Relationships between streamwater nitrogen and primary uptake compartments: an isotopic approach

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TESIS DOCTORAL

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"Relationships between streamwater nitrogen and primary uptake compartments: an isotopic approach"

Relacions entre el nitrogen de l'aigua del riu i els compartiments primaris de captació: una aproximació isotòpica

Memòria presentada per Ada Pastor Oliveras per optar al grau de doctora per la Universitat de Barcelona

Ada Pastor Oliveras
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"Given that a major component of this human-dominated period of Earth's history is a massive disruption of the global nitrogen regime, the new studies suggest that future paleontologists should look for lighter nitrogen isotopic signatures in geological strata derived from lake sediments or for traces of heavier nitrogen in plant fossils. Whether such signals are an ephemeral blip in the stratigraphic record or a sustained shift lasting millennia may, in due time, be seen as an indicator of humanity's success, or failure, in achieving planetary sustainability"

J.J. Elser (2011)

CONTENTS

AGRAÏMENTS

Aquesta tesi ha sigut possible gràcies a la implicació i ajuda de moltes persones. Amb aquestes breus línies vull fer-les partícips d'aquesta tesi, ja que, en part, també és seva. A tots i totes, aquells que surten anomenats més avall, o que per distracció m'he deixat, ben sincerament: moltes gràcies!

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Blanes, 29 d'abril 2014.

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ABSTRACT

The overarching goal of this dissertation was to explore relationships between streamwater nitrogen (N) and the most representative primary uptake compartments (PUCs) in stream ecosystems (e.g. microbial biofilm, algae, bryophytes, macrophytes). In particular, environmental factors driving these biogeochemical relationships along a strong anthropogenic gradient were explored and differences among and within PUC types were compared. To elucidate the factors controlling these relationships, we used N stable isotopes ($\delta^{15}N$; in ‰), both natural abundance (*Chapter one, two and three*) and ^{15}N labelling techniques (*Chapter four*)

First, we examined the spatial variability of $\delta^{15}N$ natural abundance of PUC types, and related this variability to $\delta^{15}N$ values of dissolved inorganic species (DIN, ammonium and nitrate) across streams differing in nutrient availability. We found that the variability of $\delta^{15}N$-PUC was mostly explained by location within the fluvial network, and was related to $\delta^{15}N$ of DIN species for PUCs living within the stream channel. The prediction power for $\delta^{15}N$-PUC was improved by stream nutrient concentrations and stoichiometry, indicating the relevance of stream nutrient environment to understand $\delta^{15}N$ values of PUCs.

Second, we analyzed the temporal variability of $\delta^{15}N$ natural abundance in PUC types and DIN species in four streams with different nutrient concentrations. Our results did not show isotopic temporal patterns over a year. However, among streams, the highest variability was found in the urban stream and, among PUC types, temporal variability tended to be higher in PUCs submerged in streamwater with faster turnover rates, such as filamentous algae.

Third, we studied the $\delta^{15}N$ variability of epilithic biofilms in different stages of development under contrasting stream nutrient concentrations. We observed that $\delta^{15}N$ variability of early-stage biofilm (colonizing artificial substrates) was lower than late-stage biofilm (attached to stream cobbles). Except at the low-nutrient stream, $\delta^{15}N$ of early-stage epilithon was lower than that of late-stage biofilm. Moreover, during biofilm colonization, $\delta^{15}N$ increased with biomass accrual. Changes between successional stages were more pronounced at the high-nutrient stream. These results suggested successional stage as a relevant factor controlling $\delta^{15}N$ variability of epilithic biofilm at the local scale.

Fourth, N and C biogeochemical interaction between the biofilm-litter compartment and streamwater during litter decomposition was evaluated by using double-labeled

(^{15}N and ^{13}C) leaves of two *Populus* species (*P. fremontii and P. angustifolia*). These species differed in their concentration of recalcitrant compounds (i.e. tannins) and were expected to influence the microbial decomposer community dependency to streamwater. Litter type strongly affected biomass and stoichiometry of microbial assemblages growing on litter, but the proportion of N and C derived from streamwater was not different. Gross immobilization of N from the streamwater was higher for the low-tannin litter, probably as a consequence of higher microbial biomass, contrasting to C fluxes which were higher for the high-tannin litter, suggesting C limitation.

Overall, this dissertation provides insights into what controls ^{15}N biogeochemical relationships between PUC types and water in fluvial ecosystems. This has implications for the use of N stable isotopes in ecological and environmental studies in aquatic ecosystems, and can help to develop successful management strategies to mitigate N excess in fluvial systems.

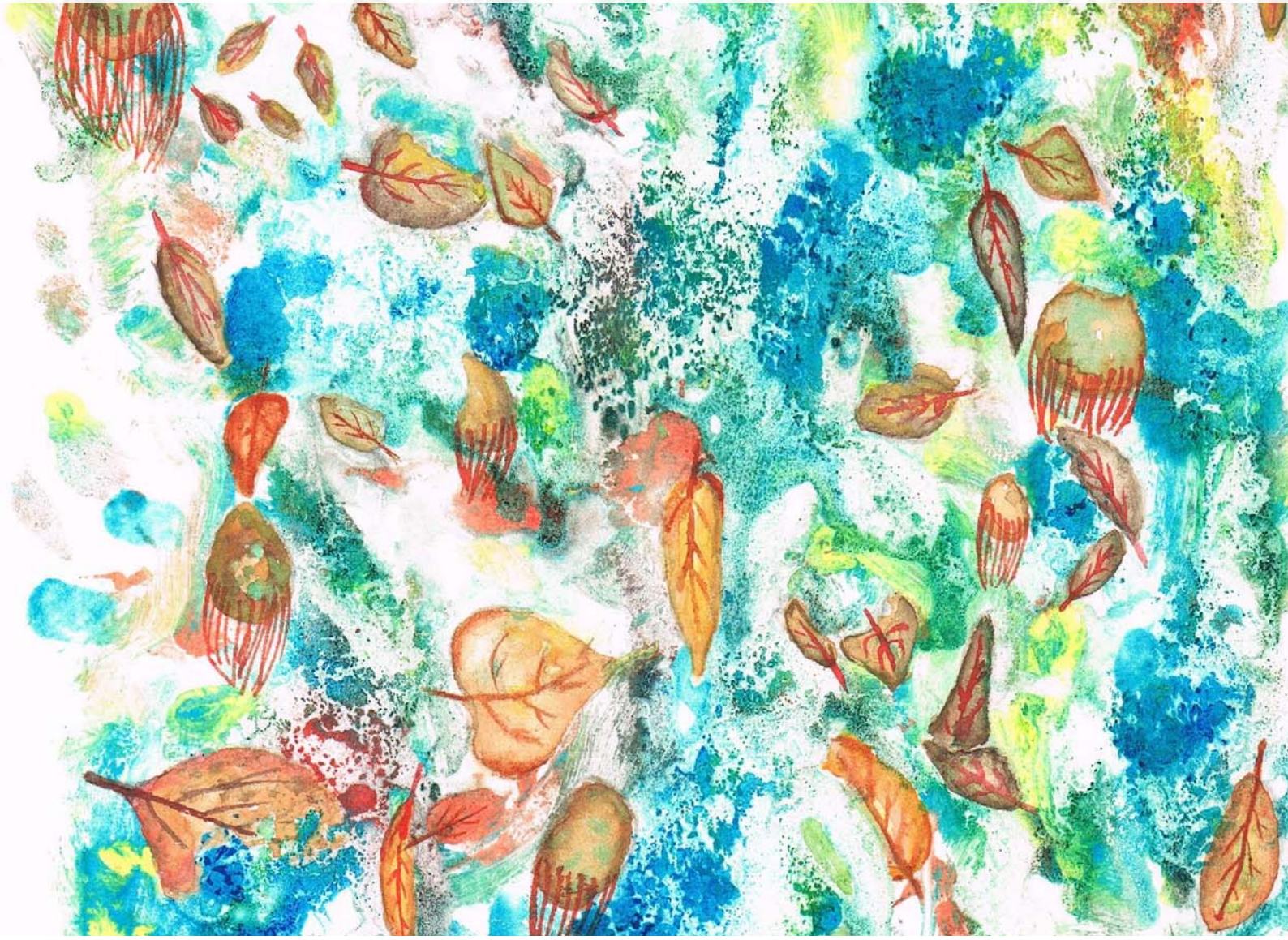
LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

acf	autocorrelation function estimation
AFDM	ash-free dry mass
AGR	agricultural stream (*chapter two*)
AICc	Akaike Information Criterion corrected for small sample size
ANOVA	analysis of variance
ANCOVA	analysis of covariance
AFDM:	ash-free dry mass
at	atom
ºC	degree Celsius
C	carbon
chl a	chlorophyll *a*
CBOM	coarse benthic organic matter
cm	centimeter
CPSIL	Colorado Plateau Stable Isotope Laboratory
D	deviance explained
DIN	dissolved inorganic nitrogen
DO	dissolved oxygen
DOC	dissolved organic carbon
DON	dissolved organic nitrogen
DW	stream reach downstream of the WWTP outfall (*chapter three*)
f	fractionation factor
Fig.	Figure
FOR	forested stream (*chapter two*)
FBOM	fine benthic organic matter
g	Earth's gravitational acceleration
g	gram
h	hour
GAM	generalized additive model
GI	gross immobilization
HOR	horticultural stream (*chapter two*)
IC	ion chromatography
IRMS	isotope ratio mass spectrometer
k	decomposition rate constant
K	half-velocity constant
L	liter
M	mass
m	meter
m a.s.l	meters above sea level
MB	microbial biomass
mg	milligram
mL	milliliter
mm	millimeter
n	number of observations
N	nitrogen
n.a.	not available
NAU	North Arizona University
NE	northeast
n.s.	not statistically significant
P	statistical significance
P	phosphorous
pNH_4^+	proportion of N in PUC derived from NH_4^+ vs NO_3^-
PUC	primary uptake compartment
r	coefficient of correlation analysis
r^2	coefficient of determination of a statistical model
RMSE	root mean-square error
s	second
SE	standard error
SD	standard deviation
SpC	specific conductivity
sp.	species
SRP	soluble reactive phosphorous
t	time
TC	total carbon
TN	total nitrogen
USGS	United States Geological Survey
UP	stream reach upstream of the WWTP outfall (*chapter three*)
URB	urban stream (*chapter two*)
U.S.A	United States of America
UTM	Universal Transverse Mercator projected coordinated system
UTM-E	easting coordinate in the UTM zone.
UTM-N	northing coordinate in the UTM zone.
WWTP	wastewater treatment plant
$\delta^{13}C$	carbon isotopic composition
$\delta^{2}H$	hydrogen isotopic composition
$\delta^{15}N$	nitrogen isotopic composition
µg	migrogram
µS	microsiemens

General introduction and objectives



GENERAL INTRODUCTION

I.1 Fluvial ecosystems and nitrogen dynamics

Fluvial networks are fascinating ecosystems which play a significant role to the biosphere (Meybeck 2003, Sponseller et al. 2013). Although rivers and streams represent only a small portion of the Earth's water, they are essential for human welfare by providing essential goods and ecosystem services (Costanza et al. 1997, Hassan et al. 2005, Allan and Castillo 2007). Streams also have accumulated multiple pressures, as a result of anthropogenic activities, especially in developed areas (Dodds et al. 2013). Due to the hierarchical nature of rivers, human disturbances in the watershed can be transferred to reaches and microhabitats in streams, and subsequently result in changes in stream community structure and function (Vitousek et al. 1997, Allan 2004, Burcher et al. 2007; Fig. I.1). These ecological changes greatly diminish the capacity of streams to provide valuable ecosystems services (Townsend et al. 2003, Dodds et al. 2013).

One major pressure fluvial ecosystems undergo is associated to nitrogen (N) enrichment. Humans have altered nutrient cycling at the global scale; in particular, for the N cycle, these alterations might have already exceeded biophysical thresholds of recovery (Rockström et al. 2009). The industrial conversion of atmospheric N_2 into reactive N for human use, together with fuel combustion, has resulted in large amounts of N reaching

the environment, adding a number of greenhouse gases to the atmosphere and polluting aquatic systems (Vitousek et al. 1997, Galloway et al. 2003, Erisman et al. 2008, Elser 2011).

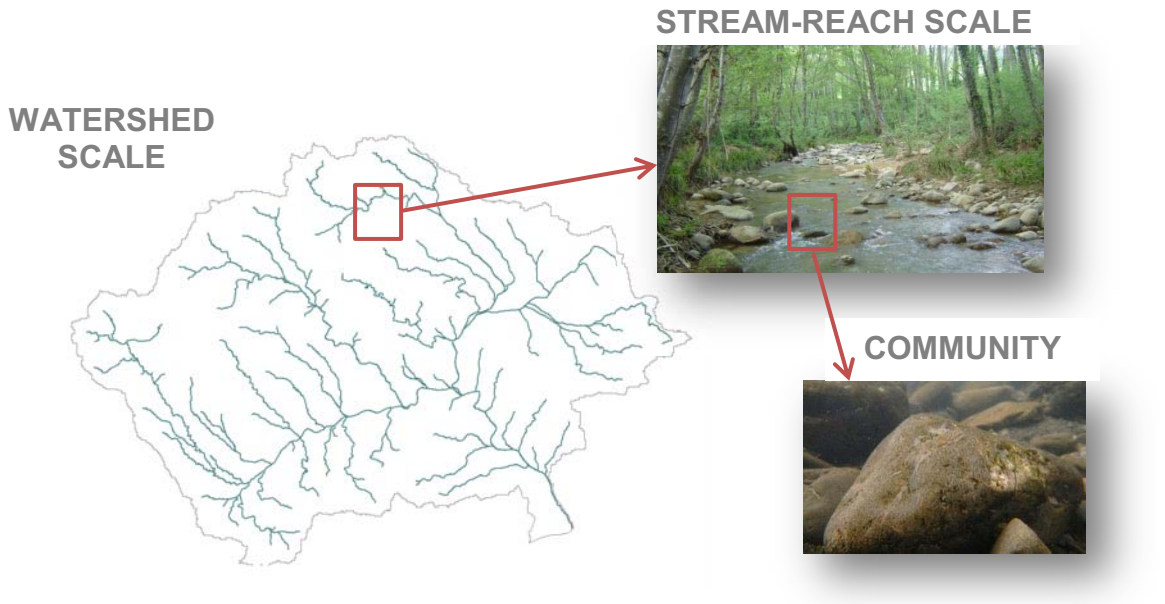


Figure I.1 Schematic illustration of the hierarchical organization of a fluvial network. Human impacts can be transferred from the large scale, the watershed, to subsequently smaller scales, eventually altering the structure and function of stream communities.

Several factors can contribute to increase N concentrations in fluvial ecosystems. First, nutrient fluxes into aquatic systems have dramatically increased due to intensive human land use in catchments world-wide (Carpenter et al. 1998, Foley et al. 2005, Scanlon et al. 2007), direct dumping of urban or industrial sewage (i.e. point sources; Martí et al. 2004, Merseburger et al. 2005), and atmospheric deposition (Bernal et al. 2013). Second, geomorphological modifications of streams, an undesired common feature in human-altered streams (Paul and Meyer 2001, Allan 2004), can

reduce streams' availability to reduce the N load and increase its availability in these ecosystems (Bukaveckas 2007, Kaushal et al. 2008). These effects of N enrichment can be amplified in Mediterranean streams because of their reduced dilution capacity, especially during summer low flow (Martí et al. 2010, Cooper et al. 2013), a factor that will likely be intensified with the effects of climate change (Whitehead et al. 2006, Wilby et al. 2006).

Dissolved inorganic nitrogen (DIN), mostly ammonium and nitrate, reaching streams can be removed by biotic uptake and by conversion of ammonium (NH_4^+) to nitrate (NO_3^-) via nitrification or NO_3^- to N_2O and N_2 via denitirification (e.g. Peterson et al. 2001, Beaulieu et al. 2011; Fig. I.2). Among these processes, biotic uptake constitutes the majority of NH_4^+ and NO_3^- removal from streamwater. Though biotic uptake does not result in a permanent removal of N, it slows down the transport of DIN and hence controls N export to downstream aquatic ecosystems. Along the river continuum, the highest (areal) uptake rates of N occur in headwaters, often accounting for more than half of the total inputs from their watershed (Alexander et al. 2000, Peterson et al. 2001). Thus, small streams are key sites to the transformation and retention of N, and should be considered priority restoration sites for N removal (Craig et al. 2008).

This dissertation examines N biogeochemical interactions between streamwater DIN and stream-riparian biota in small streams, and attempts

to elucidate some factors driving these interactions. This information can provide comprehension of terrestrial N links with stream ecosystems and biota uptake controlling factors, which affect N export downstream. Better understanding these interactions will help develop successful management strategies to enhance fluvial ecological functions.

