. Coefficient of determinations (r^2) and *p*-values are indicated in each panel. Dashed line indicates the 1:1 ratio between ^{15}N-DIN and ^{15}N-PUC signatures.

The use of ^{15}N natural abundance as a tracer between DIN and PUCs relies on an isotope mixing approach that considers the different N sources, but also the fractionation that may occur during chemical, physical, and biological uptake processes within each particular PUC. Isotope fractionation of N during uptake (Evans 2001, Robinson 2001, Bedard-Haughn et al. 2003) and assimilation (Waser et al. 1998, Casciotti et al. 2003, Cohen and Bradham 2010) may play a significant role on the divergences between ^{15}N-DIN and ^{15}N-PUCs. Fractionation dictates that ^{15}N signatures of the products and substrates in every biologically-mediated N processes will differ due to the different preferential use of the lighter N stable isotope (Fry 2008). Quantification of isotope fractionation from field samplings is complicated; and thus, estimates of N fractionation from in situ measurements have a high degree of uncertainty (Bedard-Haughn et al. 2003). For this reason, in field studies, the overall

discrimination factor, which represents the difference between the ^{15}N signatures of substrate and product (i.e., $^{15}N_{source}$–$^{15}N_{PUCs}$), is a more useful term. However, if there is more than one DIN source the distinction between isotope mixing effects and isotope fractionation is always controversial. The large number of values for discrimination factors compiled within this Thesis show that, despite of the difficult interpretation, the ^{15}N discrimination between PUCs and the two DIN species is relatively consistent both among PUCs and between pristine and urban streams (Figure 7.7). The small differences in ^{15}N discrimination factors among PUCs with such different structure and under different DIN concentrations contrasts with the reported effects of organism's metabolism and nutrient concentration on isotope fractionation during N uptake (Evans 2001, Robinson 2001, Bedard-Haughn et al. 2003). The observed narrow variation in ^{15}N discrimination factors among PUCs may indicate that isotope mixing rather than fractionation drives ^{15}N differences between PUCs and their DIN sources. Results from discrimination factors also indicate clear differences of the ^{15}N-PUCs in front of the two DIN species (Figure 7.7). In general, ^{15}N-PUC signatures were more similar to ^{15}N-NO_3 than to ^{15}N-NH_4. This suggests that most of the DIN uptake by PUCs is in the form of NO_3 (*Chapter 1 and 2*), as previously discussed, especially for PUCs that relay on DIN from the water column (i.e., epilithon, filamentous algae, bryophytes), which could be explained by the usually higher NO_3 availability in stream waters associated with higher NO_3 uptake rates compared to NH_4 (Ribot et al. 2013). The higher NO_3 discrimination factors of macrophytes, especially in urban streams, may be attributed to a large intra-plant ^{15}N variation during NO_3 uptake (Evans et al. 1996).

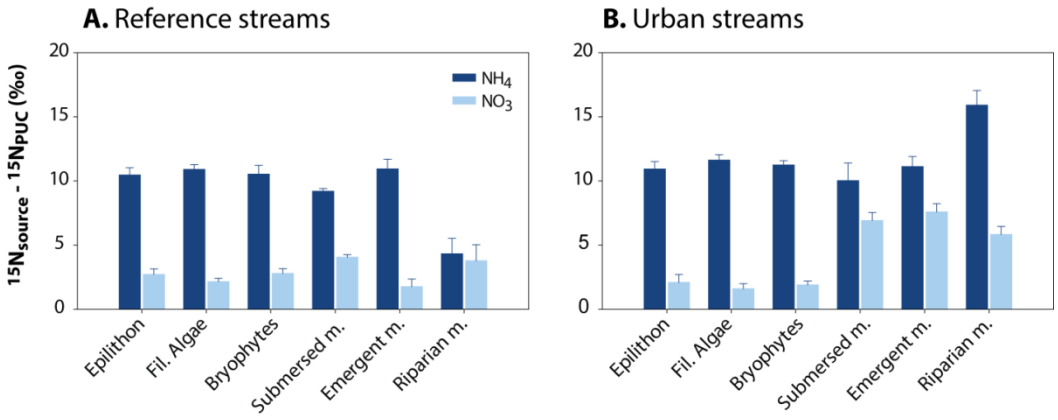


Figure 7.7: Variation in discrimination factors between ^{15}N of NH_4 and NO_3 (sources) and ^{15}N of the different primary uptake compartments (PUC) at both reference (A) and urban (B) streams. Columns and error bars are mean values and standard error of the mean, respectively.

Finally, it is worth mentioning that although human effects on the ^{15}N signatures of DIN species were already described (Panno et al. 2006, Lefebvre et al. 2007), results from this Thesis have also demonstrated a clear contrast between ^{15}N-PUC signatures from pristine and from human-impacted streams. This contrast is illustrated for epilithon in figure 7.8, but the rest of PUCs follow similar patterns. Within this context, one may be confused by the term of '*natural abundance*', which is commonly used to differentiate the ^{15}N signatures found in the environment to those resulting from tracer ^{15}N additions into ecosystems. The truth is that 'natural abundance' refers instinctively to pristine conditions, but results complied in this Thesis show that high ^{15}N signatures of anthropogenic DIN inputs are also propagated to PUCs and probably to higher trophic levels. Therefore, in many cases, the term '*natural abundance*' may also include results from altered conditions in which all PUCs exhibit ^{15}N signatures unlikely to be found under pristine conditions (Figure 7.8). For this reason, the term '^{15}N *ambient abundance*' would probably be more appropriate in order to

include the variability in ^{15}N-PUC signatures generated by different factors in both pristine and human-altered conditions, thereby minimizing the possible confusion associated with the term '*natural abundance*'. A term that unequivocally refers to both natural and anthropogenic variation in ^{15}N-PUC signatures can be particularly relevant for studies using ^{15}N ambient abundances to examine trophic webs (Coat et al. 2009, Boecklen et al. 2011). Nitrogen isotopes are typically used to separate the different trophic levels in aquatic food webs; and thus, using a clear term such as '^{15}N *ambient abundance*' may avoid misunderstandings in the use of ^{15}N isotopes in trophic ecology.

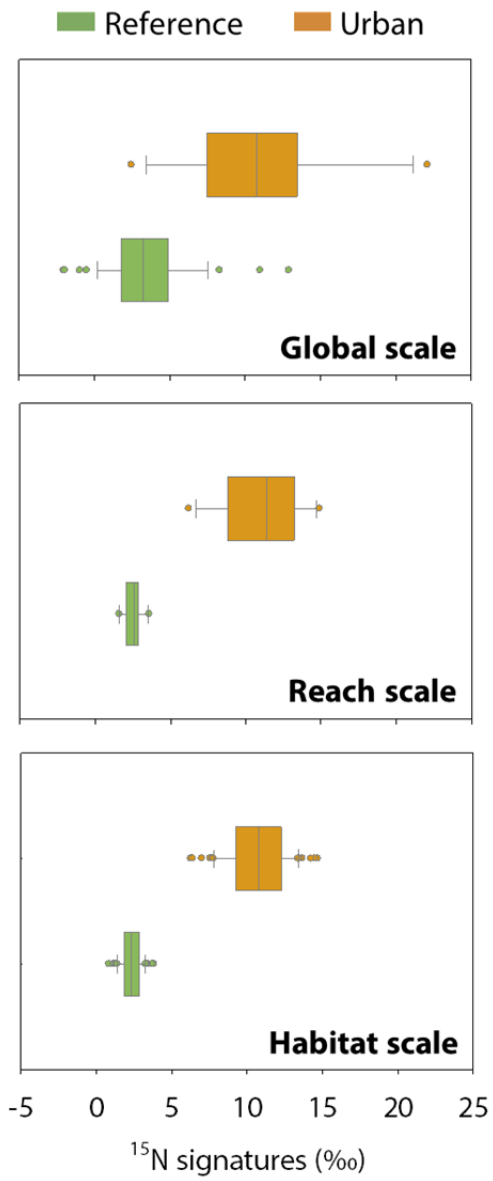


Figure 7.8: Variation in ^{15}N signatures of epilithon in reference and urban streams across different spatial scales. Data for the global scale correspond to compiled values from streams worldwide draining either forested (reference) or urbanized (urban) catchments. Data at the reach and habitat scale correspond to values from Fuirosos stream (reference) and from a WWTP-influenced reach in La Tordera river (urban).

7.2 Using ^{15}N natural abundances of primary uptake compartments to understand their contribution to stream N cycling

Results from this Thesis provide several lines of evidence showing that ^{15}N natural abundances in PUCs provide information on the contribution of these benthic compartments to in-stream N cycling. Firstly, global patterns of variability in ^{15}N signatures of DIN and PUCs suggest, in a qualitatively manner, that N uptake by PUCs is related to DIN sources from the catchments. Secondly, the clear distinction in ^{15}N-PUC signatures between reference and urban streams (Figure 7.5 and 7.8) is consistent with the enriched ^{15}N values of DIN from anthropogenic sources found in this Thesis and in other studies (Panno et al. 2008, Chen et al. 2009) or DIN transformations in the catchment (Udy and Bunn 2001, Diebel and Vander Zanden 2009), which confirms that PUCs constitute a significant N fate in stream networks. Thirdly, the observed relationships between ^{15}N signatures of DIN and PUCs among streams (Figure 7.6) or within a stream for specific PUCs (*Chapter 2*) suggest a transfer of DIN into PUCs; and thus, a strong reliance on stream water DIN by PUCs. Finally, results from the translocation experiment (*Chapter 3*) provide empirical support to previous evidences and allowed quantifying uptake and turnover rates for different PUCs under contrasting environmental conditions.

It has been shown that 60-70% of DIN inputs to streams are retained by in-stream processes (Peterson et al. 2001). These estimates were mostly based on rates associated with $N\text{-}NH_4$; and thus, information of NO_3 uptake appeared as a critical uncertainty factor on the overall picture of in-stream DIN uptake. Mulholland et al. (2008) indirectly suggested that uptake by benthic

compartments could play an important role in total NO_3 uptake at reach scale, by showing that denitrification could only account for 18% of the estimated values. Our results on ^{15}N discrimination factors discussed in the previous section (Figure 7.7) are in agreement with this previous study, pointing at NO_3 as the most important DIN source for PUCs. Higher NO_3 than NH_4 dependence by PUC uptake may be explained by the higher availability of the former DIN species and seems to be additionally favored by light availability that increase the activity of PUCs; and thus, probably their N uptake (*Chapters 2 and 4*).

N uptake by PUCs is a relevant process contributing to stream N dynamics, but to fully understand their contribution to reach scale N dynamics, it is important to also consider the turnover time of assimilated DIN by each PUC. Results on ^{15}N changes over time from the translocation experiment provided insights on this component of N cycling for each compartment (*Chapter 3*). We used the time required to achieve isotopic equilibrium under translocated conditions as a surrogate for the N turnover time in each photoautotrophic PUC. Within this context, epilithon and filamentous algae, with typically low N standing stocks and high metabolism, achieved isotopic equilibrium faster than other PUCs with more complex structures, regardless of differences in environmental conditions among sites. From these results we conclude that ^{15}N natural abundance of epilithon and filamentous algae is a good integrator of short-term ^{15}N-DIN variability in the stream water, while ^{15}N natural abundance of bryophytes and, particularly, macrophytes are better integrators of medium to long-term ^{15}N-DIN variability. This is also supported by the greater annual variation of ^{15}N signatures in epilithon and filamentous algae (Pastor et al. 2014). In addition, this finding indicates that, although the contribution to stream N uptake of bryophytes or macrophytes can be low due to their

relatively low abundance in streams and low uptake rates, their role on stream N retention may become more relevant since they can retain N for longer-time periods.

7.3 The role of primary uptake compartments on in-stream N uptake, controlling factors and their scales of operation

Through the previous sections we have indicated that ^{15}N natural abundance of PUCs is a valuable tool to examine the influences of several environmental factors on N uptake by PUCs and on their potential contribution to stream N uptake at different spatial scales. However, it is also true that the use of ^{15}N-PUC natural abundances for these purposes has more constrains at smaller spatial scales (e.g., habitat scale) and under relatively pristine conditions. It is well known that spatial heterogeneity of stream networks, as well as in other ecosystems, is hierarchically organized by a set of successional levels from global (e.g., catchment) to fine spatial scales (e.g., habitat or microhabitat; Frissell 1986, O'Neill et al. 1986). As a result, spatial heterogeneity in each hierarchical level is embedded in the upper level, so that spatial variation increases from fine to large spatial scales. We demonstrated that patterns of variability in ^{15}N natural abundances of PUCs followed this hierarchical organization by showing greater spatial variation at larger spatial scales, in both reference and human-altered streams (Figure 7.1). However, ^{15}N-PUC variation observed at fine scales does not seem to be particularly related to the N uptake or ^{15}N-DIN variation at this scale. Therefore, variation in ^{15}N-PUC natural abundance at this scale does not seem to be informative of the factors that regulate their contribution to stream N cycling. For instance, despite the

large spatial variation in microbial N uptake at the microhabitat scale observed in Fuirosos stream (*Chapter 4*), there was no trace of any functional imprints on the microbial ^{15}N natural abundances. It is in these situations when the use of ^{15}N tracer additions is strictly required in order to understand factors controlling the role of PUCs in stream N uptake from fine to larger spatial scales. The use of ^{15}N tracer additions in this Thesis also helped to corroborate some of the findings obtained from patterns of ^{15}N natural abundances.

7.3.1 Hydraulic influences across spatial scales

A principle in ecology is that examination of spatial variation requires using the appropriate scale depending on the organisms or processes of interest (Allen 1977, Pringle et al. 1988, Levin 1992). It is evident that the spatial scale at which factors influence the role of each PUC to in-stream N uptake will vary according to their particular characteristics. Moreover, it is well known that nutrient cycling in stream ecosystems is mostly driven by microbial assemblages developed in epilithon or in accumulation of detrital organic matter (Hall et al. 1998, Mulholland et al. 2000, Webster et al. 2009). Therefore, the spatial scales of variation perceived by microbial-dominated PUCs can be different from those perceived by other primary uptake compartments.

We found that spatial heterogeneity at microhabitat scale influences the response of benthic microbial assemblages to N cycling, which results in a mosaic of N uptake and turnover rates at reach scale. This mosaic is characterized by a dominance of patches with relatively low N uptake rates, with some highly active patches (i.e., hot spots). Results indicated that this functional heterogeneity of microbial PUCs was mostly attributed to the microhabitat variation in water velocity and depth. Highly active patches of epilithon were related with faster water velocities, whereas those of FBOM

were related with higher water column depths. Therefore, reductions of the spatial heterogeneity in water velocity and depth, associated with hydro-morphological changes of the stream channel, can lead to a strong alteration in the microbial contribution to stream N uptake (*chapter 4*). Interestingly, the same hydro-morphologic factors influencing microbial N uptake at fine spatial scales can be also relevant at larger scales, influencing, for instance, the spatial distribution of macroorganisms such as bryophytes and/or macrophytes (Bowden et al. 2007). At the habitat scale (i.e., pools and riffles), the abundance of macrophytes is maximized when local conditions of water velocity (0.3 – 0.5 m s^{-1}) and depth (30 – 80 cm) are combined, although these optimal ranges can vary substantially among different species (Riis and Biggs 2003). Habitat distribution of macrophytes within the streams can be critical to determine whether or not they contribute to in-stream N retention (*Chapters 2 and 3*). At the reach scale, water velocity and whole-reach N uptake follows a very similar relationship than the one observed at the microhabitat level for epilithon (Figure 7.9). Thus, water velocity seems to positively affect N uptake at both fine and larger spatial scales of stream ecosystems with relatively pristine conditions (i.e., low DIN concentrations). However, increases in water velocity or depth at the reach scale have been also associated with a decrease in nutrient uptake efficiency by reducing the residence time of nutrients (Valett et al. 1996, Butturini and Sabater 1998, Peterson et al. 2001, Hall et al. 2009). Overall, these results indicate that even though increased water velocities at the reach scale lead to greater whole-reach N uptake, this increment does not seem to be proportional to the increased in N flux in the water column associated with high flow conditions; and thus, the whole-reach uptake

efficiency decreases as showed by the positive relation between uptake length (i.e., the inverse of the uptake efficiency) and discharge (Peterson et al. 2001).