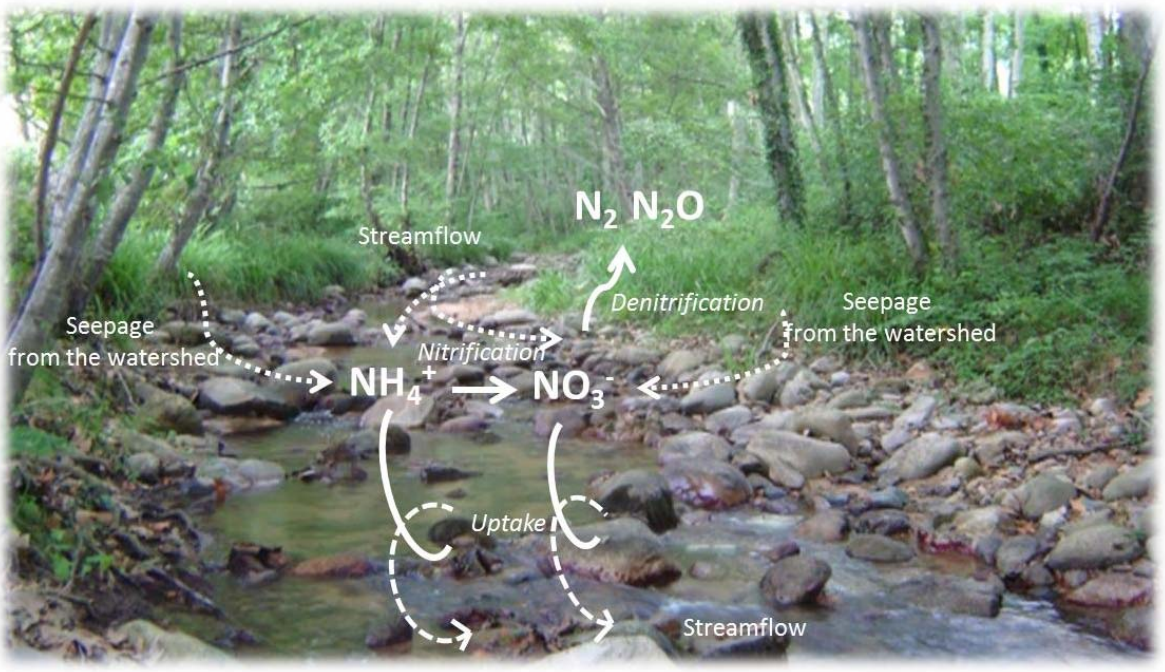


Figure I.2 Dissolved inorganic nitrogen (DIN) dynamics in a fluvial ecosystem. DIN species enter into the stream reach either from upstream flow or seepage from the watershed. NH_4^+ can be converted to NO_3^- via nitrification and NO_3^- to N_2 via denitirification. Benthic biota uptake and assimilate DIN, which is later regenerated back to the streamwater and exported downstream.

I.2 The primary biotic uptake compartments

Stream and riparian biota, hereafter referred as primary uptake compartments (PUCs; Fig. I.3), comprise multiple types of organisms that can directly assimilate N. They include both autotrophic (e.g., algae,

bryophytes, or macrophytes) and heterotrophic organisms (e.g., bacteria or fungi) and are energy sources for organisms higher up in the food web (Cummins and Klug 1979). PUCs range in body size over sixteen orders of magnitude, from the small prokaryotes, weighing less than 10^{-12}g, to large riparian trees reaching more than 10^{4}g. They represent highly diverse biological traits, from simple microbial cells, which can be grouped forming microbial biofilms, to complex biological tissues in higher organisms (e.g. structural polymers such as cellulose in plants). Within streams, they occupy a wide range of habitats, from benthic habitats, where interaction with streamwater is intense and obligate, to stream-bank habitats (see Table 1.1), where organisms' reliance on N streamwater is reduced and use of other sources, such as groundwater and soil water, is likely.

PUC attributes influence their activity. Body size has been long recognized as one of the main variables explaining the function of aquatic organisms (Hildrew et al. 2007). The metabolic theory of ecology quantitatively predicts how body-size dependence on metabolic rate controls ecological processes (Brown et al. 2007). Larger organisms are associated to higher element storage and have longer element residence times, in comparison to small organisms, which have faster metabolic rates (Allen et al. 2005). Additionally, PUC location within streams and the availability to access other N sources are likely to drive N fluxes from streams to PUCs.

A comprehensive study including a wide-range of PUC types varying in size and habitat can provide insights into differences of the biogeochemical role of each organism when interacting with streamwater, and ultimately about how N is processed in streams. In this study, the most representative PUCs in stream-riparian ecosystems were investigated, including the following eight PUC types (Fig. I.3; Table I.1): biofilm on stream cobbles (epilithon), filamentous algae, biofilm on detritus (including biofilm on fine and coarse allochthonous organic matter; FBOM and CBOM, respectively), macrophytes living in the water channel ("aquatic macrophytes"), macrophytes located farther from the stream channel in the banks of the stream ("stream-bank macrophytes"), and leaves and submerged roots of alder trees (*Alnus glutinosa*, the dominant riparian tree in these streams).

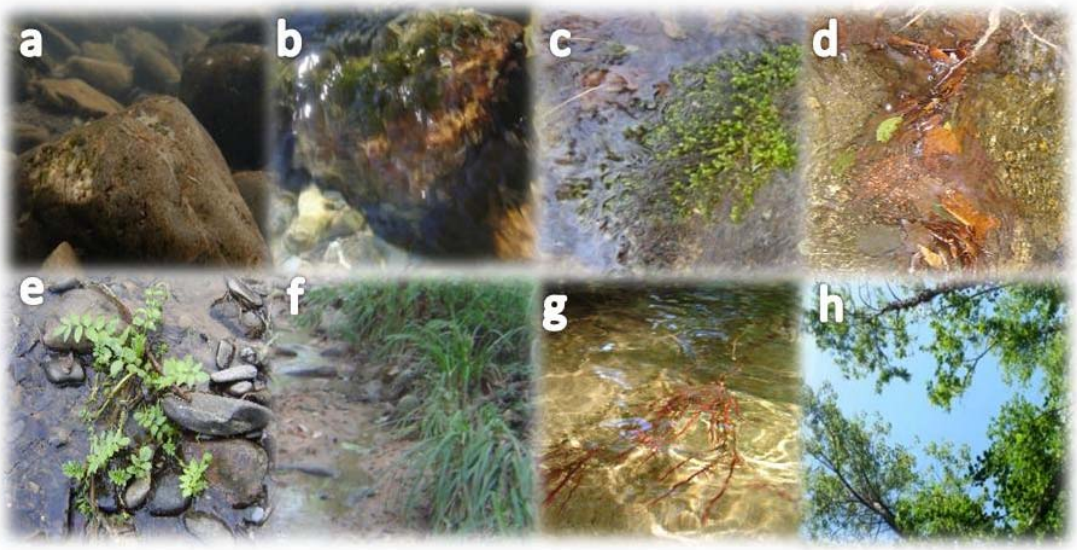


Figure I.3 Photographs of the studied PUC types of stream-riparian ecosystems. In order: a) epilithon, b) filamentous algae, c) bryophytes, d) biofilm-detritus, e) aquatic macrophytes, f) stream-bank macrophytes, g) riparian tree roots submerged in streamwater, h) leaves from riparian trees with roots submerged in the streamwater.

Chapters one and *two* examined these eight PUC types, whereas *Chapters three* and *four* focused on one specific PUC type (Table I.1). *Chapter three* focused on the epilithon compartment, and compared epilithic biofilms in early- and late- development stages. *Chapter four* studied the biofilm-detritus compartment through leaf litter decomposition, specifically leaf litter, plus the associated microbial biofilms growing on them. We used leaf litter of *Populus fremontii* and *Populus angustifolia*. These two cottonwood species differed in their recalcitrant phytochemical concentrations and are considered tree foundation species (Ellison et al. 2005) because of their broad effects on both terrestrial and aquatic ecosystem (Whitham et al. 2006, Schweitzer et al. 2008).

Table I.1 Classification of all PUCs sampled (grouped by functional type) and their habitat within the stream. For each PUC type, the chapters where it appears are denoted.

PUC type	Species included and/or description	Habitat within the stream reach	Chapters
Filamentous algae	*Cladophora* sp. *Lemanea* sp.	Submerged within stream channel	1, 2
Epilithon	Microalgae (mainly diatoms), fungi and bacteria constituting the biofilm on stream cobbles	Submerged within stream channel	1, 2, 3
Bryophyte	*Fontinalis antipyretica* *Rhynchostegium riparioides* Hepatics	Submerged within stream channel	1, 2
Biofilm-detritus	Fungi and bacteria constituting the biofilm on detritus (CBOM and FBOM) and small fractions of litter organic matter.	Submerged within stream channel	1, 2, 4
Aquatic macrophytes	*Alisma plantago-aquatica* var. *lanceolatum* *Apium nodiflorum* *Equisetum* sp. *Polygonum amphibium* *Ranunculus* sp. *Rorippa nasturtium-aquaticum* *Rumex* sp. *Typha latifolia* *Veronica anagallis-aquatica* *Veronica beccabunga* *Callitriche stagnalis*	Living into the stream channel	1, 2
Stream-bank macrophytes	*Arundo donax* *Athyrium filix-femina* *Carex pendula* *Carex remota* *Cyperus longus* *Mentha* sp. *Phalaris arundinacea*	Living into the banks of the stream	1, 2
Roots of riparian trees	*Alnus glutinosa*	Submerged within stream channel	1, 2
Leaves of riparian trees	*Alnus glutinosa*	Living into the banks of the stream	1, 2

I.3 Utility of N isotopes ratios in aquatic research

Isotopic techniques have been developed and extensively used in the last decades, conveying some of the most exciting advances in ecological and environmental research (Hobson and Wassenaar 1999, West et al. 2006). In particular, the use of stable-isotope ratios in aquatic studies has become a very strong tool to infer element sources and fluxes (Finlay and Kendall 2007, Kendall et al. 2007). Elemental stable isotopes are atoms with the same number of protons and electrons but different numbers of neutrons. N has two stable isotopes, ^{14}N, which makes up 99.635% of N abundance, and ^{15}N, the heavier form, which makes up only 0.365% in the environment (Sulzman 2007). The isotopic differences among materials are very small, so stable isotope abundances are commonly expressed using delta notation (δ; in parts per thousand [‰]; Peterson and Fry 1987), which is the ratio of the two most abundant isotopes in the sample compared to that of a standard, which is atmospheric N_2 for N (^{15}N:^{14}N=0.0036765).

Stable isotopes are typically measured by gas isotope-ratio mass spectroscopy (Sulzman 2007). The basics of this technique consist in initially converting the sample into gas (e.g. N_2) and ionizing it in an ion source to form positively charged particles. These charged molecules enter the so-called flight tube, which is bent with a magnet positioned over it. Molecules are separated according to their mass because those containing the heavier isotope bend less than those containing the lighter isotope (i.e.

the ratio of the curvature is proportional to the square root of the mass-to-charge ratio). Faraday cups measure the intensity of each beam of ions of a given mass at the end of the flight tube. The ion current flows through a resistor and generates a voltage which is used as the output from the mass spectrometer (Sulzman 2007).

In ecological research, the use of N stable isotopes generally falls into two major groups. First, *natural abundance techniques* rely on the analyses of the differences of naturally occurring stable isotopes in the environment. Second, *labelling techniques* use compounds (referred as tracer o labeled material) enriched in the heavy isotope above the natural abundance range (Robinson 2001). This ^{15}N-tracer technique follows the movement of enriched material through the system over time; sometimes the influx of ^{14}N diluting the ^{15}N labeled material is monitored and analyzed through pool dilution techniques, which have been widely applied in soil biogeochemistry to study gross nitrogen fluxes (Murphy et al. 2003).

In freshwater ecosystems, studies examining the environmental and anthropogenic influences of N loading and subsequent processing through food webs have greatly benefited from both natural abundance N isotope ratios and ^{15}N labelling techniques. In this dissertation, we took advantage of the two groups of techniques. *Chapters one, two* and *three* relied on the study of naturally occurring N stable isotopes and study the relationship between DIN in stream and the main PUCs in stream-bank ecosystems

across a nutrient concentration gradient and over time. *Chapter four* used ^{15}N enriched leaf litter material to trace N immobilization fluxes from streamwater during decomposition by applying an adaptation of the isotope dilution technique.

Natural abundance of N isotope ratios techniques

The first group of isotopic techniques, which relies on the natural abundance of stable isotopes, has been extensively used to document PUCs in aquatic ecosystems (Peipoch et al. 2012). Particularly, $\delta^{15}N$-PUC values have become extremely useful in food web studies, where it has become almost commonplace to determine $\delta^{15}N$ of PUCs as the isotopic baseline to further trace N to higher trophic levels (Peterson 1999, Finlay and Kendall 2007, Boecklen et al. 2011). To a lesser extent, but equally important, $\delta^{15}N$ has helped in the identification of anthropogenic N, because DIN often has distinct isotopic compositions depending on the source (Mayer et al. 2002, Kendall et al. 2007, Lefebvre et al. 2007), which can subsequently be transferred to the biota (Vander Zanden et al. 2005, Kohzu et al. 2008). For example, DIN derived from sewage or agricultural fields are commonly enriched in $\delta^{15}N$, in contrast to synthetic fertilizers and atmospheric deposition which have $\delta^{15}N$ values near or below zero (Kendall et al. 2007, Holtgrieve et al. 2011).

Previous natural abundance N studies have documented a high variability of naturally occurring $\delta^{15}N$ in PUCs (Gu 2009, Peipoch et al.

2012), but the underpinning factors driving $\delta^{15}N$ variability are still unclear. The isotopic variability of N is expected to be dependent on the isotopic values of streamwater DIN, because PUCs rely on DIN as their N source (Evans 2001, Kohzu et al. 2008). However, studies examining patterns of variably in $\delta^{15}N$ values in relation to the variability of $\delta^{15}N$-DIN species, especially for NH_4^+, are rather scarce in stream ecosystems. Though PUCs comprise multiple types of organisms with different physiological processes and metabolic rates, most research in streams has been restricted to single compartments (e.g. macrophytes, Kohzu et al. 2008 or algae, Kaushal et al. 2006) and comparative studies among PUC types are lacking. Understanding relationships between isotopic values of PUCs and DIN, and the factors driving them over time and across strong human gradients has ecological and environmental relevance. First, isotopic relationships provide insights into basic N processes occurring in stream and the role of each PUC type. Second, isotopic ratios can give information about the environmental and anthropogenic influences on N source. Third, knowledge of the factors affecting $\delta^{15}N$ variability can improve the accuracy of techniques applying $\delta^{15}N$ natural abundance.

^{15}N labelling techniques

Labelling techniques, which rely on the use of isotopically enriched material above ambient values, has been extremely useful to quantify simultaneously occurring N processes in fluvial ecosystems. The use of

$^{15}NH_4^+$ or $^{15}NO_3^-$ tracer isotope addition techniques has allowed quantifying different N spiraling processes simultaneously occurring in a stream at ambient nutrient concentrations. These studies have quantitatively determined indices of N spiraling processes (e.g. spiraling length or N uptake rates), which are comparable within and among streams, greatly improving our understanding of the importance of N retention in fluvial ecosystems (Stream Solute Workshop 1990, Mulholland and Webster 2010).

^{15}N-labeled organic material has been extensively used to investigate N processes during decomposition in terrestrial ecosystems, especially in agricultural fields (Voroney et al. 1989, Haynes 1997) but also forests (e.g. Holub and Lajtha 2004, Bimüller et al. 2013). Few studies, however, have traced DIN exchange by applying ^{15}N-labeled material in fluvial ecosystems (but see Cheever et al. 2013). The production of ^{15}N leaf litter is time consuming and expensive, but the use of ^{15}N-enriched material applying isotopic pool dilution can complement information provided by ^{15}N-DIN additions. By focusing on a more detailed scale, not at stream-reach but at compartment level, researchers can get a lot of high resolution information. Because all input fluxes are characterized by lower ^{14}N to ^{15}N ratios, all inputs entering into the biofilm-leaf compartment are quantified. Additionally, isotopic changes can be tracked over a longer period of time compared to relatively short ^{15}N additions.

I.4 Study sites

La Tordera catchment (NE Iberian Peninsula)

Research for this dissertation was primarily carried out in La Tordera catchment (868.5 km^2), which is located approximately 50 km northeast of Barcelona (NE Iberian Peninsula; Fig. I.4). This catchment has been the focus of multiple studies, including social (e.g. Caille et al. 2007), ecological, and biogeochemical perspectives (e.g. von Schiller et al. 2008, Vazquez et al. 2013, Ribot et al. 2013), providing a wealth of background information.

La Tordera catchment covers a remarkable altitudinal gradient from the sea level up to 1700 m within a distance of 35 km. Most of the catchment (77%) is covered by natural vegetation (mostly forest), but agricultural (16%, mostly on the northeastern plains) and urban and industrial uses (7%, mostly along the main valley) are also present, resulting in a heterogeneous land use mosaic with a large variability in the amount and apportionment of nitrogen emissions across the catchment (Caille et al. 2012). We capitalized on these characteristics to study the influence of human influence on the stream biogeochemical interactions within the same fluvial network.

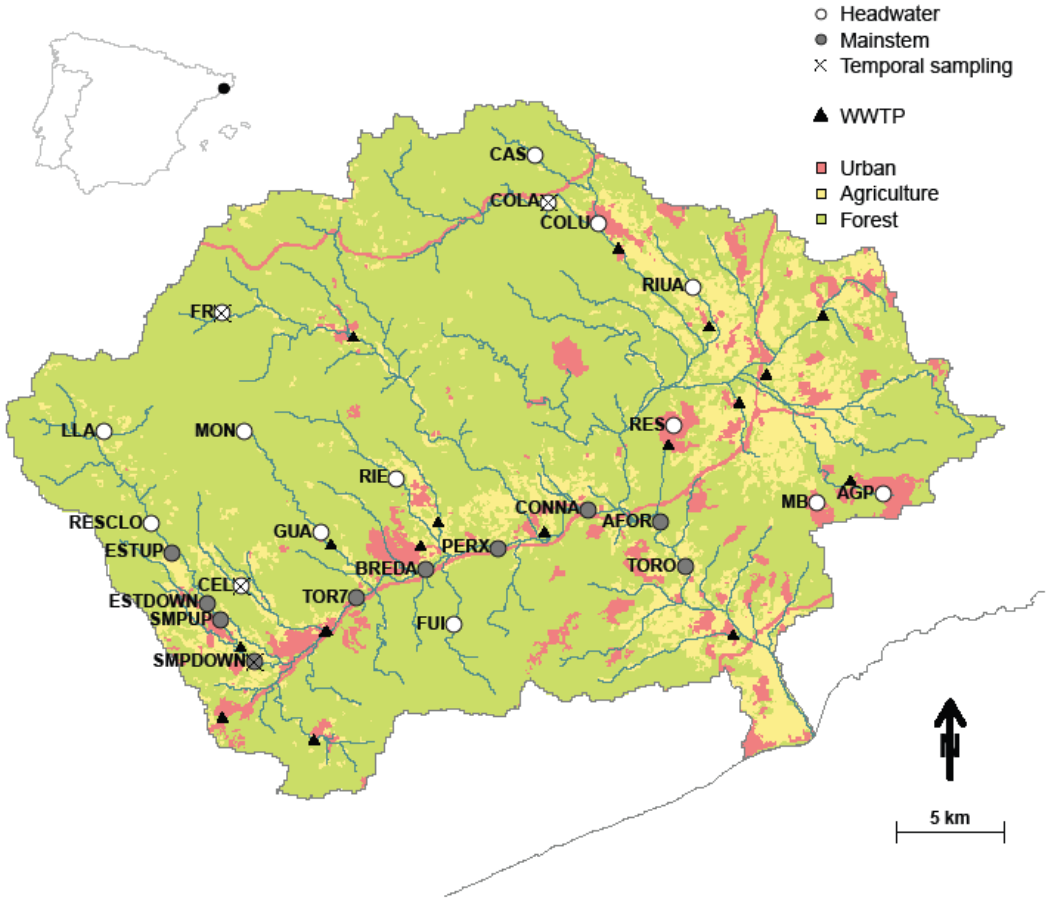


Figure I.4 La Tordera catchment in the Iberian Peninsula and associated study streams. Samples sites included in the annual monitoring (*Chapters 2 and 3*) are indicated with a cross. The type of reach (headwaters or mainstem) and the location of WWTPs are highlighted. Land uses are grouped into urban (red), agricultural (orange) and forested (green) classes.

In *Chapter one,* we selected 25 sampling sites along La Tordera (Table I.2), 15 of which were located at the headwaters and were influenced by a broad variety of human impacts. The other 10 sampling sites were located along the mainstem of La Tordera, and were largely influenced by emissions from urban wastewater treatment plants (WWTP). From these 25 sampling sites, we selected four stream reaches differing in their dominant

adjacent land use types and their concentration and $\delta^{15}N$ of NH_4^+ and NO_3^- (*Chapters 2* and *3*; Table I.2). Specifically, we selected *Font del Regàs* (FR) reach as a forested stream (FOR; low-nutrient stream), *Santa Coloma de Farners* (COLA) which is influenced by irrigated horticultural production (HOR; low/mid-nutrient stream), *Sant Celoni* (CEL) which is surrounded by non-irrigated agriculture (AGR; high/mid-nutrient stream), and *Santa Maria de Palautordera* (SMPDOWN) as and urban stream (URB; high-nutrient stream) which receives the effluent of a municipal wastewater treatment plant (WWTP).

Table I.2 Physiographic characteristics of the study sites and land use of the catchments drained by them. Headwater streams are listed in order of decreasing forested area and mainstem reaches are listed in order of decreasing subcatchment area. Land use data were from the year 2002 and obtained from the Department of Environment and Housing of Catalonia (http://www.gencat.net). Oak Creek data were obtained from LeRoy et al. (2006).

Streams	UTM-E (m)	UTM-N (m)	Altitude (m a.s.l.)	Area (km^2)	Forest. (%)	Agric. (%)	Urban (%)
UTM Zone: 31T (North)							
La Tordera catchment:							
Headwaters							
FR*	454275.6	4630618	528	12.7	99.7	0.2	0.0
MON	455296	4625149	1130	3.2	99.5	0.0	0.0
CAS	468759.86	4637853	239	8.6	99.5	0.5	0.0
FUI	464965.74	4616290	131	13.2	98.5	1.4	0.1
LLA	448792.75	4625172	498	16.3	97.5	2.2	0.0
RESCLO	450978.47	4620945	307	56.2	96.4	3.4	0.0
GUA	458804.58	4620501	168	13.4	96.4	2.2	0.2
RIE	462346.52	4623025	207	15.4	96.1	3.1	0.6
COLA*	469369.22	4635715	163	44.8	93.8	2.6	3.5
COLU	471697	4634752	129	18.9	93.2	3.2	3.2
CEL*	455165.13	4618071	240	9.1	90.9	8.3	0.0
RIUA	476039.5	4631817	89	10.3	61.3	31.3	3.9
RES	475177.35	4625434	76	1.2	22.0	10.1	67.8
AGP	484839.22	4622299	137	1.3	12.2	0.0	86.0
MB	481793.72	4621875	130	0.9	7.3	0.0	90.7
Mainstem							
ESTUP	451955.5	4619602	266	60.0	96.1	3.7	0.0
ESTDOWN	453547.37	4617271	208	67.9	93.4	5.7	0.4
SMPUP	454197.87	4616478	189	68.6	92.9	6.0	0.6
SMPDOWN*	455763.64	4614587	154	80.7	88.3	9.1	2.0
TOR7	460504.5	4617538	104	168.0	82.9	10.3	5.6
BREDA	463696.5	4618816	82	218.4	83.6	9.0	6.1
PERX	467046	4619804	66	281.8	83.8	9.0	5.9
CONNA	471214	4621586	48	424.7	85.5	8.2	5.2
AFOR	474557	4621016	41	775.3	79.4	12.5	6.6
TORO	475678.5	4618944	29	784.3	79.2	12.6	6.7
UTM Zone: 12S (North)							
Oak Creek	434629	3876974	1600	77 450[a]	-	-	-

* Study sites (FR, COLA, CEL, SMPDOWN) monitored during the annual sampling. In chapter two these stations are coded as FOR, HOR, AGR and URB, and at chapter three as *low-nutrient*, *low/mid-nutrient*, *high/mid-nutrient*, and *high-nutrient streams*, respectively. [a] Area corresponds to the total area drained by Oak Creek.

Oak Creek (Arizona, USA)

Chapter four was conducted upper Oak Creek (Fig. I5), a headwater stream situated on the southern edge of the Colorado Plateau (Arizona, USA; see Table I.2). This catchment is characterized by steep topography and sandstone/limestone bedrock (LeRoy et al. 2006). It is extensively covered by Ponderosa pine (*Pinus ponderosa*) with minor human activities in the upstream reaches. Riparian vegetation, predominately deciduous, includes Fremont cottonwood (*P. fremontii*), narrowleaf cottonwood (*P. angustifolia*), Arizona alder (*Alnus oblongifolia* Torr.), Arizona sycamore (*Platanus wrightii* S. Wats.), coyote willow (*Salix exigua* Nutt.), and Goodding's willow (*Salix gooddingii* Ball) (LeRoy et al. 2006). Thus, Oak Creek is a relatively natural stream with low nutrient concentrations, which made it a suitable stream in which to conduct a ^{15}N labeled material study.

Figure I.5 Studied reach at Oak Creek in Arizona, USA.

DISSERTATION OBJECTIVES

The overarching goal of this work was to explore relationships between N streamwater and the most representative PUC types in stream ecosystems, by using stable N isotopes, to elucidate factors controlling them. In particular, environmental factors driving these biogeochemical relationships along a strong anthropogenic gradient were explored and differences among and within PUC types were compared.

These objectives are addressed in four independent chapters focusing on different aspects of N biogeochemical interactions between DIN and PUCs, which are explored using isotopic techniques. The first two chapters comprise an inclusive approach allowing spatial (*chapter one*) and temporal (*chapter two*) comparisons among the most representative PUC types in stream ecosystems. The other two chapters focused on a particular PUC type, either epilithon (*chapter three*) or detritus (*chapter four*). Specific objectives for each chapter are as follows.

Chapter one. This chapter examined the spatial variability of the $\delta^{15}N$ natural abundance of PUC types, relating this variability to $\delta^{15}N$ values of DIN species (NH_4^+ and NO_3^-) and to the stream nutrient environment in which they grew (DIN and phosphate concentrations). In particular, two research questions were addressed: (1) whether $\delta^{15}N$-PUC was better explained by PUC type or by location in the watershed, and, (2), which

factors control $\delta^{15}N$ of PUCs across a strong gradient of nutrient concentration within the fluvial network.

Chapter two. This chapter assessed annual temporal variability of $\delta^{15}N$ of DIN species and PUC types in stream ecosystems. Specifically, we evaluated how temporal variability in $\delta^{15}N$ of DIN and of PUCs differed among streams with contrasting human impacts and PUC types.

Chapter three. This chapter examined the $\delta^{15}N$ variability of epilithic biofilm in different stages of development under contrasting stream nutrient concentrations. To test the effect of biofilm growth on $\delta^{15}N$ variability, we used two approaches. First, $\delta^{15}N$ variability was evaluated for early- and late- stage biofilm during one year in four streams reaches that differed in their nutrient concentrations. Second, $\delta^{15}N$ was examined during biofilm growth for one month under low- and high-nutrient concentrations.

Chapter four. This chapter examined the biogeochemical interaction between the biofilm-leaf litter system and streamwater during litter decomposition. In particular, we used isotopically double-labeled (^{13}C and ^{15}N) leaves to quantify the relative importance of N and C fluxes, from streamwater to biofilms on leaf litter. We also examined how these fluxes vary between two contrasting leaf litter types (*P. fremontii* and *P. angustifolia*) that differed in their concentration of recalcitrant compounds.

Overall, the understanding of N isotopic interactions between DIN and PUCs provides insights into in-stream N processes, and comprehension of the environmental and anthropogenic factors driving these relationships. This information has implications for the development of restoration and management strategies to mitigate the effects of N in fluvial systems.

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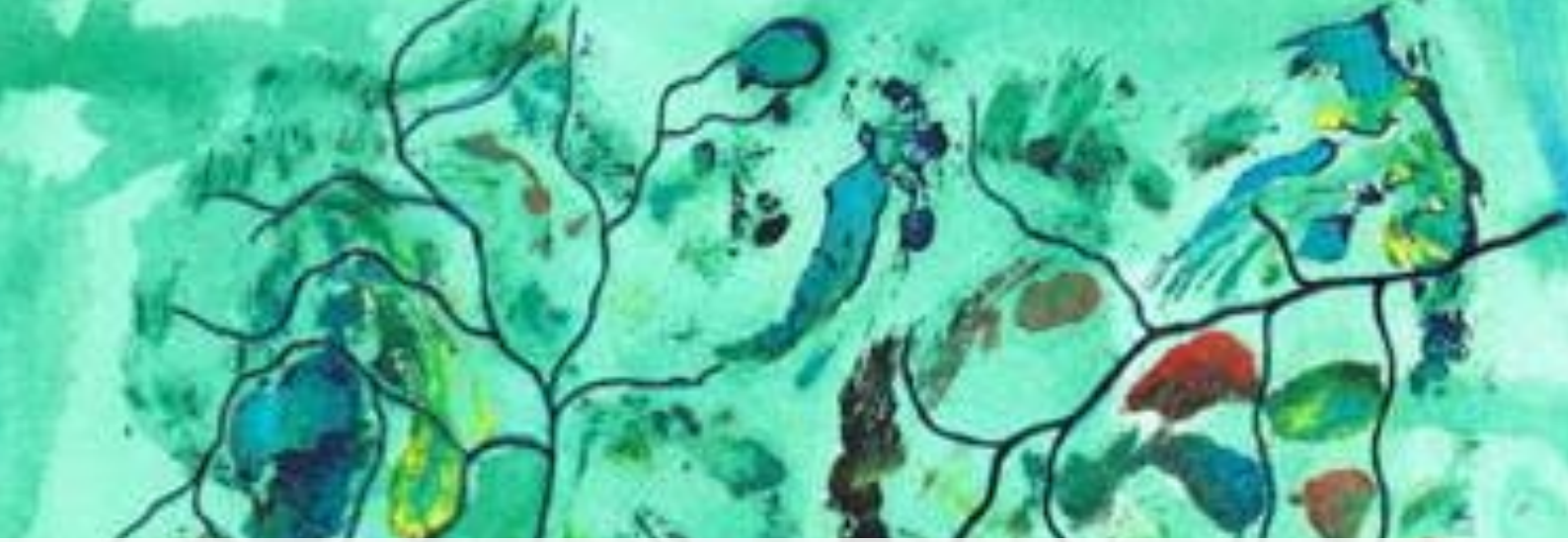

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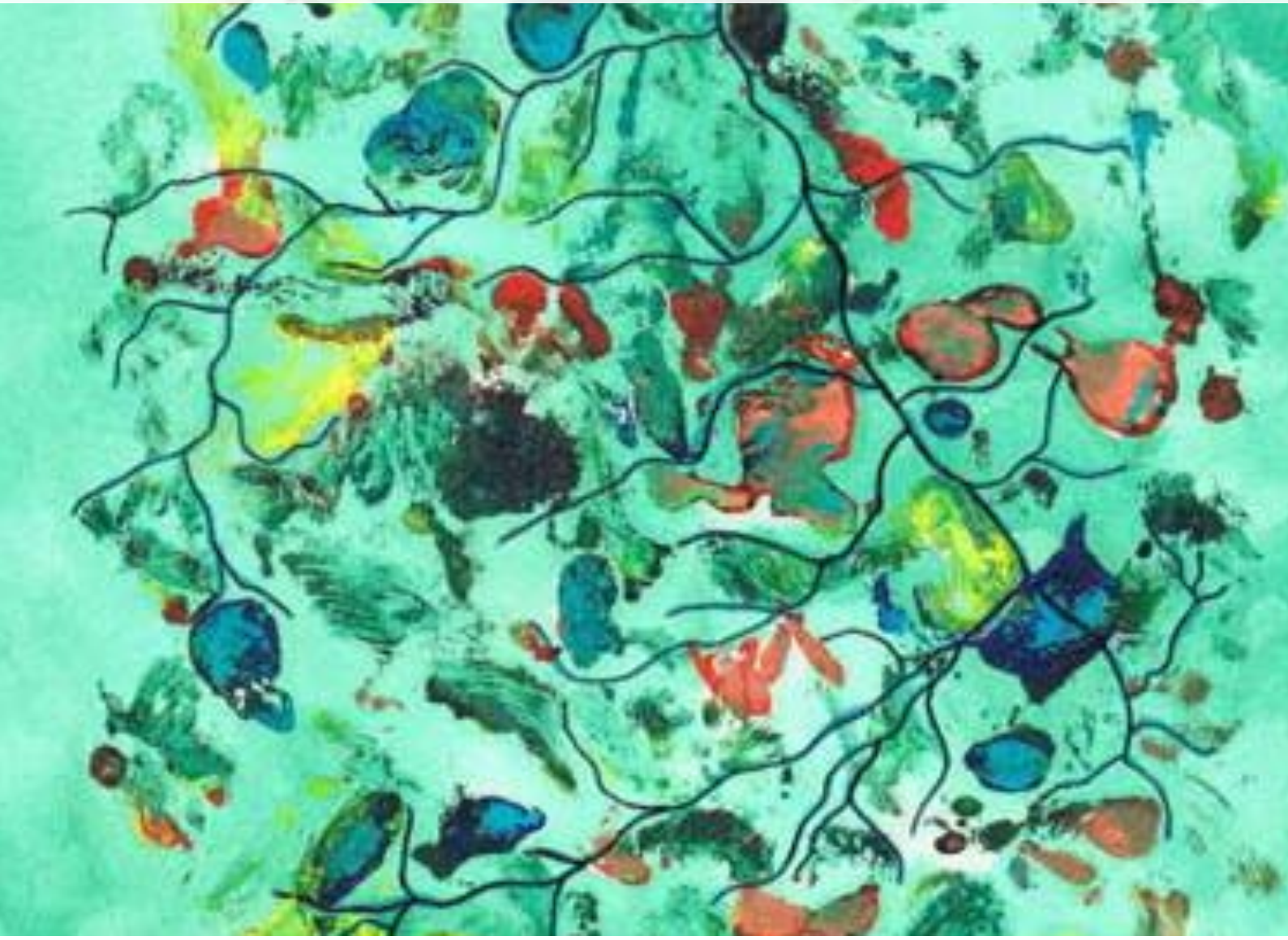
Nitrogen stable isotopes in primary uptake compartments across streams differing in nutrient availability



Supporting information is available at the supporting information of this dissertation (Appendix A). It includes information on the characteristics of the stream reaches, isotopic relationships between PUCs and DIN species, mixing model analyses and multiple linear regressions; Figure SA.1 and Tables SA.1-SA.7. This information is also available free of charge via the Internet at http://pubs.acs.org.