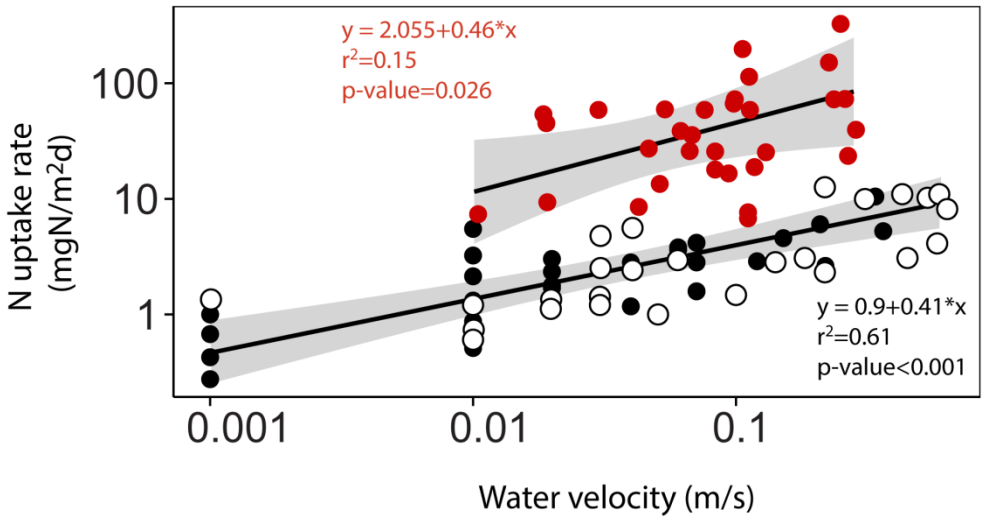


Figure 7.9: Log-log relationship between water velocity and N uptake rates by epilithon (black and with dots; data from Fuirosos stream, *Chapter 4*), and by whole-reach N uptake rate estimates (red dots, data from 32 different pristine streams across the US from *Peterson et al. 2001* and *Mulholland et al. 2008*.)

7.3.2 Influence of nutrient and light availability

Nitrogen uptake rates of all photoautotrophic PUCs considered in this Thesis responded positively to increases in DIN and light availability, evidencing the well-known linkage between energy and nutrient requirements by these organisms (Dodds et al. 2004, Allen et al. 2005, von Schiller et al. 2007). This was observed with results from the use of ^{15}N as both natural abundance (*Chapter 3*) and tracer additions (*Chapter 4*). In particular, the comparison of N cycling between an open and a shaded reach in Fuirosos stream showed almost 3-

times higher total N uptake and retention by PUCs under higher light availability (*Chapter 4* and Fig 7.10). Considering PUC-specific responses, differences in N uptake seem to be greater among reaches differing in DIN concentration (*Chapter 3*) and light incidence (*Chapter 3 and 4*) than among different photoautotrophic PUCs, even though they encompass remarkably differences in terms of structural complexity and metabolism. However, epilithon N uptake showed no response to increased light availability in Fuirosos stream (Figure 7.10). This may be explained by the much lower DIN concentrations in Fuirosos stream than in the WWTP-influenced reach in La Tordera river (*Chapter 3 and 4*), suggesting that that light effects on N uptake is subedited to the N availability. In contrast to N uptake, our results indicated that variation in the N turnover is apparently larger among the different photoautotrophic PUCs than among different reaches, suggesting a strong control by the intrinsic character of each compartment (*Chapter 3*). Therefore, the role of photoautotrophic PUCs on in-stream N cycling seems to be controlled by the balance between the influence of extrinsic factors on their N uptake and the influence of intrinsic factors on their N turnover/retention. These results are also supported by the ^{15}N tracer addition, but this experiment also showed that these patterns and controlling factors observed for photoautotrophic compartments are not as consistent for detrital-based PUCs, such as the FBOM, since both N uptake and turnover seems to be highly influenced by light availability (i.e., an extrinsic factor; Figure 7.10).

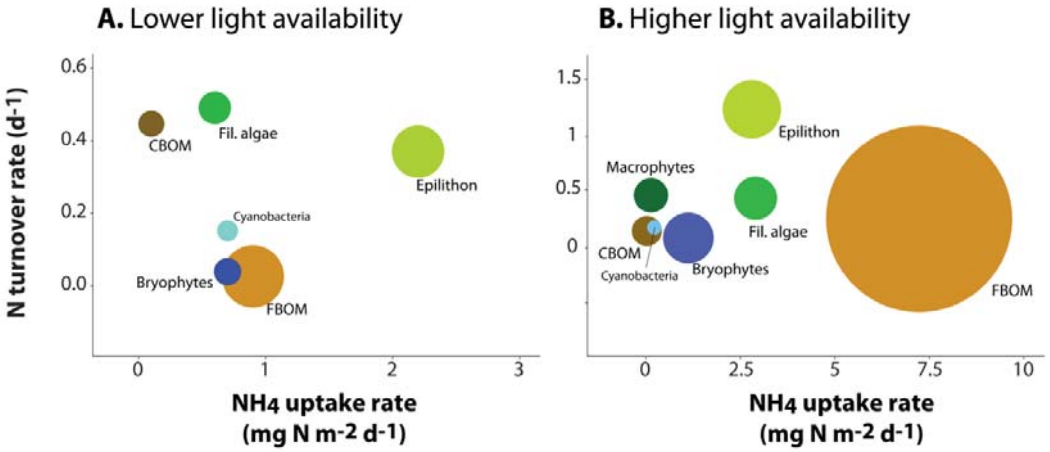


Figure 7.10: Relationship between ammonium uptake rates and N turnover rates for all different PUCs present at the shaded reach (A) and open reach (B) in Fuirosos stream. Turnover rates are calculated from the ^{15}N decay after the end of the addition and thus represent the estimated N release rate for each compartment. The size of the dots is proportional to the amount of N biomass retained in each compartment.

7.3.3 Implications within the context of global change

In the Mediterranean region, some relevant pressures on stream ecosystems derived from human-driven global change are the increased nutrient inputs and the deforestation of riparian zones. These two pressures are associated with land use changes in the catchments (Gasith and Resh 1999, Merseburger et al. 2005, von Schiller et al. 2008, Martí et al. 2010, Caille et al. 2012). In this Thesis we show that PUCs play an important role on in-stream nutrient cycling, and also show differences in relative role of each PUC on, as well as the controlling factors on this contribution. This information can be fundamental to preserve the functional integrity of stream ecosystems and find management strategies in front of changes from global change. Many results in this Thesis showed that N uptake by PUCs, especially by photoautotrophic

PUCs which are most directly linked to the stream water DIN, responds to increases of both nutrient and light availability. However, a more difficult prediction is to ascertain whether or not this increased N uptake will result in greater N retention. Variation in the N cycling within photoautotrophic PUCs (i.e., N uptake vs turnover) seems to be limited by the particular physiological constraints of each PUC, even when their N uptake varies substantially. These limitations result in greater variation in N turnover among compartments than among streams with different environmental conditions; and thus, alterations of the nutrient loads and riparian vegetation in stream ecosystems may be counterbalanced by higher photoautotrophic N uptake, but not necessarily by a greater N retention within the reach. Significant increases in the photoautotrophic contribution to total N retention (i.e., the balance between uptake and turnover) may rather be associated to greater relative abundance of fast-growing compartments such as filamentous algae, which are favored in open-canopy channels. However, in those cases where the contribution of photoautotrophic PUCs on stream N retention is not particularly influenced by changes in nutrient and light conditions, FBOM has demonstrated that can sustain a large proportion of the total N retention by PUCs.

Results from this Thesis also showed that preserving the spatial heterogeneity of the stream channel is critical to sustain the role of in-stream N cycling by microbial assemblages (epilithon and FBOM), which are the most ubiquitous PUCs among streams and have a high contribution to N uptake and retention at reach scale. For instance, the morphological alteration of the channel derived from riparian deforestation, which generates large sediment inputs to the streambed and decrease its spatial heterogeneity, may result in a reduced contribution of microbial compartments to stream N retention. In

contrast, other PUCs, such as macrophytes, may be favored by the sediment accumulation and higher light availability, and their greater abundance may partially compensate the decrease by microbial N uptake. This last example is in fact a current situation in many human-altered lowland streams, where channels are constrained, spatial heterogeneity is poor, and PUCs such as macrophytes are usually surprisingly abundant. Ultimately, channel alterations may lead to important changes in the relative abundance and distribution of different PUCs in stream ecosystems, as well as in their N uptake capacity, which, according to our results, will certainly change the capacity of streams to retain N inputs from adjacent catchments at reach scale.

8

General conclusions

Major conclusions from 3rd chapter:

- At the global scale, land use in the catchment is a key driver of the variability in ^{15}N signatures of both DIN and PUCs. Variation in ^{15}N signatures among streams draining different land use types is greater than variation within a single stream, regardless of the compartment considered.
- ^{15}N signatures of DIN and PUC are higher in streams draining catchments with agriculture and urban activities than in those draining forested catchments
- Within each stream, major among-compartments differences in ^{15}N signatures are between photoautotrophic and detrital-based compartments.
- Future studies should address data gaps on temporal variation of stream ^{15}N signatures to understand how N isotopic signatures of PUCs integrate temporal variation of ^{15}N-DIN signatures, and how ^{15}N signatures of NH_4, NO_3 and PUCs are linked to each other.

Major conclusions from 4th chapter:

- Spatial variation in ^{15}N natural abundances of DIN and of aquatic macrophytes from streams impacted by WWTP effluents indicates that direct assimilation of stream water DIN is occurring by submersed and amphibious species; while species of macrophytes located at the stream-riparian edge most likely rely on DIN sources other than those provided by stream water DIN.
- In WWTP-influenced streams, DIN uptake by submersed and amphibious macrophytes seems to be mainly in the form of NO_3.

Major conclusions from 5th chapter:

- Experiments based on translocation of PUCs from pristine sites to urban-influenced sites seem to be an efficient and accurate approach to understand the factors influencing the ^{15}N natural abundances of PUCs as well as to estimate the N cycling rates of photoautotrophic PUCs.
- Variation in DIN uptake of photoautotrophic PUCs is principally driven by nutrient concentration and light incidence, rather than by the particular characteristics of each PUC.
- Variation in N turnover is more remarkable among photoautotrophic PUCs than among study reaches, suggesting an intrinsic control by the particular characteristics of each PUC on N turnover, and being relatively independent of environmental influences.

Major conclusions from 6th chapter:

- Spatial heterogeneity of microbial N uptake at the microhabitat scale is characterized by a mosaic of patches dominated by microhabitats of low N, with hot spots of highly active N uptake accounting for the 20% of the reach coverage.
- Spatial variation of epilithon N uptake at microhabitat scale is principally driven by flow velocity.
- Spatial variation of FBOM uptake is controlled by a combination of biophysical factors, indicating the relevance of FBOM characteristics on their role of stream water N uptake.
- The extent to which spatial variation in microbial N uptake at fine scales is integrated at the whole-reach scale is potentially affected by factors

operating at the ecosystem level, such as the degree of canopy cover, which determines the relative abundance of each PUC.

- Results from this study highlights that losses of spatial heterogeneity as well as alteration of riparian canopy cover can have a significant incidence on the way that PUCs contribute to N cycling at the reach scale, which ultimately dictates the N dynamics of stream ecosystems.

9

Glossary

Primary uptake compartments: Autotrophic and/or heterotrophic organisms or microbial assemblages living on the stream benthos that can directly assimilate dissolved inorganic nitrogen forms in the water column.

Nutrient uptake: Process of taking up any inorganic, organic, dissolved, or particulate nutrient from the environment into a living organism. Normally is expressed as uptake rate with units of mass per area and time.

Nutrient retention: The capacity of organisms to retain the taken up nutrients for a period of time by using them to conduct certain processes or by generating new biomass**.**

Nutrient turnover: A term used to explain the relationship between uptake and release of nutrients in organisms. If it is expressed with units of time (i.e., turnover time), represents the average amount of time required to renew the total standing stock of a particular nutrient in an organism. If it is expressed with units of inverse time (i.e., turnover rate or Biomass-specific uptake rate), represents the uptake rate per unit of biomass in a particular organism.

Stable isotope: Non-radioactive atoms of an element whose nucleus contain the same number of protons but different numbers of neutrons (^{14}N and ^{15}N nucleus contain seven and eight neutrons, respectively).

^{14}N: The more abundant of the two stable isotopes of nitrogen, contains seven protons and neutrons in the nucleolus (atomic mass = 14).

^{15}N: The less abundant of the two stable isotopes of nitrogen, contains seven protons and eight neutrons in the nucleolus (atomic mass = 15).

Isotope abundance: The amount of an isotope relative to other isotopes, whether expressed on the atom percentage or δ scale.

Natural abundance: The abundance of an isotope in samples to which no tracer has been added (i.e., naturally found on ecosystems).

Isotopic tracer: An isotopically distinct, but chemically indistinguishable substance, added to an ecosystem to follow the substance as it is transformed and transported by abiotic and biotic processes.

δ scale: Difference in stable isotopes ratio between the sample and a nutrient-dependent standard (atmospheric N_2 for Nitrogen isotopes). Usually expressed in units of parts per thousand (‰) relative to the standard (that is 0‰).

$\delta^{15}N$: The abundance of ^{15}N on the δ scale. Positive values mean that samples are more ^{15}N enriched than the standard, while negative values indicate that samples are ^{15}N depleted compared to atmospheric N_2.

Isotope fractionation: A differential behavior of light and heavy isotopes in every kinetic reaction by which light isotopes react faster than heavy isotopes resulting in ^{15}N enriched substrates and ^{15}N depleted products after a reaction.

Isotope mixing: Combination of two different nutrient sources with or without different isotope content in one unique nutrient pool. Fractionation and mixing together control isotope cycling and circulation through all ecosystems.

Isotope discrimination: The term used to express the effects of isotope fractionation when comparing a source and a sink, or a substrate and a product of a reaction. Isotope discrimination factor is calculated as the difference in δ value between substrate and product, or vice versa, and is usually symbolized as Δ. Frequently, this term is wrongly called isotope fractionation factor.

10

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Variability in $\delta^{15}N$ natural abundance of basal resources in fluvial ecosystems: a meta-analysis

Marc Peipoch[1], Eugènia Martí[2], AND Esperança Gacia[3]

Biogeodynamics and Biodiversity Group, Centre d'Estudis Avançats de Blanes, Consejo Superior de Investigaciones Científicas (CEAB-CSIC), Blanes, Spain

Abstract. Variation in N stable isotope ($\delta^{15}N$) signatures of basal resources can influence interpretation of trophic relationships in ecosystems, and significant variation in $\delta^{15}N$ signatures has been reported in streams and rivers. However, a comprehensive understanding of the main factors driving $\delta^{15}N$ variability is lacking, and this variability confounds the consumer's trophic-level position during $\delta^{15}N$ analysis. We conducted a meta-analysis to examine the variability in $\delta^{15}N$ natural abundance of basal resources and dissolved inorganic N (DIN) in streams and rivers in relation to the environmental factors that may drive this variability. The meta-analysis was based on a literature review over the last 20 y (1989–2009) and contained signatures of $\delta^{15}N$-DIN ($\delta^{15}N$-NO_3 and $\delta^{15}N$-NH_4) and $\delta^{15}N$-basal resources ($\delta^{15}N$-detrital compartments, $\delta^{15}N$-biofilm, $\delta^{15}N$-algae, and $\delta^{15}N$-macrophytes) from >100 rivers or streams. Signatures of $\delta^{15}N$-DIN varied widely (−8.4–19.4‰), and we found fewer values for $\delta^{15}N$-NH_4 than $\delta^{15}N$-NO_3, even though NH_4^+ is assimilated rapidly by basal resources. The range of $\delta^{15}N$-basal resources was also broad (−4–16‰) within and among compartments. Human land use was the most significant factor explaining variability in $\delta^{15}N$-DIN and $\delta^{15}N$-basal resource signatures. We found significant differences between $\delta^{15}N$ signatures of photoautotrophic (i.e., autochthonous) and detrital (i.e., allochthonous) basal resources. Our results point out the difficulty in defining a baseline $\delta^{15}N$ signature of the food web, and provide a basis to explain confounding results in studies using $\delta^{15}N$ analysis to identify trophic linkages in fluvial food webs.