ABSTRACT

High variability in natural abundance of nitrogen stable isotopes ($\delta^{15}N$) has been reported for primary uptake compartments (PUCs; e.g. epilithon, filamentous algae, bryophytes, macrophytes) in human-impacted aquatic ecosystems but the origin of this variability is not well understood yet. We examined how $\delta^{15}N$ of different PUC types relate to $\delta^{15}N$ of dissolved inorganic nitrogen (DIN) species (nitrate and ammonium) and to the stream nutrient concentrations in which they grow. We selected 25 reaches located across the fluvial network of La Tordera catchment (NE Spain, 868.5 km^2), encompassing a gradient of human pressures from headwaters to the river valley. $\delta^{15}N$-PUC variability was mostly explained by location within the fluvial network and was strongly related to the $\delta^{15}N$ of DIN species, especially of ammonium. Models were stronger for PUCs growing within the stream channel, and thus using stream water as their main source of nutrients. Regression models including nutrient concentrations improved the prediction power for $\delta^{15}N$-PUCs, suggesting that nutrient concentrations and stoichiometry cannot be ignored in explaining natural abundance of nitrogen isotopes in PUCs. These results provide insights into what controls variability in $\delta^{15}N$ of PUCs within a stream network, with implications for the application of stable isotopes as an ecological tool.

1.1 INTRODUCTION

The natural abundance of nitrogen (N) stable isotopes (expressed as $\delta^{15}N$, in ‰) has been extensively used in freshwater ecosystem research (Finlay and Kendall, 2007), in particular for tracing the transfer of N from basal resources to higher trophic levels in food web studies (Peterson 1999, Boecklen et al. 2011). To a lower extent, but equally important, $\delta^{15}N$ has assisted in the identification of anthropogenic N sources, because the species of dissolved inorganic nitrogen (DIN) often differ in their $\delta^{15}N$ depending on their origin (Mayer et al. 2002, Kendall et al. 2007, Peipoch et al. 2012). For example, synthetic fertilizers and atmospheric deposition have N isotopic values (i.e. $\delta^{15}N$) close to zero or lower (Kendall et al. 2007, Holtgrieve et al. 2011). In contrast, N compounds derived from septic waste or manure are commonly enriched in ^{15}N, and thus tend to increase the isotopic values of DIN in receiving streams (Kendall et al. 2007, Ribot et al. 2012).

Changes in isotopic values of DIN from natural and anthropogenic sources of nitrogen entering aquatic ecosystems may be reflected in $\delta^{15}N$ values of autotrophic (e.g. algae, bryophytes or macrophytes) and heterotrophic (e.g. bacteria or fungi) organisms that can directly assimilate dissolved nutrients from the water column, hereafter referred to as primary uptake compartments (PUCs). The fact that PUCs can integrate changes on isotopic values of DIN over time, together with the fact that their isotopic analysis is less time-consuming and easily conducted than $\delta^{15}N$-DIN analyses, provide support for the

applicability of $\delta^{15}N$ of PUCs as an ecological tool. In addition, PUCs are the basal resources for food webs and this isotopic variability can be transferred to higher trophic levels, thus having implications for the entire stream ecosystem.

The $\delta^{15}N$ of PUCs is not only influenced by the isotopic values of the stream water DIN species (nitrate and ammonium; Evans 2001, Kohzu et al. 2008) physiological processes during acquisition and dissimilation of N can also affect the $\delta^{15}N$ value of each particular PUC through isotopic fractionation processes (i.e., preferential use of the lighter isotope; Evans 2001; Dijkstra et al. 2008). In addition, the degree of isotopic fractionation may change depending on stream DIN concentration and elemental stoichiometry relative to N demand by PUCs (e.g., Dijkstra et al. 2008, Wanek and Zotz 2011). Consequently, $\delta^{15}N$ is potentially variable both among PUC types and among stream locations (McCarthy et al. 1977, Cloern et al. 2002, Jones et al. 2004, Peipoch et al. 2012). In particular, within a catchment, $\delta^{15}N$-PUC variability among stream locations can be amplified by the diverse DIN sources from human activities with distinct $\delta^{15}N$ values.

Despite the widespread applicability of the naturally occurring $\delta^{15}N$ values, studies examining patterns of variability in $\delta^{15}N$ natural abundance of PUCs across strong environmental gradients of nutrient concentrations and relating these to the variability of $\delta^{15}N$-DIN values are rather scarce (but see McClelland et al. 1998, Kohzu et al. 2008). Moreover, most of the available data on $\delta^{15}N$-

DIN values are for nitrate, and much less data are available for ammonium (Peipoch et al. 2012), even though ammonium is commonly believed to be more easily used by PUCs than nitrate (Dortch 1990, Barko et al. 1991). Finally, despite the variability observed among PUC types (Peipoch et al. 2012), most research in streams has been restricted to single compartments (e.g. particulate organic matter [Kendall et al. 2001], macrophytes [Kohzu et al. 2008], or algae [Kaushal et al. 2006]) and comparative studies among PUCs are lacking.

This study aims to fill some of these gaps by examining the spatial variability of ^{15}N natural abundance of several stream PUC types (i.e., detritus, epilithic biofilm, filamentous algae, bryophytes, macrophytes and alder roots and leaves) and by relating this variability with the $\delta^{15}N$ values of DIN species (ammonium and nitrate) and with stream nutrient environment (DIN and phosphate). To address this goal, we selected 25 stream locations from the headwaters to the mainstem river valley within La Tordera catchment (Catalonia, NE Spain) that are subjected to different land uses and human pressures, thus covering a wide range of stream nutrient concentrations. We had two specific objectives. First, we asked whether $\delta^{15}N$-PUC was better explained by PUC type or by location. Differences in $\delta^{15}N$ among PUC types would suggest that specific PUC characteristics, such as physiological N processes or habitats preferences within the stream reach, are the main constraint on the acquisition of $\delta^{15}N$ values. In contrast, major differences in

δ^{15}N-PUC among locations would indicate the predominance of environmental controls over PUC characteristics. Second, we assessed the factors controlling δ^{15}N of PUCs. We examined how δ^{15}N values of the main potential sources (ammonium and nitrate) of N for PUCs were related to δ^{15}N values of PUCs, considering the proportion of N derived from each source. Finally, we statistically modeled the δ^{15}N of PUCs as a function of the nutrient environment in which they grow.

1.2 MATERIAL AND METHODS

Study site

This study was carried out in La Tordera catchment (868.5 km^2), which is located approximately 50 km North-East of Barcelona (NE Iberian Peninsula) (Fig. SA.1). The catchment is dominated by siliceous lithology, mostly granodiorite and some schists. It covers a remarkable altitudinal gradient from the sea level up to 1700 m within a distance of 35 km. Although most of the catchment (77%) is covered by natural vegetation (mostly forest), agricultural (16%, mostly on the north-eastern plains) and urban and industrial uses (7%, mostly along the main valley) are also present, resulting in a heterogeneous land use mosaic that translates into a large variability in the amount and apportionment of nitrogen emissions across the catchment (Caille et al. 2012). Within this catchment, we selected 25 sampling sites along the stream network (Table SA.1), 15 of which were located at the headwaters and were influenced

by a broad variety of human impacts, spanning a wide range of stream nutrient conditions (von Schiller et al. 2008, Caille et al. 2012). The other 10 sampling sites were located along the mainstem of La Tordera river, and were largely influenced by emissions from urban wastewater treatment plants (WWTP). During the sampling period, discharge was low-medium at headwaters (0.3 to 211.0 L/s). Discharge at the mainstem sites (41 to 580 L/s) did not show any clear longitudinal pattern along the river, likely because of the intensive water use in the watershed and the presence of losing reaches.

Field procedures

Field sampling was carried out in May-June 2009 (early summer). At each stream site, we collected water samples for nutrient concentration (40 mL, three replicates per station) and stable-isotopes analyses (one replicate of 0.4L to 3L for NH_4^+ and one replicate of 0.5 L for NO_3^-). Samples for $\delta^{15}N\text{-}NH_4^+$ were processed immediately (see below), whereas samples for nutrient concentrations and $\delta^{15}N\text{-}NO_3^-$ were frozen and stored at -20ºC until laboratory analysis. Stream discharge was estimated using a mass balance approach by recording changes in conductivity over time at a site located 10 to 20 meters downstream of the slug addition point where a conservative tracer (i.e., NaCl solution) was added into the stream (Gordon et al. 2004). At each site, when available, we collected samples of the following PUC types for $\delta^{15}N$ analysis: biofilm on stream cobbles (epilithon), bryophytes, filamentous algae, detritus (i.e. fine and coarse allocthonous organic matter; FBOM and CBOM,

respectively), leaves and roots of alder trees (*Alnus glutinosa*, the dominant riparian tree in these streams) growing at the stream bank, and macrophytes (a total of 18 species which were present at the sampling sites; Table SA.3). For the analysis presented here, the species of macrophytes collected were classified as either "aquatic macrophyte" (i.e. species living in the water channel with potentially high interaction with the stream water), or "stream-bank macrophyte" (i.e. species located farther from the stream channel into the banks of the stream; with potentially low interaction with the stream water).

At each station, three replicates of epilithon, biofilm on CBOM and FBOM were obtained. Epilithon from the light-exposed side of cobbles was sampled by scraping randomly selected cobbles with a soft metal brush and subsequently filtering each sample onto ashed 0.7 µm pore size FVF glass fiber filters (Albet, Barcelona, Spain). Biofilm on CBOM was sampled by collecting leaves accumulated on the stream channel and washing them in a bucket with stream water. A sample of the suspended fraction was then taken from the water with a syringe and collected on ashed 0.7 µm pore size glass fiber filters (Albet, Barcelona, Spain). FBOM samples were collected by placing a plastic corer into the surface stream sediment, which was manually agitated. An aliquot of the suspended material was obtained with a plastic syringe and, then, filtered onto ashed 0.7 µm pore size glass fiber filters (Albet, Barcelona, Spain). At each station, composite samples of bryophytes, macrophytes, and

filamentous algae, as well as root tips of alder submerged into the water, and leaves from alder trees were harvested when present and stored in a cooler until arrival to the laboratory.

Laboratory analysis

Stream water samples were analyzed for soluble reactive phosphorous (SRP) by the molybdenum blue colorimetric method (Murphy and Riley, 1962), for ammonium concentration by the salicylate method (Reardon et al. 1966), and for nitrate by ionic chromatography (761 Compact IC1.1, Metrohm, Switzerland). Total nitrogen (TN) was analyzed in a Shimadzu TOC-VCS with a coupled TN analyzer unit and dissolved organic nitrogen (DON) was calculated as the difference between TN and DIN concentrations. The $\delta^{15}N$ values of stream water NH_4^+ and NO_3^- were determined using an adaptation of the ammonia diffusion method (Holmes et al. 1998, Sigman et al. 1997) and the details of the methodology used can be found in the literature (von Schiller et al. 2009, Ribot et al. 2012).

PUCs samples were oven-dried at 60ºC and plant tissues were ground in a MM 200 mixer mill (Retsch, Germany). Encapsulated samples for the analysis of $\delta^{15}N$-NH_4^+, $\delta^{15}N$-NO_3^- and $\delta^{15}N$ of PUCs were sent to the University of California Stable Isotope Facility (Davis, California, USA) and analyzed by a continuous flow PDZ Europa 20-20 isotope ratio mass spectrometer after sample combustion in PDZ Europa ANCA-GSL on-line elemental analyzer (Sercon Ltd.,

Cheshire, UK). The natural abundance of N stable isotopes was expressed in standard notation ($\delta^{15}N$ in ‰) relative to a standard (i.e. atmospheric N_2), where $\delta^{15}N = 1000^* [(R_{sample}/R_{standard})-1]$, and R is the $^{15}N/^{14}N$ molar ratio. The analytical precision on five repeated measures of alder leaf standard was ±0.15 ‰.

Statistical analysis

Concentrations of NH_4^+, NO_3^-, SRP, DON and TN, and DIN to SRP ratio were log-transformed to meet requirements for regressions analyses. The data for the rest of variables were not transformed. Correlations among stream nutrient concentrations (NH_4^+, NO_3^-, SRP, DON and TN) were examined using Pearson correlation analysis. Relationships between $\delta^{15}N$-NH_4^+ and $\delta^{15}N$-NO_3^- as a function of nutrient concentrations were also evaluated using Pearson correlation analysis. To estimate the relative importance of location vs. PUC type in explaining the $\delta^{15}N$ of individual PUCs, a two-way factorial analysis of variance (ANOVA) was conducted using “PUC types” (8 levels: detritus, epilithon, algae, bryophyte, aquatic macrophytes, stream-bank macrophytes, alder root, and alder leaf) and “type of stream location” (2 levels: headwater and mainstem) as factors. To quantify the relative importance of each explanatory variable on $\delta^{15}N$-PUC variability, we used the LMG method in the R package ‘relaimpo’ (Grömping 2006). This method provides a decomposition of the model r^2 whereby the contribution of each independent variable is averaged over orderings among regressors.

The relationships between the $\delta^{15}N$ of each PUC type and the $\delta^{15}N$ of the DIN species were analyzed using simple linear regression. Regression slopes were compared among PUC types by testing for the significance of the interaction between PUC type and either $\delta^{15}N\text{-}NH_4^+$ and $\delta^{15}N\text{-}NO_3^-$ in linear models (ANCOVA). To estimate the proportion of N in PUC derived from NH_4^+, six different models were fitted by maximum likelihood methods for each PUC. These models, from lower to higher complexity, assumed the following: model 1) no fractionation; m.2) single fractionation term for NH_4^+ and NO_3^-, m.3) separate fractionation terms for NH_4^+ and NO_3^-, m.4) fractionation linearly dependent on concentrations, m.5) fractionation dependent on the logarithm of the concentration, m.6) fractionation dependent on the concentration with a Monod saturating function (Table SA.5). Candidate models were identified using the Akaike Information Criterion corrected for small sample size (AICc; Burnham and Anderson 2002) as those differing by less than two AICc units from the best model.

Finally, to explore the contribution of the local nutrient environment (as measured by the concentrations of NH_4^+, NO_3^-, TN, DON, SRP and DIN to SRP ratio, and their interactions) in addition to the $\delta^{15}N$ of DIN species as predictors of $\delta^{15}N$ of PUCs, we built all possible linear models involving these variables and their pairwise interactions for each of the PUC types. We controlled for model complexity by limiting models to three or fewer predictors, and by including interaction terms only if the variables involved were also included as

main terms. Candidate models were identified using the AIC as those differing by less than two AICc units from the best model. We automated this process using the R package 'glmulti' (Calcagno and de Mazancourt 2010) whereas the relative importance of each variable in the best single model was estimated by r^2 partition using the R package 'relaimpo' (Grömping 2006). All data analyses were carried out using R, version 2.15.1 (R development Core Team 2012).