Key words: stable isotopes, nitrogen, stream, river, food web, basal resources, land use.

Over the past 20 y, C and N stable isotopes have been used extensively to identify trophic relationships in different ecosystems (Michener and Schell 1994, Bluthgen et al. 2003, Premke et al. 2010). The number of stable isotope studies describing stream foodweb linkages has increased exponentially in the last 2 decades. In particular, stable isotopes of C ($\delta^{13}C$) are a powerful tool with which to identify organic-matter flows from basal resources to consumers in stream food webs and to evaluate the role of autochthonous and allochthonous food resources supporting secondary production (Rounick et al. 1982, Hamilton et al. 2004). Stable isotopes of N ($\delta^{15}N$) have been used to establish the trophic position of organisms in food webs as a consequence of the trophic-level enrichment in $\delta^{15}N$ (Minagawa and Wada 1984, Fry 1991). However, unlike $\delta^{13}C$, use of $\delta^{15}N$ in trophic ecology has generated controversy because of high variability in $\delta^{15}N$ signatures at different trophic levels. Compared to $\delta^{13}C$ signatures, much less is known about the factors influencing such variability, although it can confound interpretation of $\delta^{15}N$ signatures when measuring trophic position of organisms (Vander Zanden and Rasmussen 1999, O'Reilly et al. 2002).

Variation in $\delta^{15}N$ of consumers is strongly influenced by variation in $\delta^{15}N$ at the lower levels of the food web; i.e., by variation in $\delta^{15}N$ of basal resources rather than by variable fractionation along the food chain (Kline et al. 1990, Kling et al. 1992, Cabana and Rasmussen 1994). Many investigators using $\delta^{15}N$ signatures to identify foodweb linkages in freshwater ecosystems have pointed out that an appropriate measure of consumer's trophic position can be interpreted only relative to variation in $\delta^{15}N$ of basal resources among and within systems (Cabana and Rasmussen 1996, Vander Zanden and Rasmussen 1999).

The variability in $\delta^{15}N$ of basal resources has been related to isotopic fractionation during uptake processes (Macko et al. 1987, Handley and Raven 1992, Gannes et al. 1997) or to physiological differences among organisms that can lead to differences in N uptake and turnover rates (Cabana and Rasmussen

[1] E-mail addresses: mpeipoch@ceab.csic.es
[2] eugenia@ceab.csic.es
[3] gacia@ceab.csic.es

TABLE 1. Categorical and continuous explanatory variables included in the $\delta^{15}N$ database and used in 2 factorial analyses of variance to examine the factors influencing $\delta^{15}N$ variability in both dissolved inorganic N (DIN) and basal resources. The table shows the levels considered for the categorical variables and units for the continuous variables. Climate region was divided in 4 categories following Köppen climate classification (Peel et al. 2007). Urban and agricultural land uses were determined when >25% of the catchment was occupied by those practices, whereas cover under this percentage was considered forested. E. = epilithic, f. = filamentous, CPOM = coarse particulate organic matter, FPOM = fine particulate organic matter.

Variables type	Name	Categories or units
Categorical	Climate region	Equatorial, arid, warm temperate, continental
	Land uses	Forested, urban, agricultural
	Sampling season	Winter, spring, summer, autumn
	Stream order	1–7
	Basal-resource compartments	E. biofilm, f. algae, macrophytes, FPOM, CPOM, leaf litter
	DIN form	NO_3^-, NH_4^+
Continuous	Catchment area	km^2
	Discharge	m^3/s
	C:N stoichiometry	Unitless

1996). Moreover, variability in $\delta^{15}N$ of basal resources also can be influenced by variation in the $\delta^{15}N$ of N sources (i.e., dissolved inorganic N [DIN]), which has been associated with human density (Cabana and Rasmussen 1996, Nishikawa et al. 2009), climatic region (Fry 1991), or seasonality (Toda and Wada 1990, Yoshioka et al. 1994). However, this information is related only to discrete studies and particular organisms and is particularly scarce when examining both N sources and basal resources of fluvial ecosystems. Hence, we acknowledge a lack of a comprehensive description and quantification of the variability in the $\delta^{15}N$ signatures of organisms (basal resources) and N sources (DIN) in fluvial ecosystems, even though this knowledge could help provide a better interpretation of the trophic structure of stream food webs.

The objective of our study was to achieve a comprehensive understanding of the variability in $\delta^{15}N$ natural abundance of basal resources and DIN in fluvial ecosystems by exploring the existence of general patterns and examining the intrinsic and environmental factors that might influence such variability. Ultimately, our goal was to provide a basis for a better interpretation of $\delta^{15}N$ signatures in studies describing foodweb linkages of fluvial ecosystems. Our study is based on an extensive review of existing ecological studies in streams and rivers conducted over the last 2 decades that used the natural abundance of N stable isotopes and a meta-analysis of $\delta^{15}N$ data compiled from these studies.

Methods

Literature search and development of the database

We searched the Thomson Reuters Web of Knowledge database (http://thomsonreuters.com/) for publications in the period from 1989 to 2009 containing the key words *stable isotope*, *stream* and *river*, *nitrate* and *ammonium* (as DIN forms; Table 1), and *macrophytes*, *filamentous algae, epilithic biofilm*, *fine particulate organic matter (FPOM)*, *coarse particulate organic matter (CPOM)*, and *leaf litter* (as basal-resource compartments; Table 1). Combinations of these key words resulted in a set of >850 publications over the 20-y period. Of these publications, 54 contained values of ^{15}N natural abundance for basal resources or DIN (Appendix). We used this set of publications to generate a database for the meta-analysis of ^{15}N variability of basal resources and DIN. We used $\delta^{15}N$ signature, which is the isotope ratio (^{15}N:^{14}N) of a sample relative to the ratio of a standard (i.e., atmospheric N_2) expressed in parts per mil (‰). This ratio is the most widely used expression for the ^{15}N content of sources and organisms. In instances where data were provided only graphically, we obtained $\delta^{15}N$ signatures by digitization of the graph with GIMP 2.6 free software (GIMP, Boston, USA). When publications included data from several streams, we introduced data from each stream as a different case in the database. When publications included $\delta^{15}N$ data for the same river or stream from different sampling dates, we introduced these data as different cases. The $\delta^{15}N$ database contained a total of 147 cases, corresponding to 104 different rivers or streams, with 262 signatures of $\delta^{15}N$ natural abundance for different DIN forms and basal-resource compartments.

To examine factors that might contribute to the variability in $\delta^{15}N$ of basal resources ($\delta^{15}N$-basal resources) and DIN ($\delta^{15}N$-DIN), each data case was complemented with data for several explanatory variables, when available (Table 1). We chose these variables as surrogates of different environmental factors that potentially influence $\delta^{15}N$ variability and

of intrinsic characteristics of the compartments examined (e.g., form of DIN, compartment type, and C:N ratios of basal resources). Data introduced for each explanatory variable were mostly categorical, except for catchment area, stream discharge, and C:N ratios, which were numerical (Table 1).

Analysis of the $\delta^{15}N$ database

We characterized the variability of $\delta^{15}N$ for the different forms of DIN and basal-resource compartments with several statistical descriptors (range, mean, median, standard error of the mean [SEM], and variance). We assessed the relative importance of each explanatory variable on the variability of $\delta^{15}N$ with separate analyses of variance (ANOVAs) for basal resources and DIN data. We homogenized the database among streams and rivers by calculating mean $\delta^{15}N$ signatures for those streams having replicated data from a single sampling date. We analyzed only those explanatory variables for which data were available for >95% of the cases (climate region, land use, season, type of basal-resource compartment, and DIN form). The variables included in the analysis were all categorical, so we applied a factorial ANOVA to examine which categorical variable accounted for the largest proportion of the variance in $\delta^{15}N$ values. Assumptions of normal distribution and homogeneity of variances of the $\delta^{15}N$ data set were assessed with Kolmogorov–Smirnov and Levene tests, respectively. The model was not balanced (number of cases was not equal among categories), so we used Scheffe's test to make pairwise comparisons among categorical levels within each explanatory variable. We considered results significant when $p < 0.05$. We assessed the relative contribution of each explanatory variable to the total amount of variance explained by the full factorial ANOVA model (all explanatory variables considered) by extracting each variable from the model and comparing the variance obtained to that from the full model. The explanatory variables that were not included in the factorial ANOVA analysis were mostly numerical (i.e., discharge, catchment area, and C:N ratio) except for stream order, which was categorical. Therefore, we assessed their influence on $\delta^{15}N$ variability with univariate linear regression analysis. We ran statistical analyses in R (version 2.14.0; R Foundation for Statistical Computing, Vienna, Austria).

Results

Variability in $\delta^{15}N$ for stream basal resources and DIN

Available data for ^{15}N natural abundance of DIN and basal resources are mostly from studies conducted

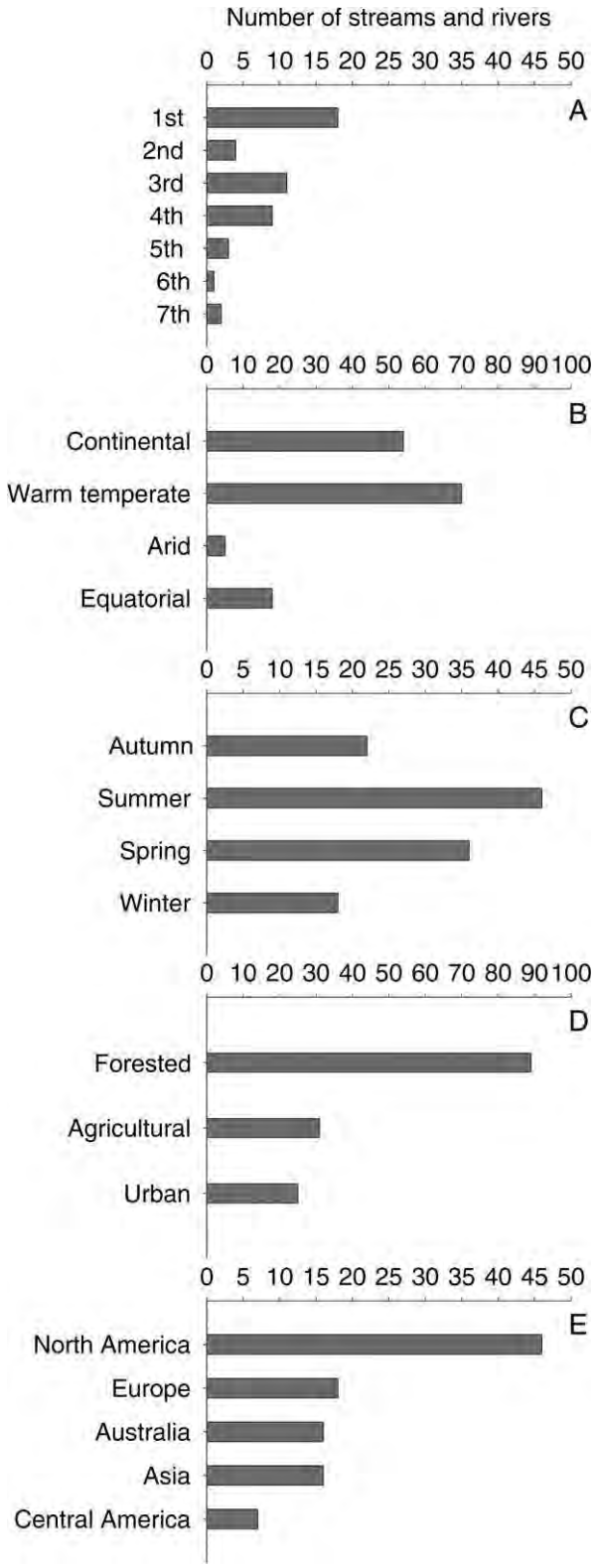


FIG. 1. Distribution of rivers and streams included in the database by stream order (available for only 48 streams) (A), climate region (B), sampling season (C), catchment land use (D), and continent (F).

in low-order streams (Fig. 1A), in warm temperate or continental biogeoclimatic regions (Fig. 1B), and during summer or spring (Fig. 1C). Most of the existing $\delta^{15}N$ data are from studies conducted in forested (i.e., relatively pristine) catchments of North America (Fig. 1D, E).

The range of $\delta^{15}N$ signatures was broad for basal resources (−4–16‰) and DIN (−8.4–19.4‰), but mean $\delta^{15}N$-DIN was higher for NO_3^- and NH_4^+ than $\delta^{15}N$-basal resources for all compartments (Fig. 2). $\delta^{15}N$-DIN mostly corresponded to $\delta^{15}N$-NO_3 signatures. $\delta^{15}N$-NH_4^+ data were scarce, but encompassed a broader range. $\delta^{15}N$ signatures of detrital compartments (leaf litter, CPOM, FPOM) differed more and had lower means than $\delta^{15}N$ signatures of compartments dominated by photoautotrophic organisms (macrophytes, epilithic biofilm, filamentous algae) (Fig. 2). Among detrital compartments, $\delta^{15}N$-leaf litter had the narrowest range (−3.9–4.7‰), the lowest variance, and the lowest mean (Fig. 2), whereas $\delta^{15}N$-FPOM and $\delta^{15}N$-CPOM had similar distributions. $\delta^{15}N$ signatures of compartments dominated by photoautotrophic organisms had similar variances and means (Fig. 2).

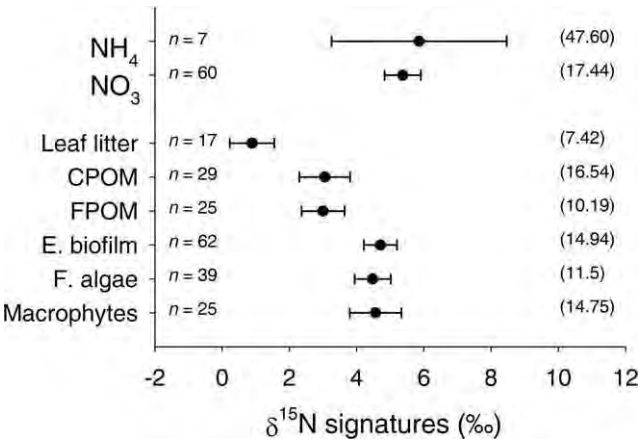


FIG. 2. Mean (±1 SEM) $\delta^{15}N$ signatures of dissolved inorganic N (DIN) and basal resources from streams or rivers compiled from the literature. The available number of $\delta^{15}N$ values for each category level is indicated in the left margin of the *y*-axis. Numbers on the right side of the plot are variances. E. = epilithic, f. = filamentous, CPOM = coarse particulate organic matter, FPOM = fine particulate organic matter.

Factors influencing $\delta^{15}N$ variability

Variation in $\delta^{15}N$-DIN was not significantly related to catchment area ($r^2 = 0.01$, $n = 59$, $p = 0.46$) or discharge ($r^2 = 0.01$, $n = 46$, $p = 0.46$). Variation in $\delta^{15}N$-basal resources was not related to catchment area ($r^2 = 0.01$, $n = 255$, $p = 0.09$), but was negatively related to discharge, although this factor accounted for a very low proportion of the $\delta^{15}N$ variance ($r^2 = 0.04$, $n = 123$, $p = 0.02$). $\delta^{15}N$ signatures of basal resources were negatively related to their C:N ratios ($r^2 = 0.27$, $n = 113$, $p < 0.001$). In particular, detrital compartments had significantly higher C:N ratios (16.17 ± 2.01‰ [SEM]) and lower $\delta^{15}N$ signatures (2.52 ± 0.42‰) than photoautotrophic compartments (8.56 ± 0.7 and 4.63 ± 0.33‰, respectively).

Land use and its interaction with season accounted for ~72% of the total variance in $\delta^{15}N$-DIN (Table 2). $\delta^{15}N$-DIN signatures from rivers or streams draining urban (9.94 ± 0.99‰) and agricultural (7.6 ± 0.47‰) catchments were, on average, 6.6× higher than those from rivers or streams draining forested catchments (2.2 ± 0.60‰). $\delta^{15}N$-DIN did not differ between streams draining urban and agricultural catchments (Table 3).

Land use, compartment type of basal resources, season, climatic region, and the season × compartment type of basal resources together explained ~58% of the total variance in $\delta^{15}N$-basal resources (Table 2). Land use accounted for the highest proportion of the total variance in $\delta^{15}N$-basal resources (Table 2). $\delta^{15}N$-basal resources signatures from rivers or streams draining urban (6.9 ± 0.57‰) and agricultural (6.5 ± 0.63‰) catchments were, on average, >2× higher than those from rivers or streams draining forested catchments (2.4 ± 0.26‰). This pattern was consistent when considering all $\delta^{15}N$-basal resources together and $\delta^{15}N$ for each basal resource individually (Table 3, Fig. 3). Differences in $\delta^{15}N$ signatures between human-dominated and forested streams were largest for CPOM ($p > 0.05$), smallest for FPOM ($p > 0.05$) and leaf litter ($p < 0.05$), and intermediate for epilithic biofilm, filamentous algae, and macrophytes (Table 3, Fig. 3). The variable accounting for the second largest proportion of $\delta^{15}N$-basal resources variance was the type of compartment (Table 2). Mean $\delta^{15}N$ of compartments dominated by photoautotrophic organisms was 1.4 to 3.8× higher than that of detrital compartments. Season accounted for 11% of the variance in $\delta^{15}N$-basal resources, and signatures were, on average, substantially higher in autumn (5.6 ± 0.72‰) than in other seasons (summer: 3.7 ± 0.37‰, winter: 2.4 ± 0.77‰, spring: 2.3 ± 0.65‰). $\delta^{15}N$-basal resources from streams in arid regions (7.2 ± 1.36‰) were, on average, higher than those from other climatic regions (equatorial: 4.78 ± 0.51‰, warm temperate: 3.93 ± 0.41‰, continental: 3.1 ± 0.42‰) regardless of the basal-resource compartment considered.

TABLE 2. Results from 2 factorial analyses of variance used to test the relative contribution of the explanatory variables to the variability of $\delta^{15}N$-dissolved inorganic N (DIN) and $\delta^{15}N$-basal resources. Only variables with significant effect in the analysis are indicated and % total variance explained by each of these variables or by the interaction between them is shown. * = $p < 0.5$, ** = $p < 0.1$, *** = $p < 0.01$.

Explanatory variables	df	Sum of squares	p	Variability explained
DIN				
Land use	2	708.9	<0.001 ***	54.7%
Land use × season	6	225.5	<0.001 ***	17.4%
Basal resources				
Land use	2	724.3	<0.001 ***	21.3%
Basal-resource compartment	5	264.9	<0.001 ***	14.8%
Season	4	157.2	<0.001 ***	11.0%
Season × basal-resource compartment	19	267.1	0.015 **	9.3%
Climate	3	139.4	<0.001 ***	3.7%

Discussion

Variability of $\delta^{15}N$-basal resources and $\delta^{15}N$-DIN in fluvial ecosystems

Our literature search showed that authors of a relatively large number of studies included data on natural abundance of N stable isotopes in fluvial ecosystems over the last 2 decades, but only a small fraction of them (54 of 850) reported $\delta^{15}N$ signatures for basal resources or DIN forms. This bias is explained by the fact that most investigators use N stable isotopes to examine food webs, and in these studies, $\delta^{15}N$ signatures for basal resources and DIN are often neglected or are used only to establish the baseline $\delta^{15}N$ value of the system (Minagawa and Wada 1984, Cabana and Rasmussen 1996). We also found that the available data on $\delta^{15}N$-basal resources and $\delta^{15}N$-DIN are not proportionally representative of the broad diversity of river and stream ecosystems. Instead, entries from small, relatively pristine streams in temperate regions of North America are most abundant. Likewise, basal-resource compartments were not proportionally represented in the $\delta^{15}N$ database. $\delta^{15}N$ data for photoautotrophic compartments, especially biofilm, were more abundant than data for detrital compartments. Similarly, $\delta^{15}N$ data for DIN forms were relatively scarce and biased toward $\delta^{15}N$-NO_3 data, whereas $\delta^{15}N$-NH_4 data were very rare. This disparity can be particularly problematic when assessing the coupling between DIN and basal resources because NH_4^+ is the preferred form of DIN for basal resources in streams (Peterson et al. 2001). Temporal variation of stream $\delta^{15}N$ signatures is not well documented in the literature, and most of the $\delta^{15}N$ data are from studies conducted during warm seasons (spring and summer) across climatic regions. Authors of studies of natural abundances of $\delta^{15}N$ signatures generally assume isotopic equilibrium between consumers and their diets. However, our results indicate that seasonal variation of $\delta^{15}N$ signatures for DIN and basal resources may be large enough to produce long-term variation in the isotopic equilibrium between consumers and basal resources. Other investigators also have reported variation in $\delta^{15}N$ at smaller temporal scales. For instance, O'Reilly et al. (2002) showed that abrupt changes in $\delta^{15}N$-DIN signatures produced changes in ^{15}N signatures of basal resources within a few days in a tropical lake.

TABLE 3. Mean (±1 SE) N isotopic signatures ($\delta^{15}N$ in ‰) of stream basal resources and dissolved inorganic N collected in the meta-analysis database separated by land use category. Within a row, means with the same letter are not significantly different. E. = epilithic, f. = filamentous, FPOM = fine particulate organic matter, CPOM = coarse particulate organic matter, * = insufficient data.

Category	Forested	Urban	Agricultural
NO_3^-	5.32 ± 0.77^A	10.38 ± 1.03^B	7.17 ± 0.36^B
NH_4^+	-1.62 ± 6.81^A	7.89 ± 3.01^A	9.73 ± 0.09^A
Macrophytes	2.98 ± 0.6^A	7.96 ± 0.78^B	6.68 ± 0.58^B
F. algae	2.5 ± 0.87^A	6.92 ± 0.77^B	6.58 ± 0.55^B
E. biofilm	3.14 ± 0.39^A	8.11 ± 0.7^B	7.7 ± 2.18^B
FPOM	2.49 ± 0.67^A	4.9 ± 0.35^A	3.83 ± 2.49^A
CPOM	1.39 ± 0.78^A	7.21*	8.99 ± 3.87^A
Leaf litter	1.43 ± 0.49^A	3.36 ± 0.68^B	2.16 ± 0.77^{AB}

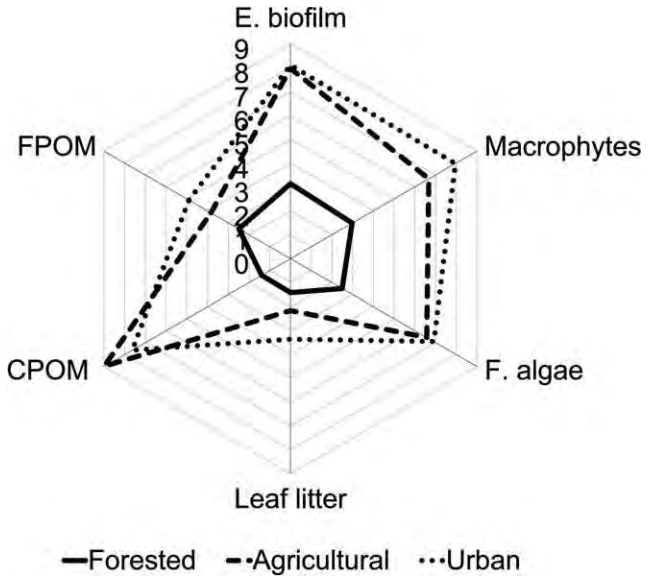


FIG. 3. Representation of the $\delta^{15}N$ signatures for each basal-resource category from streams and rivers draining the 3 landuse types considered. The lines join the mean of $\delta^{15}N$ for different basal-resource compartments within each landuse category. E. = epilithic, f. = filamentous, CPOM = coarse particulate organic matter, FPOM = fine particulate organic matter.

This result suggests that basal resources can be indicators of the short-term variation in $\delta^{15}N$-DIN signatures because of direct DIN uptake and rapid N turnover rates. In contrast, $\delta^{15}N$ signatures of higher trophic levels may integrate this short-term variation of basal resources over longer temporal scales (Cabana and Rasmussen 1996) and may be less variable over time. Changes in $\delta^{15}N$ of DIN and basal resources at different temporal scales may have important implications for studies in which N stable isotopes are used to address the dynamics of trophic interactions and to model stream food webs. Overall, the gaps observed in the existing data highlight the need of future research to broaden our knowledge on the variability of $\delta^{15}N$ signatures to improve interpretation of $\delta^{15}N$ signatures in stream food webs.

Variability in $\delta^{15}N$ signatures was high for both DIN and basal resources in streams and rivers, and most of this variability was related to differences in catchment land use. Streams draining catchments with high agricultural or urban activity had substantially higher $\delta^{15}N$-DIN and $\delta^{15}N$-basal resources than streams draining forested catchments. This pattern was consistent regardless of the compartment of basal resources considered and confirms that high variation of $\delta^{15}N$ signatures among streams is linked to anthropogenic influences. The ^{15}N-enriching effect of DIN sources in human-altered systems seems to be transferred to the different basal-resource compartments. Cabana and Rasmussen (1996) showed that $\delta^{15}N$ signatures of DIN and primary consumers in lakes increased with human population density in their catchment. Other studies in fluvial ecosystems had supported this hypothesis, but the variation in $\delta^{15}N$ signatures of basal resources was not considered explicitly in any of the previous studies. Diebel and Vander Zanden (2009) showed that variation in $\delta^{15}N$ signatures of primary consumers was explained by inputs of inorganic fertilizers, and many investigators have used $\delta^{15}N$-NO_3 signatures as tracers of N sources from different human land uses within the catchment (Chang et al. 2002, Lefebvre et al. 2007). Our results showed that human activity clearly influenced $\delta^{15}N$ signatures of both DIN and basal resources, but neither $\delta^{15}N$-DIN nor $\delta^{15}N$-basal resource signatures clearly distinguished between agricultural vs urban activity in the catchment. This result might be explained by overlapping ranges of $\delta^{15}N$ signatures of DIN sources contributed by different human activities and by the effect of isotopic fractionation associated with N-cycling transformations on $\delta^{15}N$ signatures.

Overall, the high variability observed in $\delta^{15}N$-DIN signatures (essentially represented only for NO_3^-; see above) could be explained in part by the influence of different DIN sources within the catchment, as illustrated by the variability in $\delta^{15}N$-DIN signatures in the Mississippi River (Panno et al. 2006). The range of $\delta^{15}N$-DIN signatures compiled in our study (−8.4–19.4‰) is broad and covers most of the $\delta^{15}N$ signatures of potential DIN sources to streams reported in the literature. For instance, $\delta^{15}N$ signatures from wet deposition (−15–15‰; Kendall et al. 2007) or soil N (−10–15‰; Broadbent et al. 1980) are contained relatively well within the range of variation we found for $\delta^{15}N$-DIN. Agricultural N sources that enter streams via diffuse pathways also are within the observed range. $\delta^{15}N$ of fertilizers of biological origin (i.e., guano or animal waste) range from 10 to 20‰ (Kreitler 1979), and $\delta^{15}N$ of synthetic fertilizers (i.e., N industrially fixed from atmospheric N_2) range from −4 to 4‰. In contrast, $\delta^{15}N$-DIN from point sources, such as wastewater treatment plant effluents, tend to exceed the upper limit of the $\delta^{15}N$-DIN range in our study with $\delta^{15}N$-NO_3 signatures up to 30‰ (Toda et al. 2001) and $\delta^{15}N$-NH_4 signatures as high as 28‰ (M. Ribot [CEAB-CSIC], unpublished data). The absence of records for these extreme $\delta^{15}N$-DIN signatures in streams may be explained by dilution of $\delta^{15}N$-DIN signatures when effluents enter receiving streams.

The variability in $\delta^{15}N$ signatures also has been linked to nutrient concentration because it influences the degree of isotopic fractionation of different N processes (e.g., assimilation, nitrification, or denitrification) (Paerl and Fogel 1994, Cabana and Rasmussen 1996). A 70% increase in N inputs into fluvial ecosystems since the 19th century (Seitzinger and Kroeze 1998) has caused greater availability of bioreactive N, and potentially, intensification of N cycling in terrestrial and fluvial ecosystems. This intensification could lead to higher streamwater $\delta^{15}N$ signatures via greater isotopic fractionation during N-cycling processes (Högberg 1997, Diebel and Vander Zanden 2009). Studies showing the influence of riparian soil N biogeochemical processes, especially denitrification, on streamwater $\delta^{15}N$ (Houlton et al. 2006) seem to support this last hypothesis.

Foodweb implications of variation in $\delta^{15}N$-basal resources

Basal resources sustain the entire food web. Thus, increases in $\delta^{15}N$ of basal resources in streams affected by human activity also may produce variation in $\delta^{15}N$ signatures of consumers above that caused by stepwise trophic-level enrichment in $\delta^{15}N$ signatures (Kling et al. 1992). Other factors that act within ecosystems also generate variability in $\delta^{15}N$ signatures of basal resources. For instance, Angradi (1994) showed intrasystem variation of $\delta^{15}N$ signatures associated with different basal-resource compartments, and season affected $\delta^{15}N$ signatures in 2 eutrophic lakes (Toda and Wada 1990, Yoshioka et al. 1994). We observed variation among compartments and among seasons in our $\delta^{15}N$-basal resources data.

$\delta^{13}C$ signatures also can differ significantly between photoautotrophic (autochthonous) and detrital (allochthonous) compartments (Rounick et al. 1982). Autochthonous organic C has lower $\delta^{13}C$ signatures than allochthonous organic C as a result of the $\delta^{13}C$-depleted HCO_3^- source used by photoautotrophic compartments (Rau 1980). In contrast, we found higher $\delta^{15}N$ signatures in photoautotrophic than in detrital compartments. However, unlike $\delta^{13}C$ signatures, mechanisms generating differences in $\delta^{15}N$ between photoautotrophic and detrital compartments are more difficult to ascertain. In principle, organic N in photoautotrophic compartments is derived from in-stream DIN, whereas organic N in detrital compartments is derived from allochthonous and autochthonous material colonized by stream biofilms. This duality poses a major problem when interpreting $\delta^{15}N$ signatures of detrital compartments because samples typically consist of bulk material including detrital organic matter and the microbial communities colonizing it in unknown proportions (Hamilton and Lewis 1992, Hamilton et al. 2004). Thus, description of the mechanisms generating differences in $\delta^{15}N$ among basal resources in a particular stream may be much more complex than a distinction between allochthonous and autochthonous material as described for C stable isotopes.