1.3 RESULTS

Stream nutrient environment

Consistent with our expectations, selected stream locations showed a wide range of nutrient concentrations, especially for NO_3^- (Table SA.1). The relative contribution of NO_3^- to DIN concentration (41 to 98 %) and the molar DIN:SRP ratio (7 to 292) also varied widely among all stream locations. Nutrient variability responded to the typology of streams, with lower values in headwater streams (with the exception of sites draining catchments with urban sprawl) and high values in mainstem sites, where urban impacts were stronger. The range of concentrations of NH_4^+, SRP, DON and TN were broader in mainstem locations than in headwaters locations, but not for NO_3^- (Table SA.1). There was a strong positive covariation among concentrations of stream nutrients (Table SA.2). In particular, concentration of SRP was positively correlated with concentrations of NH_4^+ ($r = 0.71$, $p < 0.001$), NO_3^- ($r = 0.45$, $p <$

0.05), DON (r = 0.59, p < 0.01), TN (r = 0.72, p < 0.001). In contrast, concentration of NH_4^+ was not correlated to concentrations of NO_3^-.

Values of $\delta^{15}N\text{-}NH_4^+$ presented a broader range and, on average, were higher (-3.3 to 36.6‰, median: 9.5‰) than $\delta^{15}N\text{-}NO_3^-$ values (1.9‰ to 15.9‰, median: 6.7‰; Table SA.1). $\delta^{15}N$ values for the two DIN species were not significantly correlated (p > 0.05). The range of $\delta^{15}N\text{-}NO_3^-$ was similar for headwater and mainstem locations, whereas the range of $\delta^{15}N\text{-}NH_4^+$ was broader in mainstem than in headwater locations. $\delta^{15}N\text{-}NH_4^+$ was positively related to NH_4^+, NO_3^- and SRP concentrations, whereas $\delta^{15}N\text{-}NO_3^-$ was positively related only to NO_3^- and SRP concentrations (Table 1.1).

Table 1.1 Coefficients (r) of Pearson correlations analysis between $\delta^{15}N\text{-}NH_4^+$ or $\delta^{15}N\text{-}NO_3^-$ and the concentrations of NH_4^+, NO_3^- and SRP.

	NH_4^+	NO_3^-	SRP
$\delta^{15}N\text{-}NH_4^+$	0.69	0.60	0.68
$\delta^{15}N\text{-}NO_3^-$	n.s.	0.49	0.50

n.s. stands for not significant correlations (p > 0.05)

Variability in $\delta^{15}N$ values among PUC types and across the fluvial network

$\delta^{15}N$ values for PUCs showed a wide range of variation, from -4.2 to 26.9‰. Of the total variance in $\delta^{15}N$ of PUCs, 54% was explained by “PUC location” and “PUC type” factors together (two-way ANOVA, p < 0.001), but location of PUCs within the fluvial network (i.e. headwaters vs. mainstem) accounted for 68% of

the total explained variance. $\delta^{15}N$ of PUCs followed similar patterns as those observed for $\delta^{15}N\text{-}NH_4^+$, with the highest $\delta^{15}N$ values and variability in mainstem locations (Fig. 1.1). Among PUC types, the range of variation was highest for macrophytes (-3.6 to 26.9‰), while alder leaves and roots showed the lowest variability (-2.7 to 3.3‰, and -1.8 to 5.2‰, respectively). Ranges of $\delta^{15}N$ were intermediate and similar for filamentous algae, epilithon, bryophytes and detritus (Fig. 1.1).

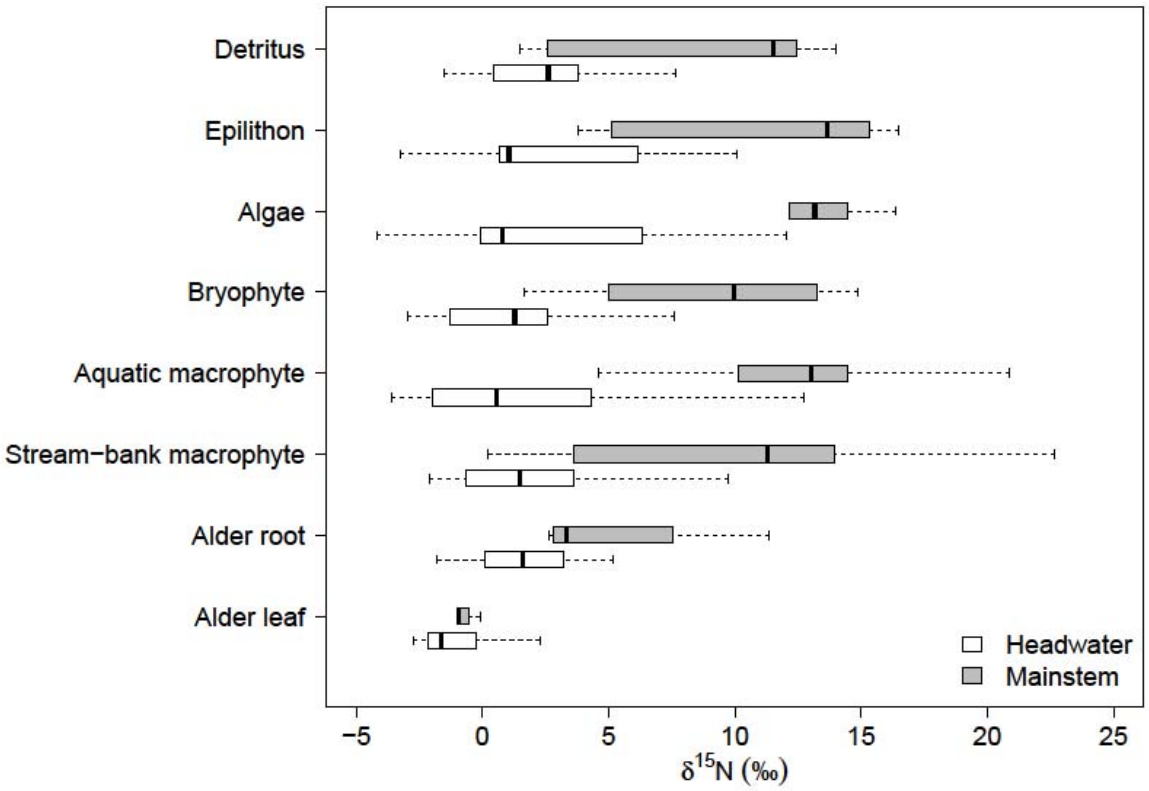


Figure 1.1 Box plots for $\delta^{15}N$ of PUCs (‰) grouped by functional type (detritus, n = 47; epilithon, n = 19; algae, n = 20; bryophytes, n = 26; aquatic macrophytes, n = 77; stream-bank macrophytes, n = 44; alder roots, n = 18; and alder leaves, n = 18) and by stream location (headwater, white boxes, n = 137; and mainstem, grey boxes, n = 132). Extreme values (values outside 1.5 times the interquartile range) are not shown.

Factors controlling the natural abundance of N stable isotopes of PUCs

δ^{15}N-PUC values were positively related to δ^{15}N of both DIN species (simple linear regression; $p < 0.01$), except for bryophytes, and for alder leaves and roots (Table SA.3). Because neither δ^{15}N-NH_4^+ or δ^{15}N-NO_3^- were related to δ^{15}N of alder leaves, this compartment was excluded from further analyses. Variability in the δ^{15}N of PUCs was always better explained by δ^{15}N-NH_4^+ (r^2 between 0.45 and 0.70) than by δ^{15}N-NO_3^- (r^2 between 0.13 and 0.28; Fig. 1.2). Values of δ^{15}N of PUCs tended to be lower than those of NH_4^+, especially when δ^{15}N-NH_4^+ was high, but tended to be closer to δ^{15}N-NO_3^- values (i.e. closer to the 1:1 line; Fig. 1.2). The slopes of the relationships between δ^{15}N of PUCs and either δ^{15}N-NH_4^+ (from to 0.33 to 0.52) or δ^{15}N-NO_3^- (from 0.54 to 0.88, Table SA.3) did not differ significantly among PUCs (ANCOVA, $p > 0.05$, Table SA.4).

Despite the fact that δ^{15}N-PUC was better correlated to δ^{15}N-NH_4^+, isotopic values of PUCs were closer to δ^{15}N-NO_3^-, which suggests that PUCs were obtaining more of their N from NO_3^- rather than NH_4^+. This is confirmed by the results of mixing models (Fig. 1.3). For all PUC types, the selection of the best-performing mixing models always included Model 2, which assumes a single fractionation term for NH_4^+ and NO_3^- (Table SA.6). Concentrations of DIN species were only included in 5 out of 13 selected models. Results from Model 2 showed that PUCs obtained from 33 to 55% of N from NH_4^+, with isotopic fractionation factors ranging from 1.8 to 4.7‰ (Fig. 1.3).

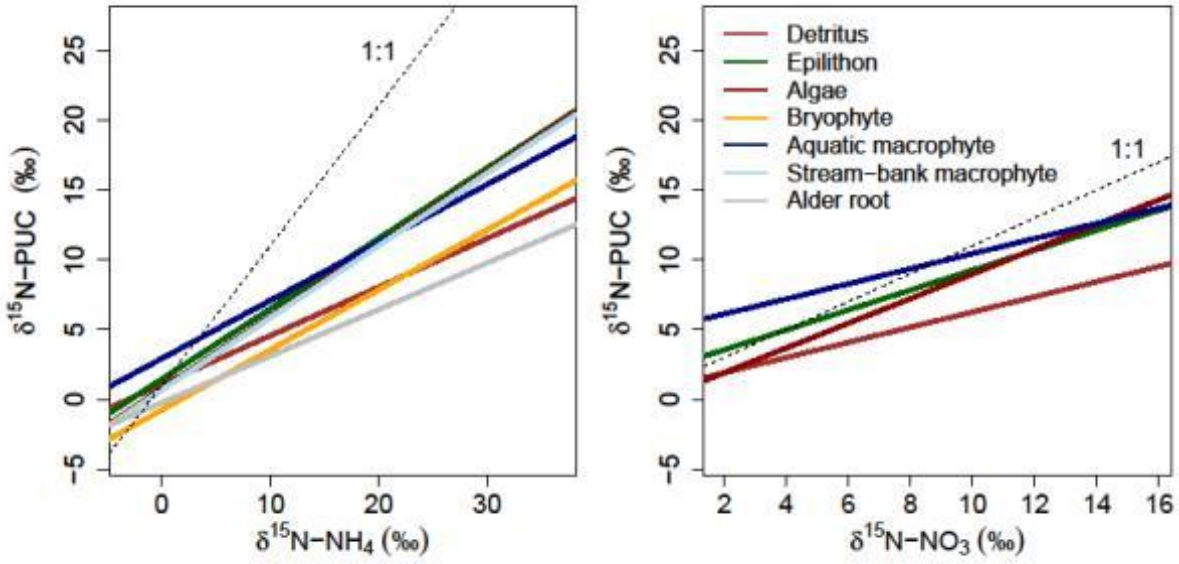


Figure 1.2 Linear regression lines between $\delta^{15}N$ of PUC and $\delta^{15}N\text{-}NH_4^+$, and $\delta^{15}N\text{-}NO_3^-$. The percentages of variance in ^{15}N-PUC explained by $\delta^{15}N\text{-}NH_4^+$ (measured as adjusted *r-square*) were: detritus, $r^2 = 0.54$; epilithon, $r^2 = 0.63$; algae, $r^2 = 0.63$; bryophytes, $r^2 = 0.70$; aquatic macrophytes, $r^2 = 0.45$; stream-bank macrophytes, $r^2 = 0.67$; alder roots, $r^2 = 0.61$. Percentages explained by $\delta^{15}N\text{-}NO_3^-$ were: detritus, $r^2 = 0.21$; epilithon, $r^2 = 0.21$; algae, $r^2 = 0.28$; aquatic macrophytes $r^2 = 0.13$. Only lines for PUCs with significant relations ($p < 0.05$) are included. The equations of the linear regressions are included in Table SA.4.

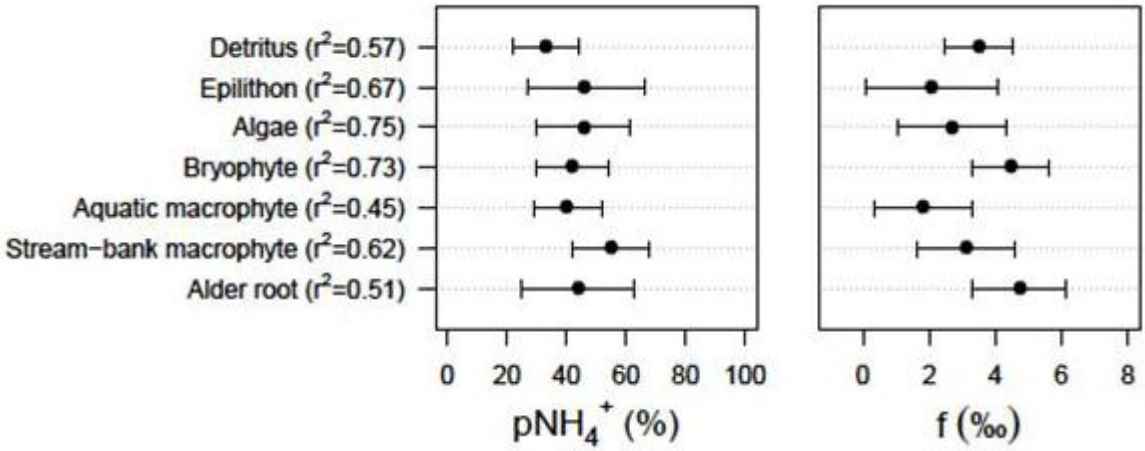


Figure 1.3 Estimates of the proportion of N in PUC derived from NH_4^+ (pNH_4^+) and fractionation factors (*f*) from Model 2 (maximum likelihood estimates with 95% confidence interval). Goodness of fit is measured as *r-square* of observed vs fitted values.

Multiple regression models including nutrient concentrations in addition to $\delta^{15}N$ of DIN species significantly improved the prediction power of univariate regression models, suggesting that stream nutrient concentrations also affect $\delta^{15}N$ values of PUCs. Selected best-performing models (i.e. with the lowest AICc) had high explanatory power (adjusted *r-squared* > 0.75, Fig. 1.4). SRP concentration and $\delta^{15}N\text{-}NH_4^+$ were selected as predictors in most of the best-performing models (seven and six out of seven, respectively) and together accounted for more than 60% of the variability explained regardless of the compartment considered. $\delta^{15}N$-PUC was positively related with these two variables (Table SA.7). When selected, concentrations of NH_4^+ and DON explained between 15 and 24% of the total variance of each model (Fig. 1.4). $\delta^{15}N\text{-}NO_3^-$ and DIN:SRP were selected only in one model each and accounted for a small fraction of the total explained variance (13% and 26%, respectively; Fig. 1.4). Interactions terms were included as significant predictive variables in three models, but with a small contribution to total r^2 (Fig. 1.4).