C:N of terrestrial material that falls into streams normally is 2 to 3× higher than that of aquatic-derived material (Elser et al. 2000, Kendall et al. 2001, Grimm et al. 2003). The result is higher C:N ratios in detrital than in photoautotrophic compartments, as we observed in our study. Allochthonous organic matter entering the aquatic ecosystem rapidly leaches soluble N in its first stages of mineralization (Zeller et al. 2000), and then is colonized by microbial communities that can incorporate DIN from the water (Mulholland et al. 2000b, Gribsholt et al. 2007). Thus, leaf litter, a less-mineralized fraction of the detrital organic matter, is expected to have a lower $\delta^{15}N$ signature than stream water. In more advanced stages of mineralization of the organic matter (coarse and fine fraction), the $\delta^{15}N$ signature should become closer to that of stream water because of DIN uptake by microbial communities colonizing this organic matter fraction (Caraco et al. 1998), as we observed in our compiled data. Unlike the photoautotrophic compartments, detrital organic matter ultimately may integrate internal and external sources of N in different proportions, which would result in considerable variation in $\delta^{15}N$ signatures among detrital compartments (i.e., leaf litter, CPOM, FPOM). Whether differences in $\delta^{15}N$ signatures between photoautotrophic and detrital compartments are relevant for the transfer of $\delta^{15}N$ signatures to the rest of the stream food web will depend on the selective assimilation of N in biofilms by consumers (Hall et al. 1998, Tank et al. 2000) and the biochemical quality of detrital organic matter (Findlay and Tenore 1982) rather than the $\delta^{15}N$ signature of the bulk material. Therefore, an appropriate estimate of the importance of microbial biomass and detrital organic matter as a source of organic N for consumers seems to be relevant when addressing detrital pathways in stream food webs and could help explain confounded results in terms of consumers' trophic level position. In general, our compiled data indicates that studies of stream food webs using N stable isotopes usually are based on samples of bulk material of detrital compartments. Separate measures of microbial $\delta^{15}N$ and detrital organic matter $\delta^{15}N$ should be a major concern in future studies because the observed differences between $\delta^{15}N$ signatures of photoautotrophic and bulk material of detrital compartments are, in some

cases, comparable to the commonly assumed 2-to-4‰ increase in $\delta^{15}N$ per trophic level (Minagawa and Wada 1984, Ehleringer et al. 1986, Peterson and Fry 1987), regardless of landuse type considered. Therefore, when differences in $\delta^{15}N$ among basal resources are within the range of trophic-level enrichment, consumers' trophic levels in the stream food web may be underestimated. This issue can be especially relevant in streams where food webs are mostly fuelled by detrital sources. In those cases, calculation of consumers' trophic levels with $\delta^{15}N$ signatures of bulk detritus material will be problematic if detritivores feed specifically on microbial N, which probably has a $\delta^{15}N$ signature closer to the streamwater DIN, and thus, more similar to photoautotrophic compartments.

In summary, our study provides a broad range of $\delta^{15}N$ values for several basal-resource compartments in streams that can be used to bracket measurements of $\delta^{15}N$ values for consumers in forthcoming studies. In addition, results from the meta-analysis highlight the importance of land use in the catchment as a key driver of the variability in $\delta^{15}N$ signatures of both DIN and basal resources. Variation in $\delta^{15}N$ signatures among streams draining different landuse types seems to be much more pronounced than variation within a single stream, regardless of the compartment considered. Therefore, difficulties would arise primarily when using N stable isotopes to compare food webs across streams without considering values of basal resources because consumers in human-altered streams feed on more ^{15}N-enriched basal resources than their counterparts in forested streams. Our results also show consistent differences in $\delta^{15}N$ between photoautotrophic and detrital compartments that should be taken into account when interpreting foodweb results based on N stable isotopes. Our study has helped elucidate data gaps that could be addressed in future studies to increase our knowledge of the use of N stable isotopes in stream food webs. One of these gaps is the need for additional data on temporal variation of stream $\delta^{15}N$ signatures. Understanding how N isotopic signatures of stream basal resources integrate temporal variation of $\delta^{15}N$-DIN signatures and how this variation is transferred to the stream food web may constitute a major step in the use of N stable isotopes in foodweb studies.

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APPENDIX. References for all publications containing values of $\delta^{15}N$ signatures used in our study. Country (state is also included in streams or rivers from the USA), name of the stream or river, and compartments sampled in each study are also indicated. E. = epilithic, f. = filamentous, CPOM = coarse particulate organic matter, FPOM = fine particulate organic matter.

References	Country	Stream or river	Compartments with $\delta^{15}N$ data available
Angradi 1993	Idaho (USA)	Henry's Fork	E. biofilm, f. algae, macrophytes, FPOM, CPOM
Angradi 1994	Utah-Arizona (USA)	Colorado	F. algae, macrophytes, FPOM, CPOM, leaf litter
Aufdenkampe et al. 2007	Peru	Vilcanota–Urubamba, Tambo, Pachitea, Ucayali–Amazonas, Marañón	CPOM
Barnes et al. 2008	Connecticut (USA)	Connecticut Creek	NO_3^-
Benson et al. 2008	Maryland (USA)	Sassafras	Macrophytes
Bergfur et al. 2009	Sweden	Rastälven, Sverkestaan, Älgängsan, Strömaran, Penningbyan, Husbyan, Hagaan, Sagan	E. biofilm, FPOM, CPOM
Burns and Walker 2000	Australia	Murray	E. biofilm, FPOM, CPOM
Buzek et al. 2006	Czech Republic	Jizera	NO_3^-
Chessman et al. 2009	Australia	Murrumbidgee, Tumut, Goobarragandra, Goodradigbee	E. biofilm
Coat et al. 2009	Guadeloupe	Grande-Anse	E. biofilm, f. algae, leaf litter
de Brabandere et al. 2007	Florida (USA)	Chassahowitzka and Homosassa	E. biofilm, macrophytes, NO_3^-
Dekar et al. 2009	Arkansas (USA)	Mulberry and Little Mulberry	E. biofilm, CPOM
Deutsch et al. 2009	Germany	Elbe	NO_3^-, NH_4^+
Evans-White et al. 2001	Kansas (USA)	Kings Creek	E. biofilm, f. algae, macrophytes, FPOM, CPOM, leaf litter
Gido et al. 2006	New Mexico (USA)	San Juan	F. algae, FPOM
Godwin et al. 2009	Pennsylvania (USA)	Spring Creek	E. biofilm
Gustafson et al. 2007	North Carolina (USA)	Neuse	FPOM, CPOM, NO_3^-
Harrington et al. 1998	Vermont (USA)	Bringo Brook, West Branch, Bethel-Gilead, Peavine, Third Branch, First Branch	F. algae, NO_3^-
Huang et al. 2007	Taiwan	Hapen Creek	E. biofilm, f. algae, macrophytes, FPOM, CPOM
Jennerjahn et al. 2008	Kerala	Kallada	FPOM, CPOM
Kao and Liu 2000	Taiwan	Langyang-Hsi	Macrophytes
Kaushal et al. 2006	Colorado (USA)	Blue	F. algae
Kohzu et al. 2008	Japan	Lake Biwa tributaries	E. biofilm
Lau et al. 2009	China	Tai Po Kau Forest, Shing Mun, Pak Ngau Shek	E. biofilm, F. algae, FPOM, leaf litter
Lefebvre et al. 2007	France	Pleine-Fougères	NO_3^-
Lofton et al. 2007	North Carolina (USA)	North Buffalo Creek	NO_3^-
Mantel et al. 2004	China	Tai Po Kau Forest	E. biofilm, f. algae, FPOM, CPOM
March and Pringle 2003	Puerto Rico	Espíritu Santo	E. biofilm, F. algae, leaf litter
Miyasaka and Genkai-Kato 2009	Japan	Kuro-kawa	E. biofilm
Mulholland et al. 2000a	Tennessee (USA)	Walker Branch	E. biofilm, FPOM, leaf litter
Panno et al. 2006	Illinois (USA)	Mississippi	NO_3^-
Peterson et al. 1993	Alaska (USA)	Kuparuk	E. biofilm, f. algae
Piatek et al. 2005	New York (USA)	Archer Creek	NO_3^-
Raikow and Hamilton 2001	Michigan (USA)	Eagle Creek	E. biofilm, FPOM, leaf litter

APPENDIX. Continued.

References	Country	Stream or river	Compartments with $\delta^{15}N$ data available
Rasmussen and Trudeau 2007	Canada	Ste Marguerite, Epinette, Big Pool	E. biofilm
Reid et al. 2008	Victoria	Pranjip, Joyce's Creek	E. biofilm, f. algae
Rezanka and Hershey 2003	Minnesota (USA)	Amity Creek	E. biofilm, f. algae, CPOM
Ribot (unpublished data)	Spain	La Tordera	E. biofilm, macrophytes, NO_3^-, NH_4^+
Rock and Mayer 2006	Canada	Oldman	NO_3^-
Saito et al. 2008	California (USA)	Truckee	E. biofilm
Sharpe and Nichols 2007	Tennessee (USA)	Conasauaga	NO_3^-
Steffy and Kilham 2004	Pennsylvania (USA)	Valley Creek	E. biofilm, F. algae, leaf litter
Stögbauer et al. 2008	Germany	Ruhr	NO_3^-
Thorp et al. 1998	Kentucky (USA)	Ohio	FPOM, CPOM
Toda et al. 2001	Japan	Chikuma	E. biofilm
Townsend-Small et al. 2005	China	Lujiang, XitiaOxi, Dongtiaoxi, Yili, Baidu, Liangxihe	NO_3^-
Udy and Bunn 2001	Australia	Bamboo, Bromfield, Clemenson, Fishers, Tranters, Wrights, Beatrice R., Dirran, Kittabah, Goolagan, Opposum, Djallan	F. algae, macrophytes
Ulseth and Hershey 2005	North Carolina (USA)	North Buffalo Creek	F. algae, FPOM, leaf litter
von Schiller et al. 2007	Spain	Santa Fe, Santa Coloma, Gualba, Sant Celoni	E. biofilm, leaf litter, NO_3^-, NH_4^+
von Schiller et al. 2009	Spain	Santa Fe, Santa Coloma, Gualba, Sant Celoni	E. biofilm, leaf litter, NO_3^-, NH_4^+
Wayland and Hobson 2001	Canada	Wapiti	F. algae
Williams and Trexler 2006	Florida (USA)	Shark, Taylor Slough	E. biofilm, f. algae, macrophytes
Zah et al. 2001	Switzerland	Roseg	E. biofilm
Ziegler and Brisco 2004	Arkansas (USA)	Moore Creek, Huey Hollow	F. algae, CPOM, leaf litter

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Aquatic Sciences

RESEARCH ARTICLE

Contrasts among macrophyte riparian species in their use of stream water nitrate and ammonium: insights from ^{15}N natural abundance

Marc Peipoch · Esperança Gacia · Alba Blesa · Miquel Ribot · Joan L. Riera · Eugènia Martí

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Abstract We examined the relevance of dissolved inorganic nitrogen (DIN) forms (nitrate and ammonium) in stream water as N sources for different macrophyte species. To do this, we investigated the variability and relationships between ^{15}N natural abundance of DIN forms and of four different macrophyte species in five different streams influenced by inputs from wastewater treatment plants and over time within one of these streams. Results showed that ^{15}N signatures were similar in species of submersed and amphibious macrophytes and in stream water DIN, whereas ^{15}N signatures of the riparian species were not. ^{15}N signatures of macrophytes were generally closer to ^{15}N signatures of nitrate, regardless of the species considered. Our results showed significant relationships between ^{15}N signatures of DIN and those of submersed *Callitriche stagnalis* and amphibious *Veronica beccabunga* and *Apium nodiflorum*, suggesting stream water DIN as a relevant N source for these two functional groups. Moreover, results from a mixing model suggested that stream water DIN taken up by the submersed and amphibious species was mostly in the form of nitrate. Together, these results suggest different contribution to in-stream N uptake among the spatially-segregated species of macrophytes. While submersed and amphibious species can contribute to in-stream N uptake by assimilation of DIN, macrophyte species located at stream channel edges do not seem to rely on stream water DIN as an N source. Ultimately, these results add a functional dimension to the current use of macrophytes for the restoration of stream channel morphology, indicating that they can also contribute to reduce excess DIN in streams.

Keywords Macrophyte · Streams · Stable isotopes · Nitrogen · Stream restoration

M. Peipoch (✉) · E. Gacia · M. Ribot · E. Martí
Integrative Freshwater Ecology Group, Centre d'Estudis Avançats de Blanes, Consejo Superior de Investigaciones Científicas (CEAB-CSIC), Accés a la Cala Sant Francesc 14, 17300 Blanes, Girona, Spain
e-mail: marc.peipoch@mso.umt.edu

A. Blesa
Consejo Superior de Investigaciones Científicas. Center of Molecular Biology "Severo Ochoa", Autonomous University of Madrid, C/Nicolás Cabrera 1, Campus de la Universidad Autónoma de Madrid, 28049 Madrid, Spain

J. L. Riera
Department of Ecology, University of Barcelona (UB), Diagonal 643, 08028 Barcelona, Spain

Introduction

Aquatic plants are an important element of river landscapes from contrasting climatic zones such as temperate (Larned et al. 2006), tropical (Sand-Jensen and Mebus 1996), subtropical (De Brabandere et al. 2007) or Pampean regions (Giorgi et al. 2005; Acuña et al. 2011), but their functional role in fluvial ecosystems has often been neglected. Aquatic plants (generally referred to as macrophytes) are irregularly distributed in stream channels depending on channel morphology, hydrologic regime (e.g., the frequency of flood events; see Riis and Biggs 2003), water velocity and light availability. In headwater streams, macrophyte development is usually restricted to stream-riparian margins because of water velocity, while lowland streams allow the development of macrophytes within the stream channel because of gentle slopes, slow flowing environments, and a lower degree of riparian canopy.

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Although abiotic factors in streams dictate the presence and distribution of macrophytes, they can also modify their surrounding habitat by promoting fine sediment and organic matter accumulation (Clarke 2002; Schulz et al. 2003; Larned et al. 2006), by increasing drag and reducing flow velocity and turbulence (Sand-Jensen and Pedersen 1999). The capacity of macrophytes to create and maintain different stream habitats has been commonly investigated and frequently used in stream restoration programs to stabilize stream beds and margins, and to provide physical habitat for invertebrates and fish (Harrison et al. 2004; Larned et al. 2006; Suren 2009). In addition, macrophytes also have an important demand for nutrients and thus may contribute to in-stream nutrient uptake, as has been reported in other freshwater systems, such as wetlands (Riis and Biggs 2003; Bowden et al. 2007), lakes (Gacia et al. 1994; Marion and Paillisson 2003) or pond fringes (Biggs et al. 2005). Despite this, the functional role of macrophytes as filters of dissolved nutrients has seldom been considered as a relevant part of stream ecosystem functioning or management, probably because studies of in-stream nutrient uptake have focused mainly on forested headwater streams, where macrophytes are not abundant (but see Pelton et al. 1998; Feijoo et al. 2011; Riis et al. 2012).

Species of macrophytes are distributed within the stream channel across a gradient of water exposure from the main channel to the flood plain according to structural traits related to how they tolerate water flow. These traits allow the differentiation of functional groups of aquatic plants based on their interaction with the stream water environment, including species totally submersed and with floating leaves, amphibious species, and riparian species located in the stream-riparian edge with roots in the reed zone (Bowden et al. 2007). These macrophyte species from different functional groups may thus have different interactions with stream water dissolved inorganic nitrogen (DIN) as their potential source of N (Fig. 1).

Natural abundance of N stable isotopes (^{15}N signatures, expressed as $\delta^{15}N$ in units of ‰) in macrophytes has been used to trace human-derived N inputs into aquatic ecosystems (Benson et al. 2008; Costanzo et al. 2001). Anthropogenic DIN sources are generally characterized by high ^{15}N signatures, and thus elevated ^{15}N signatures in macrophytes can indicate uptake of DIN sources derived from human activities (Cole et al. 2005; Udy et al. 2006; Peipoch et al. 2012). However, factors such as intra-plant isotopic discrimination (Evans et al. 1996), plant age (Evans 2001), or plant stress (Handley et al. 1999) can sometimes strongly influence the variation in plant ^{15}N signatures compared to their N sources. For instance, isotopic discrimination during uptake and assimilation of both ammonium (NH_4) and nitrate (NO_3) can be either small (Evans et al. 1996) or large (Yoneyama et al. 2001; Mariotti et al. 1982) depending on the plant species, although large discrimination effects have been mostly observed at high concentrations (i.e., 3–100 mg N-NH_4 L^{-1}, ~150 mg N-NO_3 L^{-1}) that are rarely found in natural or even slightly polluted streams (De Brabandere et al. 2007; Cohen and Bradham 2010). While recognizing the influence of these factors on uptake-associated and intra-plant discrimination, plant ^{15}N may still integrate the ^{15}N signatures of its N sources (Evans 2001) since ^{15}N signatures of macrophytes are strongly influenced by ^{15}N-DIN signatures (Kohzu et al. 2008; Pastor et al. 2013).

The aim of this study was to gain knowledge on DIN transfer from stream water to different macrophyte species to better understand the relevance of stream water N as a source for macrophytes, and thus their potential role in in-stream N uptake. To achieve this goal, we examined the spatial and temporal variability in ^{15}N natural abundance of four species of macrophytes representative of different functional groups: submersed macrophytes (*Callitriche stagnalis*), amphibious macrophytes (*Veronica beccabunga* and *Apium nodiflorum*), and riparian macrophytes located at the stream-riparian edge (*Carex pendula*), and then

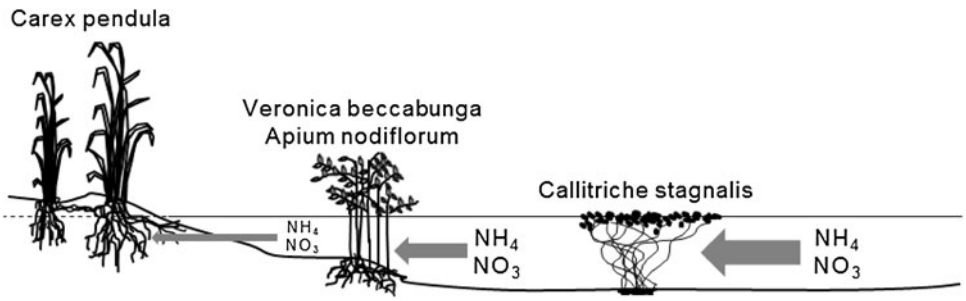


Fig. 1 Representation of the distribution of different functional groups of macrophytes across a section of the stream channel. There is a gradient of exposure to stream water from the totally exposed *Callitriche stagnalis* to the intermittently exposed *Carex pendula* depending on the stream water level. The thickness of the *arrows* indicate the expected dependence of each macrophyte species on dissolved inorganic nitrogen forms (NH_4 and NO_3) from the stream water

explored how it relates to the variability in the ^{15}N natural abundance of stream water DIN forms (i.e., NH_4 and NO_3). Finally, we used mixing models with ^{15}N signatures of the two DIN forms (NH_4 and NO_3) to assess the most relevant DIN source for those species showing significant correlations with $\delta^{15}N$-DIN values.

We conducted this study in 5 streams receiving effluents from wastewater treatment plants (WWTP) in order to enlarge the range of concentrations of DIN forms (see Marti et al. 2004) as well as the range of ^{15}N signatures for both NH_4 and NO_3. Previous studies indicated that in WWTP-receiving streams DIN can exhibit remarkable longitudinal gradients of ^{15}N signatures across relatively short stream distances (i.e., <1 km) due to intense cycling of NH_4 and NO_3 (Toda et al. 2001; Ribot et al. 2012). In addition, effluents from WWTP may also increase the abundance of aquatic macrophytes in receiving streams (Carr and Chambers 1998; Gucker et al. 2006) by increasing nutrient availability. Therefore, the expected large variation in both DIN concentration and $\delta^{15}N$-DIN values at a reach scale in these streams provided suitable conditions to examine whether or not the ^{15}N of different macrophyte species responded to these changes in the water column, which would suggest they are actively incorporating nitrogen from stream water DIN. We hypothesized that the relevance of DIN in the stream water as an N source for macrophytes would vary among functional groups due to differences in plant-stream interactions and in preferential uptake of NH_4 or NO_3 by the different species. We expected that $\delta^{15}N$ of macrophytes would be positively correlated with $\delta^{15}N$-DIN if this was a relevant N source, and that these correlations would gain strength among macrophytes as their degree of interaction with stream water increased (i.e., from riparian to submersed species). Ultimately, these results may have implications for stream management and restoration programs because macrophytes are currently used in restoration of stream channel morphology for their physical properties in substrate stabilization.

Materials and methods

Study sites

The study was conducted in La Tordera catchment (NE Barcelona, Catalunya, Spain), which has an area of 868.5 km^2 and is dominated by siliceous bedrock. This catchment is subjected to a large variety of land use practices, although urban activities are predominant in the catchment (von Schiller et al. 2008; Caille et al. 2012). The seasonal hydrologic regime of the streams in this basin typically shows high and permanent flow from late autumn to early spring and low or intermittent flow during summer. This temporal hydrologic variability results in a large variation in the relative contributions of WWTP effluents to stream water DIN within this catchment (Merseburger et al. 2005), and also in ^{15}N-DIN signatures of the receiving stream (Merbt et al. 2011; Ribot et al. 2012). Our study was based on a correlative approach in which we examined both spatial and temporal variation in the relationships between ^{15}N signatures of NH_4, NO_3, and macrophytes.

To assess spatial variation, we selected five stream reaches (ca. 1 km) within La Tordera catchment that were located downstream of inputs from urban WWTPs effluents. These streams were similar in terms of channel geomorphology, light availability and hydrologic regime. However, they differed in their dilution capacity of the WWTP input (i.e., stream discharge relative to WWTP outflow), in their nutrient loads, and in the contribution of WWTP inputs to in-stream NH_4 and NO_3 concentrations (Table 1). The study reaches were sampled once in autumn (October, 6–26) 2009. To avoid high discharge typical from spring and early summer in this Mediterranean climate, we waited for medium flow and mild temperatures to guaranty active leaf growth, the presence of *Callitriche stagnallis* and a significant but not excessive ratio of WWTP input to stream flow. These conditions were met in October 2009.

To assess temporal variation, we sampled seasonally one of the reaches (Sta. Maria de Palautordera) on 4 different dates from August 2010 to May 2011. We also included additional ^{15}N data for DIN and macrophytes from a previous monitoring (January 2008 and September 2008). Therefore, we had a data set consisting of longitudinal variation (see details below) within a reach for 5 streams on a single sampling date and for 1 stream on 6 different sampling dates.

Field sampling

On each sampling occasion, eight to 10 equidistant sampling stations were selected along a 670–850 m reach located immediately downstream of the WWTP inputs to maximize the gradients in DIN concentration and $\delta^{15}DIN$ signal. At each station, we measured longitudinal variability in physical and chemical variables and in $\delta^{15}N$ of NH_4, NO_3 and macrophyte species. We measured conductivity, water temperature, and dissolved oxygen concentration using WTW (Weilheim, Germany) 340i portable probes. Discharge was estimated using NaCl slug additions at the bottom end of each study reach and on each sampling date in SMP (Gordon et al. 2004). Surface water samples were collected at each sampling station from the stream thalweg for the analysis of nutrient concentrations

Table 1 Geographic, physical and chemical characteristics of the five study sites in Fall 2009

Stream	GUA	BRE	SMP	COL	CEL
Latitude 41°N	43′43″	44′14″	41′7″	51′6″	41′31″
Longitude 2°E	30′34″	34′3″	27′33″	40′9″	30′23″
Discharge (L s^{-1})	4.6	5.2	23.4	37.6	69.4
Temperature (°C)	18.7 ± 0.1	18.7 ± 0.4	19.8 ± 0.1	18 ± 0.1	21 ± 0.2
O_2 (mg L^{-1})	7.2 ± 0.4	5.4 ± 0.1	8.2 ± 0.3	8.7 ± 0.1	6.4 ± 0.2
NH_4-N (mg N L^{-1})	1.3 ± 0.4	0.9 ± 0.2	0.2 ± 0.1	0.9 ± 0.04	1.5 ± 0.1
NO_3-N (mg N L^{-1})	1.1 ± 0.2	5.9 ± 0.6	2.2 ± 0.1	1.5 ± 0.05	0.6 ± 0.1
NH_4/NO_3	2.1 ± 0.9	0.2 ± 0.1	0.1 ± 0.03	0.6 ± 0.05	3 ± 0.5

Values are mean ± SE of data from eight to 10 sampling stations along the 670–850 m reaches. Discharge corresponds to a single measure at the bottom of the stream reach

GUA Gualba, *BRE* Breda, *SMP* Santa María de Palautordera, *COL* Santa Coloma de Farners, *CEL* Sant Celoni

(3 replicates per station, 45 ml each) and $\delta^{15}N$ of NH_4 and NO_3 (1 L sample per station). All water samples were immediately filtered in the field through pre-ashed Albet (Barcelona, Spain) FVF glass fiber filters (0.7 µm pore size) into pre-washed plastic containers, stored on ice, and brought to the laboratory. Samples for the analysis of ^{15}N-NH_4 were immediately processed (see "Laboratory analyses" section), while filtered samples for nutrient concentrations and ^{15}N-NO_3 analyses were stored at −22 °C until further processing.

At each reach station and sampling date, the four macrophyte species were sampled when present. The presence and abundance of the each of the selected species at a particular site varied in response to the large inter-annual variability in rainfall and temperature that is characteristic of Mediterranean climates. Phenologically, all species were active at the time of sampling either because they are perennial (*Carex pendula*) or because they are annual but may grow until late fall under mild Mediterranean weather (*Callitriche stagnalis*). The two hemicryptophytes, *Veronica beccabunga* and *Apium nodiflorum* have biennial rhizomes and leave emergence is subjected to inter-annual variation. In the study area, *V. beccabunga* behaves more as an annual while *A. nodiflorum* shows perennial growth. In addition to phenology, *C. stagnalis* has fine roots and is thus susceptible of being washed away after torrential rains frequent under Mediterranean climate. Indeed, the interaction between emergence phenology and the occurrence of flooding events have been described as an important driver of spatial and temporal variation in plant composition of floodplain environments (Blom and Voesenek 1996). *Carex pendula* is a flood-resistant species that grows along stream banks. Typically, it exhibits waterlogged roots in the reed zone and perennating rhizomes beneath the soil surface. We only sampled individuals that were close to the stream channel, which were more likely to take up water and DIN from the stream. For each species and at each reach station, we collected leaves from 3 to 6 randomly selected plants to generate a composite sample. Samples were rinsed with water, immediately stored on ice in the field and brought to the laboratory for further processing.

Laboratory analyses

Stream water samples were analyzed for N-NH_4 and N-NO_3 + NO_2 concentrations following standard colorimetric methods (APHA 1995) on a Bran + Luebbe AA3 Autoanalyser (Nordersted, Germany) at the Institute of Marine Sciences (ICM-CSIC, Barcelona, Spain). The ^{15}N content of stream water NH_4 and NO_3 was determined following procedures adapted from Holmes et al. (1998) and Sigman et al. (1997), respectively. For the analysis of ^{15}N-NH_4 signatures, samples were amended with 3 g L^{-1} of MgO and 50 g L^{-1} of NaCl and a Teflon filter packet. The Teflon filter packets consisted of a pre-combusted Whatman GF/D glass fiber filter (1 cm diameter), which was previously acidified by adding 25 µl of K_2HPO_4 to trap the diffusing NH_3, and sealed within a packet formed by two pieces of Teflon tape. Immediately after adding the packets, the samples were tightly capped and incubated at 40 °C in a shaker table over 3 weeks. For ^{15}N-NO_3 analysis, NH_4 was initially removed by boiling the samples with 3.0 g of MgO and 5.0 g of NaCl. The resulting samples (0.1 l) were then transferred into acid-washed HDPE plastic bottles that were amended with 0.5 g MgO, 0.5 g Devarda's alloy and a Teflon filter packet. Immediately after adding the Teflon filter packets, samples were tightly capped and incubated at 60 °C for 48 h to reduce NO_3 to NH_4, and then incubated in a shaker table for 7 days at room temperature. At the end of the incubation, filter packets from ^{15}N-NH_4 and ^{15}N-NO_3 samples were removed from the bottles, placed in scintillation vials, and dried in a desiccator until further processing. Further details on these methods can be found in Von Schiller et al. (2009).

For determination of ^{15}N content of macrophytes, all samples were dried at 60 °C, ground with a MM 200 mixer mill (Retsch, Germany), and then subsamples (3–4 mg) were weighed on a Mettler-Toledo (Greifensee, Switzerland) MX5 microbalance. All samples (NH_4, NO_3 and macrophytes) for ^{15}N analysis were encapsulated in tins and analyzed at the UC Davis Stable Isotope Facility (California, USA). The stable isotope signatures of N for both the water samples and macrophytes were determined using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). All ^{15}N signatures are expressed in terms of delta values (δ), which are parts per thousand (‰) differences in the $^{15}N/^{14}N$ ratio between the sample and a standard (atmospheric N_2).

Statistical analysis

We characterized the variability of $\delta^{15}N$ values over space (i.e., among streams) and time (i.e., within SMP stream) for the DIN forms and the different macrophyte species by examining the mean, the range (i.e., minimum and maximum values), and the coefficient of variation (calculated from atom ^{15}N % values instead of $\delta^{15}N$ to avoid negative values, following procedures described in Fry (2008)). To describe how $\delta^{15}N$ values of NH_4, NO_3, and macrophyte species varied across streams, and among sampling dates within a stream, we used univariate permutational analysis of variance (using 999 permutations; hereafter referred to as pANOVA) using PRIMER v6 (Clarke and Gorley 2006) with the PERMANOVA+ add-on (Anderson 2001). We examined spatial and temporal variability separately; thus we conducted one pANOVA with macrophyte and DIN species (5 levels) and stream (5 levels) as fixed-effects factors and another pANOVA with macrophyte and DIN species (6 levels) and sampling date (6 levels) as fixed-effects factors. We performed pANOVA tests on reach-averaged $\delta^{15}N$ values of each macrophyte and DIN species at each stream or sampling date to handle the unbalanced data set. We also examined how spatial and temporal variation of macrophytes $\delta^{15}N$ was related to $\delta^{15}N$-NH_4 and $\delta^{15}N$-NO_3 using Spearman rank correlations. The relationships were examined with data from each reach (either from different streams or sampling dates) separately, as well as with data from all streams or sampling dates together. Results were considered significant at $P < 0.05$. Finally, in those cases where $\delta^{15}N$ values of macrophytes were significantly correlated to both $\delta^{15}N$-NH_4 and $\delta^{15}N$-NO_3 values, we estimated the relative proportions of NH_4 and NO_3 taken up by these macrophytes. This was done by fitting five mixing models differing in the isotopic discrimination factors considered: assuming no discrimination (mixing model 1), a single discrimination factor for NH_4 and NO_3 (mixing model 2), separate discrimination factors for NH_4 and NO_3 (mixing model 3), separate discrimination factors for NH_4 and NO_3 depending linearly on nutrient concentration (mixing model 4), and separate discrimination factors for NH_4 and NO_3 depending on concentration with a Monod equation (mixing model 5). A detailed description of these models can be found in Pastor et al. (2013). Models were fitted by maximum likelihood estimation using the 'bblme' R package (Bolker and Team 2012) and compared using the Akaike information criterion corrected for small sample size (AICc). The goodness-of-fit of each model was examined by comparing observed and predicted values of $\delta^{15}N$ for macrophytes using simple linear regressions. All statistical tests were done using R 2.14.0 (R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/) except for the pANOVA tests.