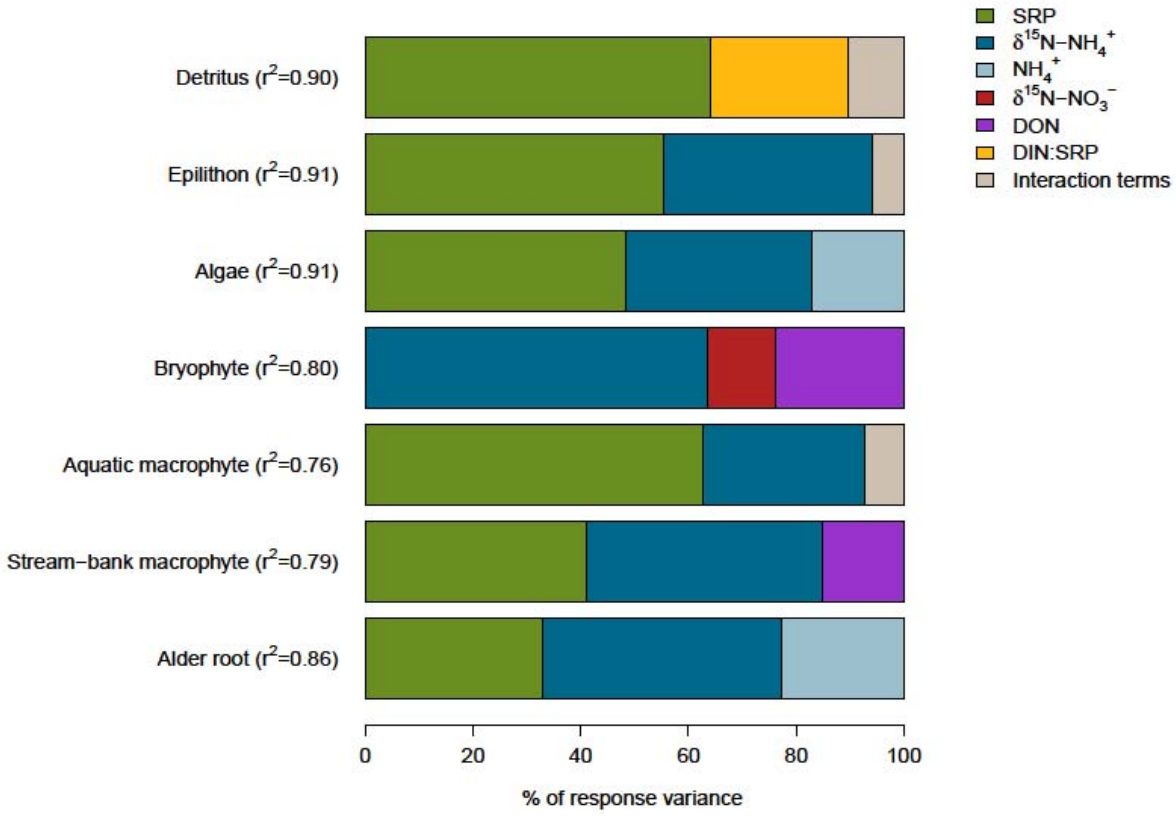


Figure 1.4 Relative contribution of independent variables (i.e., nutrient concentrations and ^{15}N signature of DIN species) to variance of ^{15}N of the different primary uptake compartments (PUC), based on the results of the best-predicting multiple regression model for each PUC type. Percentages of total variance in ^{15}N-PUC explained by the models (expressed as the adjusted *r-square*) are given in brackets next to PUCs categories in the Y axis

1.4 DISCUSSION

$\delta^{15}N$ variability across a fluvial network: location vs PUC type

Selected stream locations within the study catchment covered a wide range of nutrient concentrations as well as of ^{15}N natural abundance of the DIN species. In particular, the range of variation of $\delta^{15}N$ for the two DIN species was broader than that found for streams worldwide in a recent meta-analysis (Peipoch et al. 2012). Our data indicated that point source inputs in the watershed (i.e. WWTP

particularly concentrated at mainstem locations), usually characterized by increasing NH_4^+ and SRP concentrations, are the main responsible for the high variability in nutrient concentrations. This is in agreement with results from a nutrient emission model for La Tordera (Caille et al. 2012) and previous studies that show the large influence of WWTP effluents on the stream chemistry of this catchment (Merseburger et al. 2005, Jarvie et al 2006).

WWTP effluents can also influence the $\delta^{15}N$ value of the DIN species in stream water, especially for ammonium (Ribot et al. 2012), which may explain the high values observed at some of the mainstem sites and the positive relationships with nutrient concentrations. Moreover, the effects of point sources on receiving streams are amplified in streams from the Mediterranean region, such as La Tordera, because of their reduced dilution capacity, especially during summer low flow (Martí et al. 2010), when this study was conducted. It is worth noting that the $\delta^{15}N$ of DIN species can also vary temporally, especially in urban streams where N sources may change strongly due to runoff variability (Kaushal et al. 2011).

Consistently with the large variation in $\delta^{15}N$-DIN, especially for NH_4^+, we found a wide range of variation in $\delta^{15}N$-PUC (from -4 to 27 ‰), which is also slightly broader than that reported in the extensive compilation of $\delta^{15}N$ values of PUCs from stream ecosystems worldwide (-4 to 16‰; Peipoch et al. 2012). Differences in N use among PUC types can result in differences in their $\delta^{15}N$ signal (e.g., Evans 2001, Aberle et al. 2007), but the wide range of variation in

the $\delta^{15}N$ of the two DIN species within the fluvial network swamped differences among PUC types, resulting in higher differences of $\delta^{15}N$ values among locations than among PUC types. Absence of distinct N isotopic values among specific PUC types has been previously reported in other ecosystems, such as estuaries (Cloern et al. 2002), lakes (Jones et al. 2004) or wetlands (Chang et al. 2009), and also for organisms of higher trophic levels (Vander Zanden et al. 2005), suggesting the prevalence of environmental modes of variability over physiological differences among biota.

Isotopic relationships among PUCs and DIN species in the stream

For all PUC types, variability in the $\delta^{15}N$ of PUCs was more strongly related to $\delta^{15}N\text{-}NH_4^+$ than to $\delta^{15}N\text{-}NO_3^-$, yet isotopic values of PUCs were generally closer to the isotopic values of nitrate and mixing models indicate that PUCs take up proportionally more nitrate than ammonia (around 60% vs. 40%). However, isotopic values were considerably more variable among locations for ammonium that for nitrate. Therefore, even though PUCs may be taking up more nitrate than ammonium, their isotopic values among sites vary mostly with the isotopic values of ammonium.

The proportion of N used by PUC as NH_4^+ (33 to 55%) clearly exceeded the ammonium to DIN ratio in the water (on average 12%), which indicates higher demand for ammonium relative to its availability. This is in agreement with previous studies which argued that NH_4^+ is taken up preferentially over NO_3^-

due to its lower energetic assimilation costs and NO_3^- uptake inhibition by NH_4^+ (Dortch 1990, Barko et al. 1991). Preferential uptake of NH_4^+ over NO_3^- also contributes to explaining why isotopic values of PUCs covary with those of ammonium and not (or only weakly) with those of nitrate. It is worth noting that previous studies looking at stream PUCs ^{15}N values focused mostly on nitrate. Our results show how, at least in streams dominated by point sources, ammonium cannot be ignored.

Even though PUCs differed only weakly in their $\delta^{15}N$ value, it is interesting to note that patterns of variability clearly differed between PUCs growing in the stream channel and PUCs growing at the stream bank (i.e., stream-bank macrophytes and alder trees). The $\delta^{15}N$ of macrophytes, especially of those located at the stream bank, and of alders showed weaker relationships with stream water $\delta^{15}N$-DIN than the $\delta^{15}N$ of in-stream PUC compartments (e.g., epilithon, detritus, and filamentous algae). This might be because macrophytes and trees have larger individual biomasses and more complex biological structures than in-stream PUCs, which were mostly composed of microbial assemblages. Consequently, $\delta^{15}N$ of macrophytes and trees is expected to integrate the variation in $\delta^{15}N$-DIN over a longer temporal span, and this would weaken the relationship between their isotopic value and concurrent $\delta^{15}N$-DIN measurements. In addition, the observed differences among PUC types may reflect access to N pools other than stream water DIN (phreatic or soil water),

which may have different $\delta^{15}N$ values than $\delta^{15}N$-DIN of the stream (Sebilo et al. 2003).

In this study, macrophytes constituted a heterogeneous category including 18 species that covered a wide range of life strategies and preferential habitats, and are therefore potentially exposed to alternative DIN sources from groundwater and riparian zones. A detailed comparison of $\delta^{15}N$ among the different species of macrophytes was limited by the uneven presence of the species at the stream locations (Table SA.2). Only 2 out of 18 species occurred in more than half of the sites, *Apium nodiflorum* (which occurred in 20 sites), and *Carex pendula* (which was found in 17 sites). Data for these two most frequent species indicated some inter-species differences in $\delta^{15}N$. On average, *A. nodiflorum* was more isotopically enriched in ^{15}N than *C. pendula*, and values of $\delta^{15}N$ for *A. nodiflorum* were related to $\delta^{15}N$-NH_4^+ while those for *C. pendula* were neither related to $\delta^{15}N$-NH_4^+ nor to $\delta^{15}N$-NO_3^-. These results suggested a different interaction of these macrophytes with stream water DIN, which could be explained by their specific habitat preferences. *A. nodiflorum* develops at the margins of the wetted stream channel and is usually rooted in the streambed sediments, whereas *C. pendula* develops at the stream bank and has rhizomes that serve as reservoir organs.

The isotopic behavior of alder trees also differed markedly from that of in-stream PUCs. Although they live closed to the streamwater, and even have some of their roots submerged into the water, there are N isotopically

disconnected to the stream, suggesting the use of other N sources but stream DIN, such as N in the groundwater or atmospheric N. Alder trees can establish endosymbiotic relationships with N–fixing bacteria of the genus *Frankia*, which live in root nodules (Huss-Danell, 1997). These microorganisms can supplement alder trees with N fixed from the atmosphere, reducing their reliance on root-derived DIN that may come from the stream (Millet et al. 2012). This would probably result in lower $\delta^{15}N$ values than those of stream water DIN species because the $\delta^{15}N$ of atmospheric N_2 is zero. We also found that alder leaves were ^{15}N-depleted compared to roots. Previous studies have suggested that intra-plant isotopic differences are caused by organ-specific use of N and their physiological function or by N reallocation within the plants (Evans 2001, Dijkstra et al. 2008). In this study, root samples corresponded to visually active root tips submerged in the stream. Thus, isotopic differences between roots and leaves might be explained mainly by the fact that root tips were directly exposed to stream water DIN, whereas leaves integrated the $\delta^{15}N$ signal from different sources (i.e. phreatic or soil water) and their signature was also affected by N processing from roots to leaves. This would explain the lack of isotopic relationships between alder leaves and $\delta^{15}N$-DIN species, whereas $\delta^{15}N$ of alder roots was clearly associated to $\delta^{15}N$-NH_4^+. Alternatively, despite our best efforts to rinse out the microbial biofilm that develops on the stream-water exposed root tips, the isotopic value of this biofilm might have interfered with the $\delta^{15}N$ value of the roots *per se*, resulting in similar patterns to those observed in other stream benthic compartments.

δ^{15}N-PUC as a function of stream nutrient environment in which PUCs grow

Our results also indicate that variability in the natural abundance of N stable isotopes of PUCs was better explained (i.e. higher r^2) when both the δ^{15}N-DIN and the nutrient concentrations were considered in the models. In particular, SRP and NH_4^+ concentrations were, together with δ^{15}N-NH_4^+, the variables that explained the highest proportion of the δ^{15}N variance of PUCs. The fact that SRP concentration was included in most of the models was particularly surprising. One explanation is that the concentration of SRP was highly correlated with the concentration of DIN species and also with their δ^{15}N values. A more intriguing alternative is that fractionation processes can be influenced by the availability of DIN in relation to the availability of other nutrients, such as SRP. High SRP concentrations may enhance PUC stoichiometric demand for DIN, which may increase the N assimilated from DIN pool, thus reducing net fractionation (McKee et al. 2002, Wanek and Zotz 2011). Unfortunately, this study does not allow a direct test of this explanation, which would require an experimental approach using tracers. In addition, the interaction term between SRP concentration and δ^{15}N-NH_4^+ had a significant weight on several of the δ^{15}N PUC models (Table SA.6). Selection of interaction terms suggests that the variability of δ^{15}N-PUC reflects more complex pathways than those examined in this study, and probably demand an experimental approach to tease apart the influence of different factors (i.e. nutrient concentrations and δ^{15}N-DIN species). Interaction terms tended to be negative,

implying nonlinearities that tended to limit high values of $\delta^{15}N$. For example, the slopes between the $\delta^{15}N$ PUC and $\delta^{15}N\text{-}NH_4^+$ tended to be lower as SRP concentration increased. The interpretability of specific models is hampered by the fact that some predictors showed some collinearity, thus making it more difficult to differentiate among the effects of individual variables. In addition, N demand of PUCs might be affected by factors other than nutrient concentrations, such as the physiological behavior of each particular PUC type. Most of the study sites, especially in the mainstem of La Tordera, are not likely to be nutrient-limited, and notably reduced light availability in most sites, due to riparian shading, might play a more important role than nutrients as a controlling factor of DIN uptake by primary producers (von Schiller et al. 2007). Despite these caveats, our models do suggest that, besides the isotopic values of DIN species, nutrient concentrations and their interactions can play an important role in determining the natural abundances of N stable isotopes of the different PUCs in stream ecosystems, and that the acquisition of a nitrogen isotopic value is a more complex process than generally acknowledged.

In conclusion, our study shows a high spatial variability in $\delta^{15}N$ of different stream PUC types within the same catchment. This variability was more strongly explained by location than by PUC type, with the highest $\delta^{15}N$-PUC values corresponding to human impacted streams (i.e. mostly mainstem locations). The nitrogen isotopic value of PUCs was strongly explained by the

$\delta^{15}N$ of DIN species, especially of ammonium, and models were stronger for PUCs growing within the stream channel, and thus using stream water nutrients to satisfy their nitrogen demands. Finally, the isotopic value of PUCs did not simply reflect the $\delta^{15}N$ of the DIN sources, but was also influenced by the nutrient concentrations in which they grew, suggesting that nutrient concentrations and stoichiometric constraints cannot be ignored in explaining natural abundance of nitrogen isotopes in primary uptake compartments.

Finally, these results have two major implications. Firstly, they suggest that, if $\delta^{15}N$-DIN values are linked to nitrogen sources within the catchment, in particular those derived from urban activities, the ^{15}N natural abundances of different PUC types could be successful integrators of human impacts on stream ecosystems. The fact that PUC isotopic values were more dependent on location than on PUC type further supports the use of PUCs as indicators of nitrogen sources in streams. Secondly, the use of ^{15}N natural abundance in food web studies in streams to identify trophic levels among consumers should take into account the fact that the high variability in ^{15}N natural abundance of basal resources (i.e. PUCs) responds to $\delta^{15}N$ of DIN species and to the nutrient environment in which PUCs grow, suggesting that this variability can be incorporated into models to better understand the relationship between resources and consumer.

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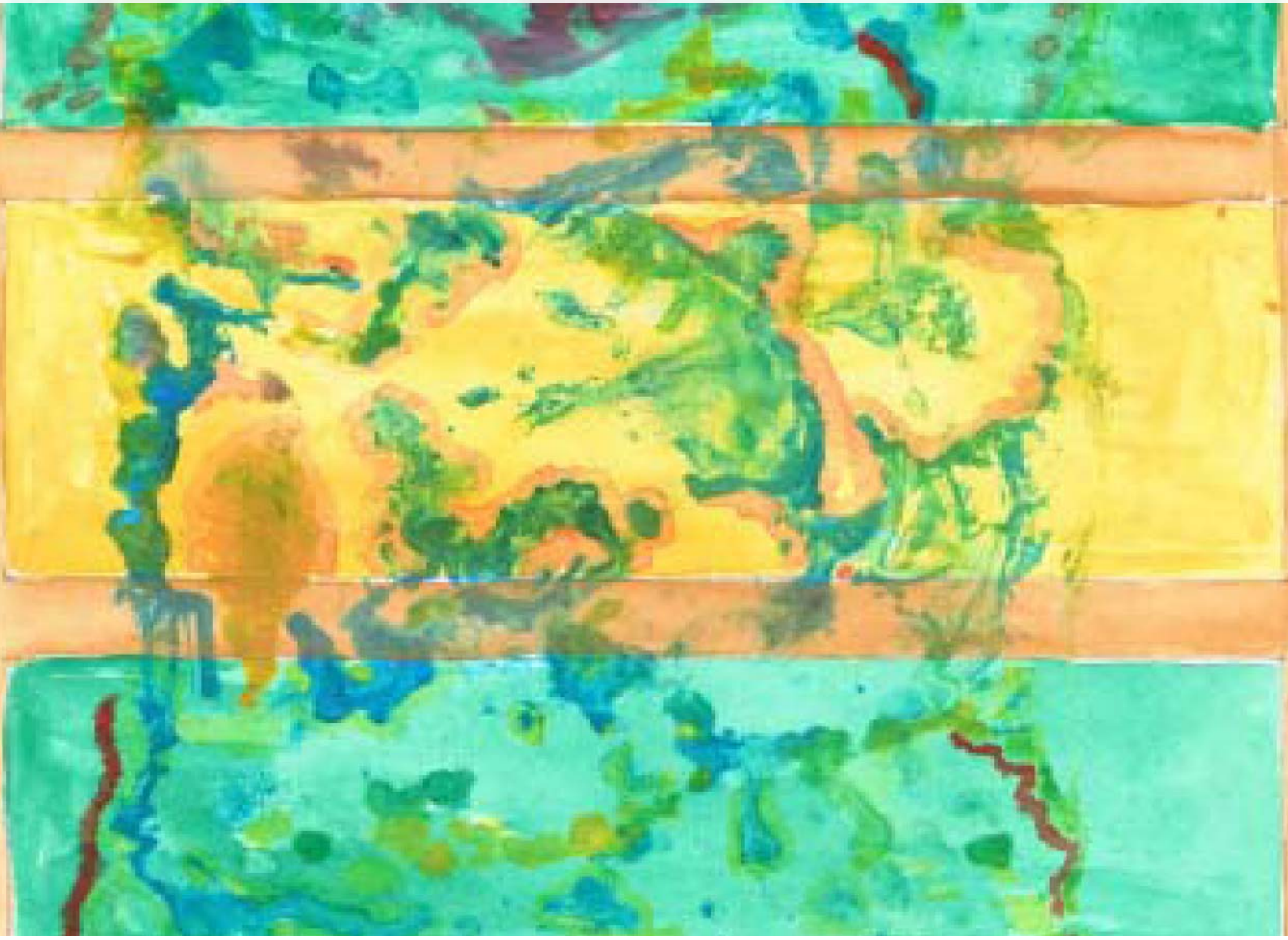
2

Temporal variability of nitrogen stable isotopes in primary uptake compartments in four streams differing in human impacts



Supporting information is available at the supporting information of this dissertation (Appendix B). It includes information on Information on the temporal correlation analyses, relationships between stream environmental variables and δ^{15}N-DIN species, temporal versus with-in reach variability, isotopic relationships between DIN species and PUCs, and cross-correlations between δ^{15}N-PUC and δ^{15}N-DIN species; Figures SB.1-SB.9 and Tables SB.1-SB.4.