Results

Physical and chemical characteristics of the streams

Stream water temperature was similar among streams, while discharge and dissolved oxygen concentration showed larger variation (Table 1). DIN concentration varied among streams, ranging from 2.1 to 6.8 mg L^{-1} as well as the relative proportion of DIN as NH_4 and NO_3 (Table 1). Seasonal variation of stream water temperature in SMP followed the climatic pattern of this Mediterranean region with high values in summer and minimum in winter (Table 2). Stream discharge was low during summer and autumn dates and increased during the spring dates (Table 2). Dissolved oxygen concentration was relatively high on all sampling dates except in summer 2008, while DIN concentration in the receiving stream varied widely among all dates (Table 2). Significant temporal shifts in the relative dominance of the two DIN forms were found at the different sampling dates (Table 2).

Variability in ^{15}N signatures of DIN forms and macrophyte species

Natural abundance of $\delta^{15}N$ for the two DIN forms and for the macrophyte species showed high spatial variability within and among stream reaches (Table 3; Fig. 2). Both stream (two-way pANOVA, Pseudo-$F_{(4,20)} = 7.58$, $P < 0.01$) and species (two-way pANOVA, Pseudo-$F_{(4,20)} = 17.86$, $P < 0.01$) had a significant effect on isotopic signatures. Values of $\delta^{15}N$ of DIN forms and macrophytes were significantly lower in GUA than in the other streams (Pair-wise test, $P < 0.05$; Table 3), while $\delta^{15}N$-NH_4 was significantly higher than $\delta^{15}N$ values of

Table 2 Physical and chemical characteristics in SMP at the different sampling dates

Sampling date	Winter 2008	Summer 2008	Summer 2010	Autumn 2010	Winter 2010–2011	Spring 2011
Discharge (L s^{-1})	73.3	13.6	24	17.9	93	342.9
Temperature (°C)	10.4 ± 0.1	22.6 ± 0.6	22.8 ± 0.4	13.6 ± 0.2	9.3 ± 0.3	16.9 ± 0.5
O_2 (mg L^{-1})	9.9 ± 0.1	4.9 ± 0.3	8.7 ± 0.1	10.2 ± 0.1	11.7 ± 0.1	10.4 ± 0.1
NH_4-N (mg N L^{-1})	3.6 ± 0.1	0.4 ± 0.1	0.1 ± 0.02	2.3 ± 0.3	0.5 ± 0.02	0.2 ± 0.03
NO_3-N (mg N L^{-1})	1.9 ± 0.1	2.4 ± 0.2	1.5 ± 0.1	2.1 ± 0.1	2.3 ± 0.03	1.5 ± 0.2
NH_4/NO_3	1.9 ± 0.1	0.4 ± 0.3	0.1 ± 0.01	1.2 ± 0.2	0.2 ± 0.01	0.2 ± 0.02

Values are mean ± SE of data from eight to 10 sampling stations along the 670–850 m reach. Discharge corresponds to a single measure at the bottom of the stream reach

NO_3, *Apium nodiflorum*, and *Carex pendula* (Fig. 2). Variability of δ^{15}N-NH_4 values was consistently higher (i.e., higher coefficients of variation; CV) than that for δ^{15}N-NO_3 (Table 3; Fig. 2). δ^{15}N values of macrophytes were generally bracketed between those of NH_4 and NO_3, except in BRE, where they were lower than δ^{15}N values of the two DIN forms (Table 3).

Temporal variation in δ^{15}N values of NH_4, NO_3 and macrophytes in SMP was high (Fig. 2). The two-way pANOVA on data from the different sampling dates showed that both sampling date (Pseudo-$F_{(5,26)} = 7.31$, $P < 0.01$) and the macrophytes and DIN species (Pseudo-$F_{(5,26)} = 11.03$, $P < 0.01$) had a significant effect on δ^{15}N values. In winter 2008, δ^{15}N values of macrophytes were significantly lower and showed lower variation than in both summer dates (Pair-wise test, $P < 0.05$ for 2008; Pair-wise test, $P < 0.05$ for 2010) regardless of the species considered (Table 3). Similar to the results from spatial variability, δ^{15}N-NH_4 values were significantly higher than those for NO_3 and macrophytes species, except for *Callitriche stagnalis* and *Veronica beccabunga* (Fig. 2). Variation in δ^{15}N-NH_4 values was higher and variation of δ^{15}N-NO_3 values was lower than the ^{15}N variability of any species of macrophytes considered (Table 3; Fig. 2). Values of δ^{15}N for macrophytes were generally bracketed between δ^{15}N values of the two DIN forms, but clearly displaced towards δ^{15}N-NO_3 (Table 3; Fig. 2). Overall, *Callitriche stagnalis* was the only species showing values of δ^{15}N similar to those of NH_4 and NO_3 for both spatial and temporal variation (Fig. 2).

Relationships between ^{15}N signatures of DIN and macrophytes

Considering data within each stream reach separately, we did not find any significant correlation between δ^{15}N of the macrophyte species and δ^{15}N of the two DIN forms, with the exception of δ^{15}N for *Apium nodiflorum*, which was correlated to δ^{15}N-NH_4 in BRE (Spearman correlation, $r = 0.95$, $P < 0.01$). When δ^{15}N data from all five study streams were pooled together to enlarge the ranges of both DIN concentration and isotopic signal, there was again a significant positive correlation between δ^{15}N for *Apium nodiflorum* and δ^{15}N-NH_4 (Spearman correlation, $r = 0.59$, $P < 0.001$; Fig. 3). No other significant correlations were found among reaches.

Considering data for each sampling date separately, we found positive significant correlations between δ^{15}N-NO_3 and δ^{15}N of *Callitriche stagnalis* ($r = 0.9$, $P < 0.001$), *Veronica beccabunga* ($r = 0.89$, $P < 0.001$), and *Apium nodiflorum* ($r = 0.72$, $P < 0.001$) in the summer of 2008 when the range of δ^{15}N values within the reach was the broadest (Table 3). When δ^{15}N data from all sampling dates in SMP were pooled, the δ^{15}N of all the macrophyte species, except for *Carex pendula*, exhibited significant and positive correlations with one or both δ^{15}N-NH_4 and δ^{15}N-NO_3 values (Fig. 4). No clear influences of either NH_4 or NO_3 concentrations on the correlation between DIN and macrophytes δ^{15}N values were observed (Fig. 4). However, we found that low NH_4 concentrations (corresponding to summers 2008 and 2010) coincided with a high variation of δ^{15}N-NH_4, whereas high NH_4 concentration (corresponding to winter 2008) coincided with low variation of δ^{15}N-NH_4 (Fig. 4).

Relative proportion of NH_4 and NO_3 taken up by macrophytes

We examined the proportion of the two DIN forms that accounted for the ^{15}N signals of those macrophyte species showing significant correlations with ^{15}N of both DIN forms (i.e., *Callitriche stagnalis* and *Apium nodiflorum*). The results from the models indicated that the relative proportion of NO_3 taken up by *Callitriche stagnalis* and *Apium nodiflorum* was much higher than the proportion of NH_4 (Table 4). Specifically, the estimated proportion of NO_3 taken up by *Callitriche stagnalis* and *Apium nodiflorum* ranged between 60.4 and 83.5 %, and between 63.7 and 73.5 %, respectively (Table 4). For both macrophyte species, the best-fit models (i.e., lower AICc values) were

Table 3 Mean (‰), minimum (Min) and maximum (Max) values, and coefficient of variation (CV) of $\delta^{15}N\text{-}NH_4$, $\delta^{15}N\text{-}NO_3$ and $\delta^{15}N$-macrophytes in each study stream during fall 2009 and at SMP on different sampling dates

DIN form/species	Study streams														
	GUA			BRE			SMP			COL			CEL		
	Mean	Min–Max	CV	Mean	Min–Max	CV	Mean	Min–Max	CV	Mean	Min–Max	CV	Mean	Min–Max	CV
Ammonium	18.3	–1.9 to 35	12.9	22.3	11.1 to 38.5	9.1	34.2	24.2 to 36.4	4.2	28.9	28.6 to 30	0.5	33.6	30.8 to 35.4	1.6
Nitrate	3.6	–2.4 to 6.9	3.7	13.1	7.7 to 14.6	2.1	12.2	10.4 to 13.5	1.1	8.6	7.9 to 9.4	0.6	12.2	10.4 to 13.5	1.1
Callitriche stagnalis							20.7	17.9 to 23.2	2.1				15.1	10 to 15.8	3.1
Apium nodiflorum				9.0	1.7 to 29	8.2	18.2	6.4 to 24	5.4	21.5	17.7 to 24.2	2	18.4	14.1 to 22.4	2.6
Carex pendula	6.6	3.6 to 8.3	1.8	4.7	2.8 to 6.2	1.1	12.7	10.2 to 20.7	4.4	16.5	7.1 to 21	5.1	19.2	11.5 to 24.5	4.2

DIN form/species	Sampling dates in SMP																	
	Winter 2008			Summer 2008			Summer 2010			Autumn 2010			Winter 2010–2011			Spring 2011		
	Mean	Min–Max	CV	Mean	Min–Max	CV	Mean	Min–Max	CV	Mean	Min–Max	CV	Mean	Min–Max	CV	Mean	Min–Max	CV
Ammonium	13.2	12.7 to 13.8	0.4	45.1	29.3 to 55.5	8.8	17.3	15.2 to 25.6	3.7	28.9	26.3 to 31.9	2.0	30.0	28.5 to 31.6	1.1	26.2	20.6 to 27.9	11.1
Nitrate	7.7	6.3 to 9.6	1.0	15.4	11.6 to 18.1	2.1	11.9	11.6 to 14.8	1.0	10.0	9.6 to 10.6	0.3	11.8	11.2 to 12.1	0.3	9.8	9.1 to 12.5	1.9
Callitriche stagnalis	2.1	–0.5 to 4.9	1.7	18.4	10.6 to 29	6.4										8.1	8.1 to 8.2	0.1
Veronica beccabunga	10.3	9.2 to 12.5	1.1	18.5	12.8 to 24.6	3.8												
Apium nodiflorum	8.0	6.3 to 14.8	3.1	22.6	14.7 to 28.7	4.1	15.6	8.7 to 25.1	5.2	12.0	2.2 to 13.3	3.7	16.2	12.3 to 20	3.0	13.3	9.4 to 16.9	3.3
Carex pendula							11.5	1 to 23.5	6.9	12.6	7.2 to 14	2.6	11.3	4.1 to 15.4	5.0	9.8	6.1 to 14.9	2.7

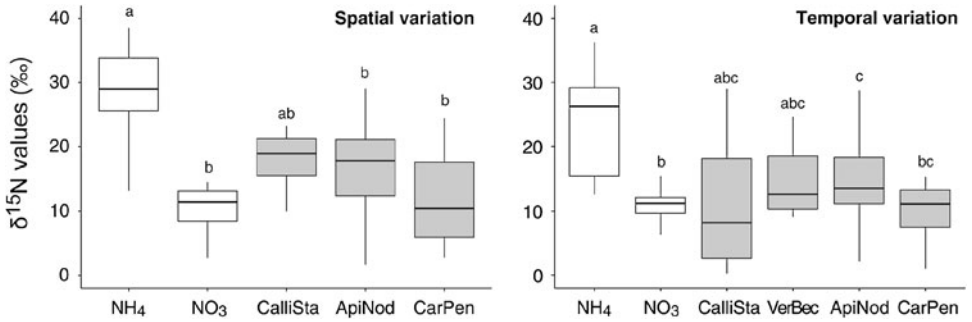


Fig. 2 *Box* plot of $\delta^{15}N$ values of NH_4, NO_3, *Callitriche stagnalis* (CalliSta), *Veronica beccabunga* (VerBec), *Apium nodiflorum* (ApiNod) and *Carex pendula* (CarPen) from the study streams in fall 2009 (spatial variation) and from all different sampling dates in SMP (temporal variation). *Letters* indicate significant differences from two-way pANOVA test ($P < 0.05$)

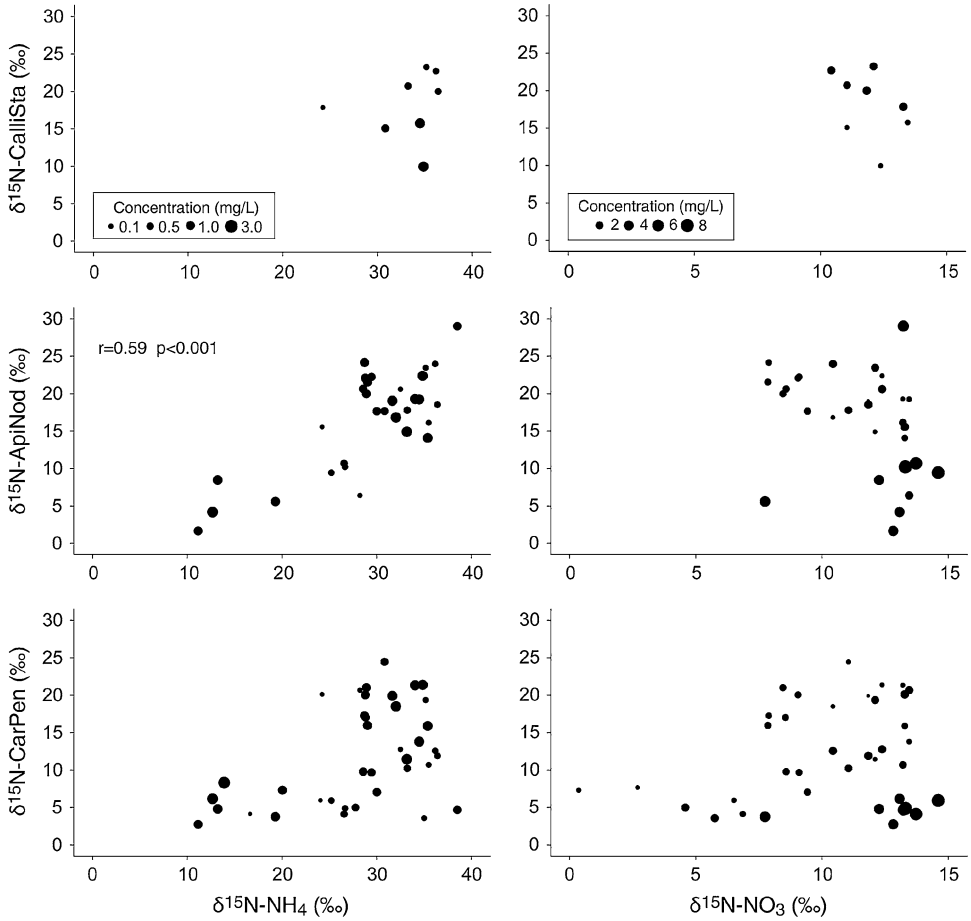


Fig. 3 Relationships between $\delta^{15}N$ values of DIN (NH_4 and NO_3) and *Callitriche stagnalis* (CalliSta), *Apium nodiflorum* (ApiNod) and *Carex pendula* (CarPen). Each panel includes all $\delta^{15}N$ data from the five study streams. The Spearman's rank correlation coefficient is shown in the *top left corner* of each panel for all significant correlations. Size of circles is proportional to NH_4 or NO_3 concentration respectively

those assuming a relationship between isotopic discrimination and nutrient concentration (i.e., models 2–5), although for *Callitriche stagnalis* all fitted models showed a high prediction power (Table 4).