ABSTRACT

Understanding the variability of the natural abundance in nitrogen stable isotopes (expressed as $\delta^{15}N$) of primary uptake compartments (PUCs; e.g. epilithon or macrophytes) is important due to the multiple applications of stable isotopes in freshwater research and can give insights into environmental and anthropogenic factors controlling N dynamics in streams. While previous research has shown how $\delta^{15}N$ of PUCs varies with $\delta^{15}N$ of dissolved inorganic N (DIN) among streams, less is known about how $\delta^{15}N$ of PUCs varies over time. Here, we examined monthly variation of $\delta^{15}N$ of PUCs and of DIN species (nitrate and ammonium) over a year, and compared it among streams with contrasting human impacts and PUC types. Our results showed no evidence of isotopic seasonal patterns. Temporal variability in $\delta^{15}N$- PUCs increased with human impact, being the highest in the urban stream, probably influenced by the high variability of $\delta^{15}N$- DIN. Among compartments, in- stream PUCs characterized by fast turnover rates, such as filamentous algae, showed the highest temporal variability in $\delta^{15}N$ values (from -3.6 to 23.2 ‰). Our study elucidates some of the controls of temporal variability of $\delta^{15}N$ in streams and highlights aspects that should be taken into account when using stable isotopes as an ecological tool.

2.1 INTRODUCTION

Understanding the temporal variability of the natural abundance of nitrogen stable isotopes (^{15}N:^{14}N, expressed as $\delta^{15}N$) in freshwater ecosystems can provide insights into how environmental and anthropogenic factors influence ^{15}N dynamics in biotic compartments. This has implications for establishing proper isotopic baselines of basal resources, which are crucial to improve isotopic food web models (Cabana and Rasmussen 1996, Zeug and Winemiller 2008, Woodland et al. 2012a, Dethier et al. 2013, Jardine et al. 2014). In addition, isotopic temporal variability should be considered when applying isotopic techniques as ecological monitoring tools, as this would allow more accurate assessments of anthropogenic impacts on nitrogen in freshwater ecosystems, and better predictions of ecosystems responses to these impacts over time (Gartner et al. 2002, Page et al. 2012).

In freshwater ecosystems, the biotic compartments that can directly assimilate dissolved nutrients from the water comprise multiple types of organisms. These include both autotrophs (i.e., primary producers such as algae, bryophytes, or macrophytes) and heterotrophs (e.g. bacteria or fungi), which hereafter will be collectively referred to as primary uptake compartments (PUCs).

Previous research in streams has shown that spatial variability in $\delta^{15}N$ of PUCs can be remarkable and that it is mostly explained by the $\delta^{15}N$ of their N sources, in particular dissolved inorganic nitrogen species (DIN, mostly

ammonium and nitrate) in the water, which in its turn varies depending on human influences (Kohzu et al. 2008, Peipoch et al. 2012, Pastor et al. 2013, Peipoch et al. 2014). However, less information is available on the temporal variability of $\delta^{15}N$ of PUCs and their N sources. In lotic ecosystems, temporal patterns of stable isotopes of PUCs and their relation with their elemental sources have been recently studied for carbon (Finlay 2004, Gu 2006, Gu et al. 2011) and, to a lesser extent, for nitrogen (Gu 2009, Ferber et al. 2004). These studies have mostly relied on compilations of data from the literature; in contrast, empirical data sets directly assessing temporal variation in $\delta^{15}N$ of PUCs and of DIN are scant, especially for stream ecosystems. This has resulted in limited knowledge of the magnitude and controls of temporal variability of $\delta^{15}N$.

Several factors can potentially contribute to the temporal variability in $\delta^{15}N$ of PUCs. First and foremost, PUCs rely on streamwater DIN as an N source, and previous studies based on spatial variability among streams have shown a good relationship between $\delta^{15}N$ values of PUCs and of DIN (Kohzu et al 2008, Pastor et al. 2013). Thus, the temporal variation of $\delta^{15}N$- PUCs can be expected to mirror, to some extent, that of the $\delta^{15}N$ of DIN species. In its turn, temporal variation in $\delta^{15}N$ of DIN can be due to temporal changes in the dominant sources of N from the catchment, both natural and anthropogenic, which may vary in their isotopic values. For instance, $\delta^{15}N$ values of synthetic fertilizers and atmospheric N deposition are close to or lower than zero (Holtgrieve et al. 2011). In contrast, N compounds derived from septic waste or manure are

often isotopically enriched, and thus tend to increase δ^{15}N- DIN in receiving streams (Kendal et al 2007, Xue et al. 2009). Secondly, the dominant biogeochemical processes occurring in the stream, including nitrification and denitrification, can also contribute to δ^{15}N- DIN variability within stream reaches and over time Finlay and Kendall 2007, Ribot et al. 2012). Finally, because the temporal variation of both N sources and in-stream processes is subject to the human activities on land adjacent to the streams, streams draining catchments with high human pressures can be expected to show larger temporal variability in δ^{15}N- DIN (Kaushal et al. 2011).

In addition to environmental drivers, the temporal variation of δ^{15}N-PUC can also be due to physiological differences. Within PUCs, this variation may be a result of differential isotopic fractionation (i.e., the preferential use of the lighter isotope over the heavier isotope) associated with the assimilation and dissimilation of N, which can vary temporally depending on the magnitude of these processes and the external nutrient availability (Evans 2001, Dijkstra et al. 2008). For example, seasonal variation in δ^{15}N values of lake consumers has been associated with their seasonal anabolism- catabolism dynamics, which causes organisms to be ^{15}N depleted during summer due to the predominance of anabolic growth (Woodland et al. 2012b). Among PUCs, assimilation, storage, and release of N occur at different rates over time, and therefore these processes may mask temporal variation of δ^{15}N- PUCs relative to the temporal variation of δ^{15}N from their DIN sources. Differences among organisms in biological traits, such as biomass, biological complexity, and activity, might

result in differences in the time span at which $\delta^{15}N$ of DIN is being integrated by PUCs, and will eventually result in differences in $\delta^{15}N$ temporal variability among PUCs (Cabana and Rasmussen 1996, Gartner et al 2002).

The main objective of this study was to assess the temporal variability of $\delta^{15}N$ of DIN species and of the most representative PUC types in stream-riparian ecosystems (i.e. filamentous algae, epilithon, bryophytes, biofilm-litter, macrophytes, and alder roots and leaves). Specifically, we evaluated how temporal variability in $\delta^{15}N$ of DIN and of PUCs differed among streams with contrasting human impacts and among PUC types. We predicted that the temporal variability of the $\delta^{15}N$ of DIN species and that of $\delta^{15}N$- PUCs would increase with the degree of human activity adjacent to the stream. Moreover, we predicted that PUCs characterized by fast N turnover (e.g. algae) would show a temporal variability more closely associated with that of $\delta^{15}N$- DIN than PUCs with low N turnover rates (e.g., macrophytes) because the latter would integrate the temporal variability in $\delta^{15}N$- DIN over a longer time span. To address these objectives, we examined monthly variation of $\delta^{15}N$ for PUCs and for DIN over one year in four stream reaches within a Mediterranean catchment (La Tordera, NE Iberian Peninsula). The selected streams differed widely in their dominant adjacent land use type (forest, irrigated and non-irrigated agriculture, urbanization) and consequently, in their DIN concentrations (von Schiller et al. 2008, Pastor et al. 2013).

2.2 MATERIAL AND METHODS

Study site

This study was conducted in La Tordera catchment (868.5 km^2), which is located in the North- East of Barcelona (Catalonia, NE Iberian Peninsula). The basin is mostly covered by forest, with significant agricultural and urban land use on the plains and along the main valley. Nutrient concentrations differed widely among tributaries affected by these different land uses (von Schiller et al. 2008, Pastor et al. 2013, Caille et al. 2012). We selected four stream reaches that differed in their dominant adjacent land use type (Table 2.1): forested (FOR), influenced by irrigated horticultural production (HOR), surrounded by non-irrigated agriculture (AGR), and receiving the effluent of a wastewater treatment plant (WWTP; URB).

Field procedures

Streams were sampled monthly from July 2010 to July 2011. In the field, we collected water samples for nutrient analysis and for the determination of $\delta^{15}N$ of NH_4^+ and NO_3^-. All water samples were immediately filtered through pre-combusted (4h, 450ºC) glass fiber filters of 0.7 µm pore size (Albet, Barcelona, Spain). Samples for $\delta^{15}N$- NH_4^+ were processed immediately (see below), whereas samples for nutrient concentrations (40 mL, three replicates per stream) and $\delta^{15}N$- NO_3^- (0.5 L, one replicate per stream) were frozen and stored at - 20ºC until analysis. In addition, conductivity and water temperature were measured

with a portable 340i sensor meter (WTW, Germany). Stream discharge was assessed using slug additions of NaCl in FOR, HOR and AGR sites (Gordon et al. 2004). Discharge data for URB was provided by the Catalan Water Agency (http://www.gencat.cat/aca/) from a gauging station at Sant Celoni, approximately 2 km downstream of the sampled reach.

On each sampling date, we also collected samples of the main PUC types in streams for $\delta^{15}N$ analysis. Biofilm on stream cobbles (hereafter "epilithon") was obtained by scraping the light- exposed side of a cobble using a soft metal brush and collecting the detached material on a filter (three cobbles as replicates). Biofilm on decomposing leaf- litter (hereafter "biofilm-litter") was sampled by collecting leaves accumulated on the stream channel and washing them in a bucket with streamwater. Subsequently, the suspended fraction in the bucket (i.e. including biofilm on decomposing leaf-litter but also small fractions of litter organic matter) was filtered until saturation, and three replicate filters were obtained. Samples were obtained for the following PUC types: bryophytes, filamentous algae, alder (*Alnus glutinosa*) leaves, alder root tips submerged in the water, and three species of macrophytes, *Ranuculus* sp. and *Apium nodiflorum*, which live in the wetted channel (hereafter "Aquatic macrophytes"), and *Carex pendula,* located at the stream bank (hereafter "Stream- bank macrophyte"). These PUC types were harvested when present and rinsed with streamwater. In each case, a composite sample from fragments of several individuals was obtained to smooth out within PUC variability.

Laboratory analysis

Water samples were analyzed colorimetrically for soluble reactive phosphorous (SRP) by the molybdenum method (Murphy and Riley 1962) and for ammonium concentration by the salicylate method (Reardon et al. 1966). The concentration of nitrate was determined by ionic chromatography (761 Compact IC1.1, Metrohm), and the concentration of dissolved organic carbon (DOC) by high- temperature catalytic oxidation (Shimadzu, TOC analyzer). The $\delta^{15}N$ of NH_4^+ and NO_3^- were determined following an adaptation of the ammonia- diffusion method (Holmes et al. 1998, Sigman et al. 1997) following the same procedure described in the literature (von Schiller et al. 2009).

Samples and filters of biotic compartments were oven- dried at 60ºC and stored. Dry samples of plant tissues were ground in a MM 200 mixer mill (Retsch, Germany) to homogenize the sample. Subsamples were weighed on a MX5 microbalance (Mettler-Toledo, Switzerland) before being packed into tin capsules for $\delta^{15}N$ and C:N analysis. Biotic samples in filters were also weighed and encapsulated after clipping a smaller section, usually a 1 cm^2-diameter circle or one half of the filter, with enough N content to be analytically detected.

Isotopic and elemental analyses were carried out by the University of California Stable Isotope Facility (Davis, California, USA) by continuous-flow isotope-ratio mass spectrometry (20-20 mass spectrometer; PDZ Europa, Northwich, UK) after sample combustion in an on-line elemental analyzer (PDZ

Europa ANCA-GSL, Sercon Ltd., Cheshire, UK). The natural abundance of N stable isotopes was expressed in standard notation ($\delta^{15}N$ in ‰) relative to the international standard of atmospheric N_2, where $\delta^{15}N = 1000 \times [(R_{sample}/R_{standard}) - 1]$, and R is the ^{15}N:^{14}N molar ratio. The analytical precision on repeated measures of our alder leaf standard was ±0.31 ‰ (measured as standard deviation).

Statistical analysis

Temporal patterns of $\delta^{15}N$ of the two DIN species were tested by examining their autocorrelation with time lags of one up to five months using autocorrelation function estimations in R (i.e. 'function acf' in R's base package). Because $\delta^{15}N$ values of NH_4^+ or NO_3^- did not show significant temporal autocorrelation, we chose the following statistical approach. For each stream, the relationships between $\delta^{15}N$- NH_4^+ and $\delta^{15}N$- NO_3^- with stream environmental parameters (discharge and the concentrations of DOC, NH_4^+, NO_3^+ and SRP, previously log-transformed) were evaluated by building all possible linear models involving these variables and their pairwise interactions using the iteratively reweighted least squares method. We controlled for model complexity by including interaction terms only if the variables involved were also selected as main effects in the model. Candidate models were identified using the Akaike Information Criterion corrected for small sample size (AICc) as those differing by less than two units from the best models (lowest AICc). We automated this process using the R package 'glmulti' (Calcagno et al. 2010).

Selected models were further evaluated for influential outliers using Cook's distance. Points with Cook's distance values greater than 1 were excluded from the analyses, and the model selection procedure was performed again. The relative importance of each variable in the best model was estimated by r^2 partitioning using R package 'relaimpo' (Grömping 2006).

Temporal patterns of δ^{15}N- PUC values were examined by autocorrelation analysis, as described above for δ^{15}N- DIN species. We also attempted to identify nonlinear trends over time using generalized additive models (GAM), with day number as the explanatory variable and cubic regression spline as the smoothing function. For this, δ^{15}N- PUC values were first standardized to a common scale by subtracting from them their mean value by PUC type and stream. These standardized values or residuals were analyzed separately for each stream. Temporal trends can be meaningfully modeled only if within-date variability in δ^{15}N is small relative to among-date variability. To check for this, we used δ^{15}N replicate values (n = 3) for epilithon and biofilm-litter samples. For each PUC and each stream, a mixed model was fitted using "date" as random effect and the intercept as fixed effect. This approach allowed us to handle the unbalanced data set due to missing values.

Having found no significant autocorrelation, relationships between δ^{15}N of each PUC type and δ^{15}N of DIN species were examined using a Pearson correlation. The analysis were conducted both with data for each stream separately, and with data for all streams pooled together. Cross-correlations

between $\delta^{15}N$ of each PUC type and $\delta^{15}N$ of both DIN species for time lags from one to five months were conducted to determine the extent to which the $\delta^{15}N$ of DIN and PUCs exhibited concordant periodic variations. Sample lag autocorrelation and cross-correlation estimates were considered not significant when they fell within 95% confidence intervals for an uncorrelated series.

Finally, to compare temporal variability among PUC types we used the standard deviation (SD) of the residuals of $\delta^{15}N$ (calculated as described above). To evaluate the effect of PUC types on isotopic variability, average SD values per PUC type were plotted against their C:N average, as a proxy for N turnover rate of each PUC type (Dodds et al. 2000). The relationship between SD and C:N was evaluated using Spearman nonparametric correlation. All data analyses were carried out using free R software, version 2.15.1 (R Development Core Team 2012).

2.3 RESULTS

Stream environmental parameters

Water discharge was similar among streams, except in URB, where it was significantly higher (Table 2.1), and showed no apparent seasonal pattern. Water temperature was lower at sites located at higher altitudes (Table 2.1) and followed the expected seasonal pattern, with higher values during summer (data not shown). The four sampled streams spanned a wide range of nutrient concentrations, from low values in FOR, to intermediate values in HOR and

AGR, to high values at URB (Table 2.1). The widest range of variability among streams was for NO_3^-, and NH_4^+ was more than 40 times higher, on average, in the urban stream compared to the other streams (Table 2.1). Within-stream temporal variability in nutrient concentrations increased with average nutrient concentrations.

Table 2.1 Average and standard deviation of physical and chemical characteristics of monthly data averaged over one year for each study stream. In parenthesis, below the stream code, the dominant land use adjacent to the stream is indicated. Latitude and longitude refer to the UTM zone 31N coordinate system.