Discussion

This study shows high variation in ^{15}N signatures of macrophytes in streams receiving inputs from WWTPs and reveals that this variability is significantly related to the variation of ^{15}N-DIN signatures, except for the riparian species (i.e., *Carex pendula*) growing on the stream bank. This coupling suggests a transfer of the ^{15}N-DIN signature to submersed and amphibious species, and supports evidence that these macrophyte species rely on stream water DIN as an N source. In the study streams, the range of observed $\delta^{15}N$-NO_3 values in WWTP-receiving streams (from −2.4 to 18.1 ‰) almost overlapped the range encompassed by a compilation of values from human altered streams world-

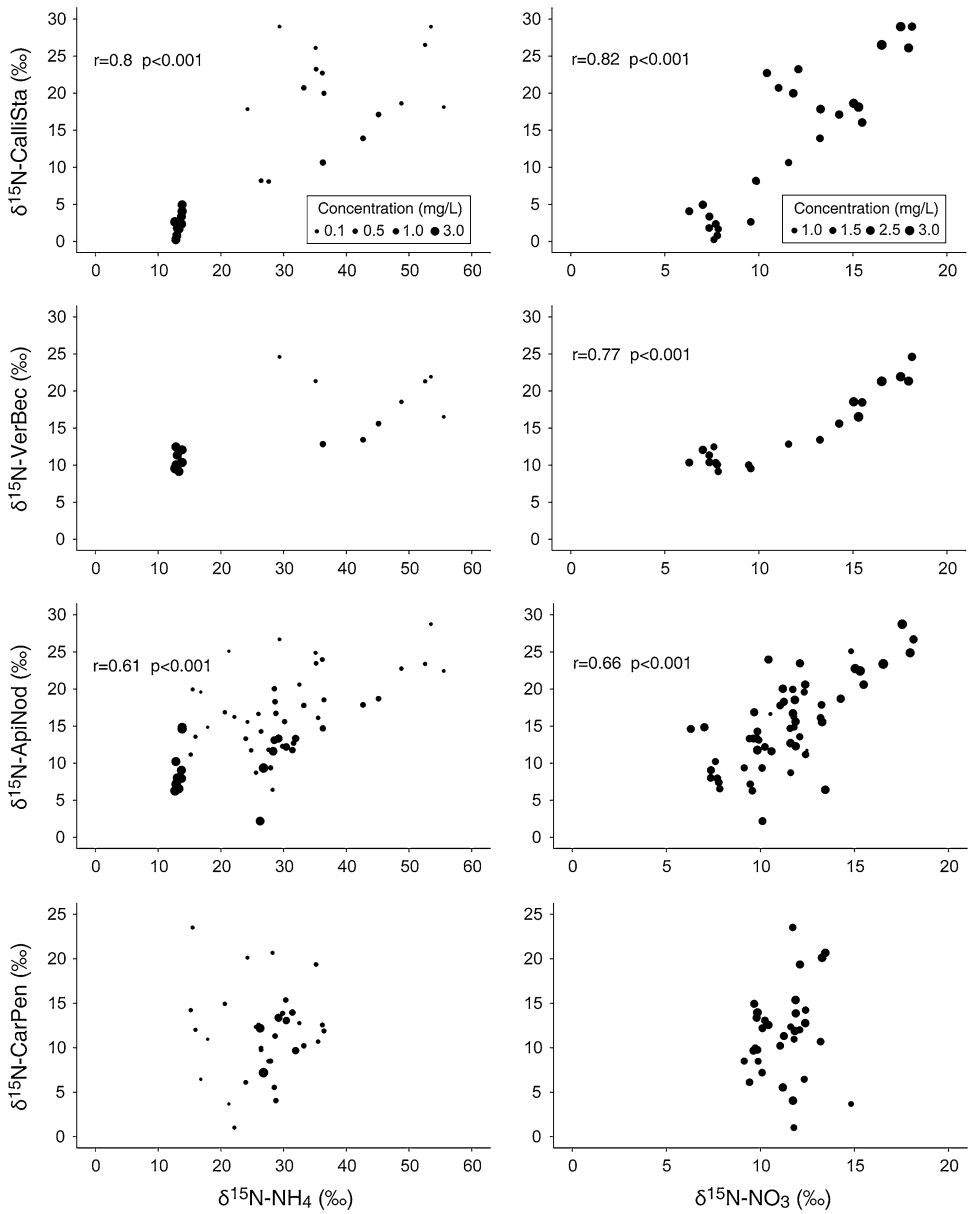


Fig. 4 Relationships between $\delta^{15}N$ values of DIN (NH_4 and NO_3) and *Callitriche stagnalis* (CalliSta), *Veronica beccabunga* (VerBec), *Apium nodiflorum* (ApiNod) and *Carex pendula* (CarPen). Each panel considers all $\delta^{15}N$ data from different sampling dates in SMP. The Spearman's rank correlation coefficient is shown in the *top left corner* of each panel for all significant correlations. Size of circles is proportional to NH_4 or NO_3 concentration respectively

Table 4 Results from the five mixing models performed with temporal $\delta^{15}N$ data of *Callitriche stagnalis* and *Apium nodiflorum*

	Callitriche stagnalis						*Apium nodiflorum*					
	AICc	df	r^2	pNH_4	pNO_3	*P* value	AICc	df	r^2	pNH_4	pNO_3	*P* value
Mixing model 1	188.20	2	0.78	0.16	0.84	**<0.01**	480.12	2	0.36	0.26	0.74	**<0.001**
Mixing model 2	181.26	3	0.74	0.40	0.60	**<0.001**	481.22	3	0.38	0.32	0.68	**<0.001**
Mixing model 3	183.97	4	0.74	0.40	0.60	**<0.001**	483.44	4	0.38	0.32	0.68	**<0.001**
Mixing model 4	171.74	4	0.82	0.22	0.78	0.42	474.56	4	0.45	0.36	0.64	**<0.001**
Mixing model 5	180.75	6	0.80	0.32	0.68	**<0.001**	480.63	6	0.43	0.36	0.64	**<0.001**

Values shown in the table are the Akaike Information Criterion corrected for small sample size (AICc), the number of parameters estimated (df), the variability explained by each model (r^2 of linear regression between observed and fitted values), the estimated relative proportions of NH_4 and NO_3 taken up by macrophytes (pNH_4 and pNO_3, respectively), and the *P* value for the estimated parameter (pNH_4 was estimated and tested H_0: $pNH_4 = 0$; pNO_3 was parameterized as $1-pNH_4$)

Values highlighted in bold indicate significant estimation of model parameters (i.e., *P* value < 0.05)

wide (4.6–19.4 ‰; in Peipoch et al. (2012)), and that of δ^{15}N-NH_4 clearly expanded the upper limit reported in the same study (2–11.9 ‰). The intensive N processing (especially nitrification and denitrification) within the batch reactors of the WWTP is subjected to isotopic discrimination and results in high ^{15}N-DIN signatures in the effluent and in a subsequent increase of ^{15}N signatures in receiving streams as observed in our data and in previous studies (Gammons et al. 2010; Merbt et al. 2011). The variability in concentration and ^{15}N signatures of the two DIN forms found in this study, both across streams and over time within a stream, could be partly explained by differences in the relative contribution of the WWTP effluent to the DIN load among receiving streams or over time within a stream. However, in-stream processes in the receiving streams can also contribute importantly to enlarge the variability of ^{15}N signatures of the two DIN forms, especially in those seasons when microbial biological activity in streams is higher (Ribot et al. 2012). In fact, in our study the largest variability in δ^{15}N-DIN within the SMP reach coincided with base flow conditions in the stream (i.e., summer), when the relative contribution of the effluent and the influence of in-stream processes on DIN are both maximized. Because biogeochemical processes affecting concentration and ^{15}N signatures of DIN are the same in the WWTP and within the stream, it is reasonable to expect that they could be somehow exposed to similar seasonal variation.

Macrophyte species showed remarkable spatial and temporal variability in their δ^{15}N values. As with δ^{15}N-DIN signatures, the observed ranges of δ^{15}N for the study macrophytes clearly expanded the upper limit of those previously reported for freshwater macrophytes affected by anthropogenic disturbances (up to 20, 8, and 15 ‰ in Finlay and Kendall (2007), in Benson et al. (2008), and in Kohzu et al. (2008), respectively). However, patterns of variability in δ^{15}N values of macrophytes differed among species. Variation of ^{15}N signatures of *Callitriche stagnalis* was tightly coupled to that of δ^{15}N-DIN, but this coupling was less pronounced in species of macrophytes located away from the stream channel (i.e. *Carex pendula*). Amphibious species rooted in the stream sediments (i.e. *Apium nodiflorum* and *Veronica beccabunga*) are known to use other additional N sources from hyporheic water and groundwater (Svendsen and Kronvang 1993; Madsen and Cedergreen 2002) that may have distinct ^{15}N signatures, which can explain why ^{15}N variation in *A. nodiflorum* and *V. beccabunga* was less coupled to δ^{15}N-DIN variation. Moreover, DIN uptake and translocation via the rhizome system of *Carex pendula* could also be an important factor influencing its δ^{15}N variation as it has been observed for other species of *Carex* (Brooker et al. 1999).

In general, both the spatial and temporal δ^{15}N variability of all macrophytes exceeded that of δ^{15}N-NO_3, but not that of δ^{15}N-NH_4. However, δ^{15}N values of selected macrophytes were consistently more similar to δ^{15}N-NO_3 than to δ^{15}N-NH_4 values. Previous studies have also found small differences between δ^{15}N-NO_3 and δ^{15}N of macrophytes in streams where NO_3 was the dominant DIN source ($\Delta\delta^{15}$N; from 0 to 6 ‰ in Kohzu et al. 2008 and from 1.9 to 3.6 ‰ in De Brabandere et al. 2007). However, these studies did not measure stream water δ^{15}N-NH_4 signatures even though aquatic plants are known to assimilate both DIN forms (Taiz and Zeiger 2010). In this study, the large data sets of paired ^{15}N signatures for the two DIN forms and for macrophytes allowed examining in detail how δ^{15}N values of the two DIN forms and of macrophytes are related. Although no clear relationships were found along single stream reaches, when we considered data from different sites or from different dates within a stream, we found significant relationships between the ^{15}N signatures of DIN and those of macrophytes. Correlations between δ^{15}N values of DIN forms and macrophyte species were strongest for *Callitriche stagnalis*, showing the relevance of stream water DIN as a N source for submersed species. Previous studies have demonstrated that nutrient uptake through floating leaves of *Callitriche* genus, and other submersed species, can be sufficient to satisfy their N demand (Madsen and Cedergreen 2002). Nichols and Keeney (1976) revealed that availability of DIN in the water column enhanced foliar uptake to supplement root uptake in submersed macrophytes such as *Myriophyllum spicatum*. Considering the high DIN concentration of the study streams, it is likely that N uptake through leaves occurred in *Callitriche stagnalis,* which explains why ^{15}N signatures of *Callitriche stagnalis* were strongly coupled to ^{15}N signatures of the two DIN forms. Surprisingly, amphibious species showed similar relationships to those for submersed macrophytes, which was not expected considering the conspicuous structural and physiological differences between species from these two functional groups. Nitrogen uptake through leaves of *Veronica beccabunga* and *Apium nodiflorum* is unlikely because in these particular streams their leaves are rarely submersed within the stream water (M. Peipoch, *personal observation*). However, their roots are shallow and commonly observed above the streambed, which could explain the significant relationships with ^{15}N-DIN signatures. Further studies examining the uptake and translocation of ^{15}N by roots and shoots of amphibious macrophytes may provide evidence on how they incorporate the ^{15}N signature of stream water DIN into their leaves, and thus confirm the in-stream DIN reliance by amphibious species to fulfill their N demand. Finally, the lack of significant correlations between δ^{15}N values of DIN and *Carex pendula* suggest that species located at the stream-riparian margin may rely more on other N sources, such as hyporheic and/or groundwater

rather than on stream water DIN. Variation in the strength of the relationships between ^{15}N signatures of DIN and macrophyte species is in agreement with the degree of stream water exposure and structural traits of the different functional types of macrophytes. The less complex structure and higher water exposure of submersed and amphibious species may allow these species to better integrate the short-term variability of ^{15}N-DIN signatures and thus increase the chances of observing correlations between ^{15}N signatures of DIN and macrophytes. On the other hand, the more intermittent water exposure and the existence of perennating buds in *Carex pendula* may result in long N turnover compared to submersed and amphibious species (Blom and Voesenek 1996), and lead to a lower capacity by this species to integrate the short-term variability of ^{15}N-DIN signatures.

Overall, the relationships observed between $\delta^{15}N$ values of DIN and macrophytes in this study suggest that macrophyte species can contribute differently to in-stream N uptake depending upon their functional group. Recent studies using nutrient additions of ^{15}N-labelled NH_4 in streams have demonstrated how submersed and amphibious macrophytes can have a relevant contribution in stream N uptake in macrophyte-rich streams (Simon et al. 2007; Riis et al. 2012). Results from our study support these previous findings, but also indicate that this contribution might be mostly restricted to macrophytes that develop within the stream channel, whereas those macrophytes that develop along stream-riparian edges seem to have a lower influence on in-stream N uptake. In addition, the consistent displacement of $\delta^{15}N$ values of submersed and amphibious species towards ^{15}N-NO_3 signatures provides strong evidence that the contribution of NO_3 to the ^{15}N pool of these macrophytes in the study streams is greater than that of NH_4. This is further confirmed by the results from the mixing models using isotopic signatures, which indicate that in these particular WWTP-influenced streams the contribution of submersed *Callitriche stagnalis* and *Apium nodiflorum* to in-stream N uptake is predominantly in the form of NO_3. The relative proportions of each DIN source taken up by macrophytes were quite consistent among mixing models with and without discrimination, and with different equations for sources mixing. Preferences in the inorganic N form used by plants have involved some controversy in the past, and although some studies have shown a preference for NH_4 (Cedergreen and Madsen 2002), other studies indicate that this may not be general (Melzer 1999; Gherardi et al. 2013), which is in accordance with our findings. The correlative approach used in this study is not sufficient to reveal which mechanisms are controlling the relative proportions of each DIN source taken up by macrophytes in these particular WWTP-influenced streams. However, it is worth mentioning that average NH_4 and NO_3 contribution to macrophytes N uptake estimated from the different models was similar to the proportion of these two DIN sources found in water. These results, therefore, suggest that the proportion of each DIN source taken up by macrophytes in these WWTP-influenced streams may be influenced by their concentration in the water, which is in agreement with the greater predictive power (i.e., higher r^2 values) for mixing models considering DIN concentration observed in this study. Studies showing how higher NO_3:NH_4 ratios in stream water stimulate the uptake rates of NO_3 by the biota support our results (O'Brien et al. 2007; von Schiller et al. 2009).

In conclusion, our results contribute towards understanding ^{15}N variation in aquatic macrophytes of streams impacted by WWTP effluents, and more importantly, highlight the potential influence of macrophytes on in-stream N uptake and how it may vary according to different species of aquatic plants. In this regard, this study indicates that direct assimilation of stream water DIN is occurring by submersed and amphibious species and, in the study streams, it seems to be mainly in the form of NO_3. In contrast, our results also indicate that macrophytes species at the stream-riparian edge most likely rely on N sources other than those provided by stream water DIN. The functional attributes of different macrophytes can be of interest to water managers when evaluating the use of macrophytes to restore channel morphology of high nutrient-loaded streams since this study shows that macrophytes, especially the species developing in the stream channel, not only influence channel morphology but they can also contribute to retain excess DIN in streams with high loads.

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