	FOR (forested)	**HOR (irrigated horticultural)**	**AGR (non- irrigated agricultural)**	**URB (urban)**
Longitude	454275	469369	455165	455763
Latitude	4630617	4635715	4618071	4614587
Altitude (m a.s.l.)	528	163	240	154
Discharge (L/s)	65±40	64±51	40±30	311±337
Temperature (ºC)	10.8±3.9	13.7±4.8	12.3±4.8	15.8±5.3
Conductivity (µS/cm)	181±24	294±38	102±12	296±107
SRP (µg P/L)	7±3	17±6	27±21	481±606
NH_4^+ (µg N/L)	9±5	10±3	12±6	496±770
NO_3^- (µg N/L)	240±196	666±257	688±333	2053±788
DIN:SRP	44±23	48±29	35±22	16±20
DOC (mg/L)	1.0±0.4	1.6±0.6	1.3±0.3	2.7±1.4
$\delta^{15}N$- NH_4^+ (‰)	3.6±1.8	5.0±3.9	6.6±3.9	28.4±11.8
$\delta^{15}N$- NO_3^- (‰)	- 0.1±0.7	5.2±1.0	3.9±2.0	10.4±2.6

Temporal variability in $\delta^{15}N$ of DIN species

In general, $\delta^{15}N$- NH_4^+ values presented a broader range (-1.9 to 49.6‰) and were on average 6.4 ‰ higher than $\delta^{15}N$- NO_3^- values (- 1.7‰ to 17.3‰, Fig. 2.1). Among streams, averages and temporal variability of $\delta^{15}N$ of NH_4^+ and NO_3^- showed patterns similar to those described above for nutrient concentrations, with the narrowest ranges of $\delta^{15}N$ in the forested site (FOR), intermediate

ranges in AGR and HOR, and the largest variability in URB (Table 2.1; Fig. 2.1). Time series of $\delta^{15}N$ of NH_4^+ or NO_3^- showed no significant autocorrelations at any time lag, nor any apparent seasonal patterns (Fig SB.1-SB.4).

Selected best-performing models for predicting $\delta^{15}N$ of DIN species (i.e. with the lowest AICc) were significantly related to stream environmental parameters ($p < 0.05$, in all streams except for $\delta^{15}N$- NH_4^+ in HOR, Table SB.1, Fig. SB.5) and the variance explained ranged from 41% to 82%. Variables selected in the best-performing models differed among streams. Concentrations of NH_4^+ and NO_3^- were selected in three and two models, out of eight, respectively. The $\delta^{15}N$ of NH_4^+ was positively related to the concentration of both DIN species, whereas the $\delta^{15}N$ of NO_3^- was negatively related to the concentration of NO_3^-, and also negatively to DOC concentration (Table SB.1), which accounted for 7% and 35% of the variance explained by these models (Fig. S5). Discharge was selected in models for URB, with a negative coefficient, and contributed 19% and 35% to the variance explained by models for $\delta^{15}N$ of NH_4^+ and NO_3^-, respectively. SRP and interaction terms between selected variables were only included as significant predictors in one model (Fig. SB.5).

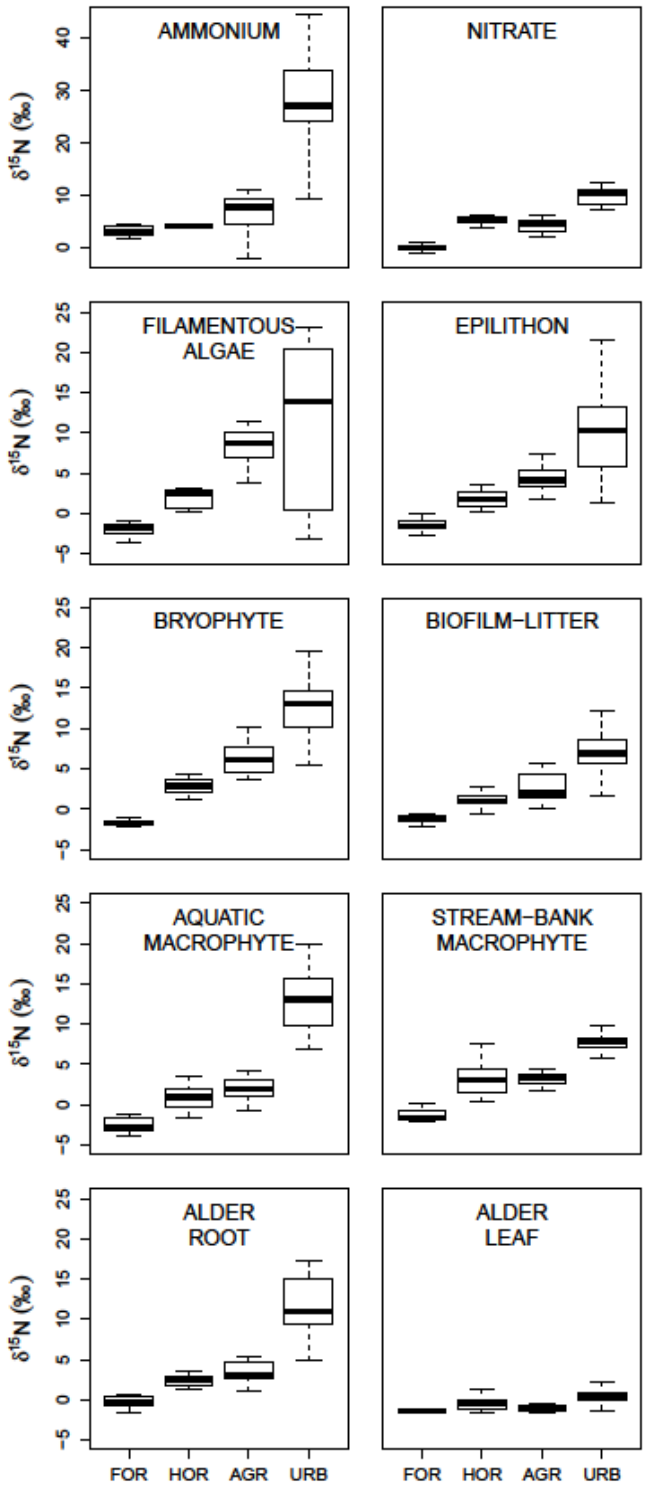


Figure 2.1 Box plots for $\delta^{15}N$ of DIN species and for each PUC type (‰) during the sampling period grouped by site (FOR, HOR, AGR, URB). Extreme values (values outside 1.5 times the interquartile range) are not shown. Note the different scale for $\delta^{15}N\text{-}NH_4^+$ and $\delta^{15}N\text{-}NO_3^-$.

Temporal variability in $\delta^{15}N$ of PUCs

Overall, $\delta^{15}N$ values for PUCs showed a wide range of variability, from -4.0 to 25.0‰ (Fig. 2.1), with the lowest values and the narrowest variability at the forested site (FOR; from -4.0 to 0.9 ‰, average: -1.4‰). The two agricultural sites had similar ranges of $\delta^{15}N$ values (from -1.6 to 11.2 ‰ in HOR, and from -1.5 to 11.4 % in AGR), but on average, $\delta^{15}N$ was lower in HOR (mean: 1.9‰) than in AGR (mean: 3.6‰). URB, the urban impacted site, showed the highest variability and the highest mean value (from -3.1 to 25.0‰, mean = 9.3). For all PUC types, both the average and the temporal variability of $\delta^{15}N$ consistently increased with mean nutrient concentration in the stream, except for alder leaves and stream-bank macrophytes (Fig. 2.1). The lowest temporal variability was found at FOR, with intermediate ranges at HOR and AGR, and the highest variability at URB.

There was no strong evidence of temporal autocorrelation for $\delta^{15}N$ of PUCs (Fig. SB.1-SB.4). Only biofilm-litter and stream macrophytes were autocorrelated at AGR at lags of 1 and 5 months for the former and a lag of 2 months, for the latter (Fig. SB.3), and alder leaves at URB at a lag 2 months (Fig. SB.4). The analysis of residuals of $\delta^{15}N$ of PUC values from the means by PUC type and stream using GAM analyses showed weak but significant nonlinear trends (Fig. 2.2). $\delta^{15}N$ residuals displayed asynchronous variation at the four studied streams. FOR, HOR and AGR showed smooth temporal variation with the lowest values found in FOR during summer, and in HOR and AGR during spring. In URB, the amplitude of $\delta^{15}N$ variability was the highest, and also

showed the highest variability among PUCs, with two ^{15}N depletion periods during spring and early winter (Fig. 2.2). The proportion of variance of $\delta^{15}N$ within sampling date (i.e., the variability within a stream-reach) versus among sampling dates for epilithon and biofilm-litter replicate samples was low (10 to 16%) at AGR and URB sites, but higher at FOR and HOR sites (from 34 to 71%, Table SB.2), where it may have masked the signal for a temporal trend. This high-variability within a stream-reach is likely to be driven by particular sampling dates with high dispersion (Table SB.3).

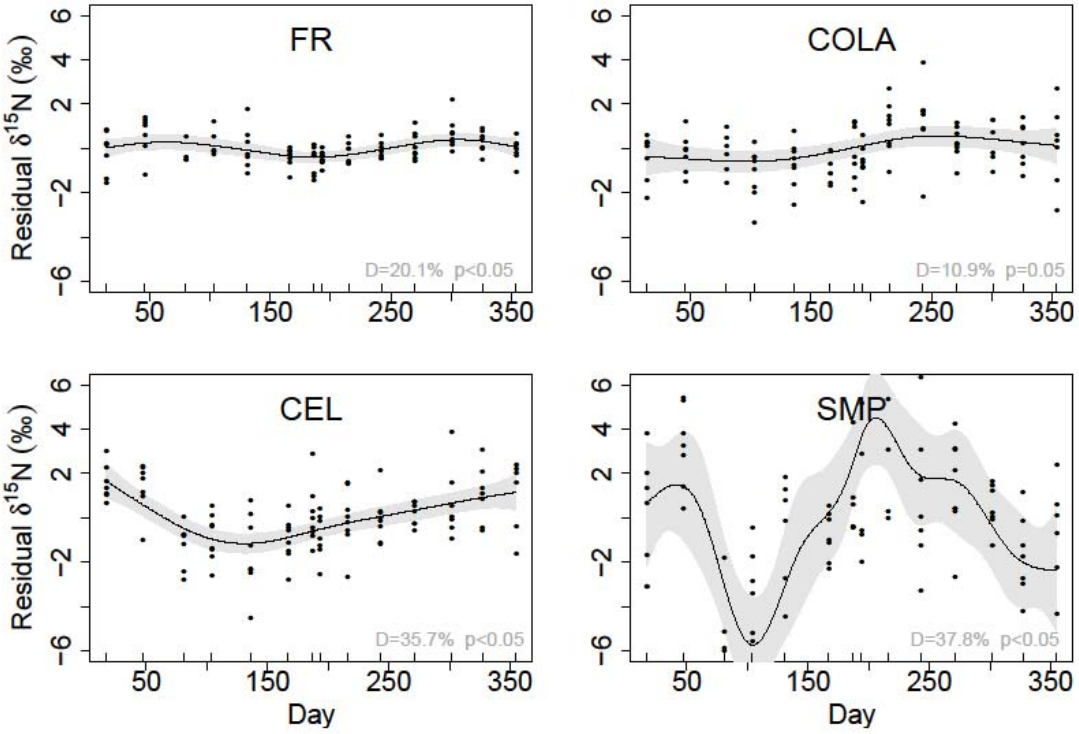


Figure 2.2 The residuals of $\delta^{15}N$ of PUC values from the mean for each PUC type and site plotted against time for each site (FOR, n = 98; HOR, n = 101, AGR, n = 104; URB, n = 102). The predicted temporal trends obtained from GAM analyses are represented by a line (gray regions stand for the confidence interval of the spline). Deviance explained (D) and p-values are included for each model.

The temporal variability of $\delta^{15}N$- PUC (measured as range of values including all streams) was smaller than the variability of $\delta^{15}N$- NH_4^+, but larger than that of $\delta^{15}N$- NO_3^-, except for biofilm-litter, stream-bank macrophytes, and alder compartments (Fig. 2.1). When stream sites where considered separately, $\delta^{15}N$ PUC was not related to the $\delta^{15}N$ of either DIN species at any site and for any PUC type (Pearson correlation; $p > 0.01$; Table SB.4). No cross-correlations were found with $\delta^{15}N$ of DIN species for up to 5 months before sampling date (only 3% of all the cross-correlation estimates were significant; Fig. SB.6-SB.9). In contrast, when data were pooled together for the four sites, $\delta^{15}N$ of most of the PUC types showed strong positive correlations with both $\delta^{15}N$- NH_4^+ and $\delta^{15}N$- NO_3^- at the sampling time (Pearson correlations from 0.51 to 0.82; $p < 0.01$; except for filamentous algae with $\delta^{15}N$- NH_4^+; Table SB.4).

Finally, the standard deviation of $\delta^{15}N$ residuals of PUCs showed a weak pattern among PUC types with respect to C to N ratios as a proxy for N turnover rates (Fig. 2.3; Spearman's rank correlation, $r = -0.67$, $p = 0.07$). This relationship was, however, consistent with our expectations. The highest temporal variability was held by filamentous algae, with low C:N ratio. In contrast, biofilm-litter, stream bank macrophytes and alder leaves showed the lowest temporal variability (Fig. 2.3).

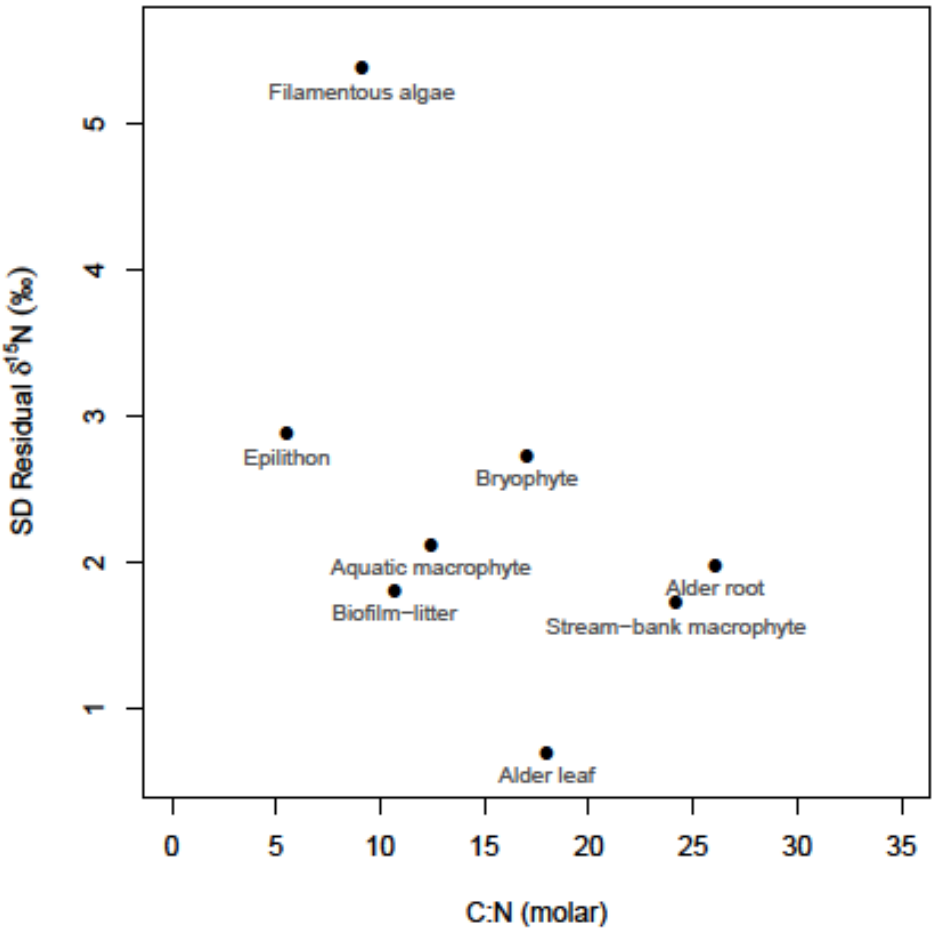


Figure 2.3 Standard deviation (SD) of the residuals of $\delta^{15}N$ for each PUC type across all studied streams versus their C:N average. Spearman correlation coefficient was $r = -0.67$ with $p = 0.07$.

2.4 DISCUSSION

Temporal patterns in $\delta^{15}N$ of DIN and PUCs

The natural abundance of $\delta^{15}N$ of DIN species varied substantially over time, although ranges of annual variability at the four sites did not exceed the ranges of spatial variability reported in a synoptic study of 25 reaches sampled

in the same watershed in summer (Pastor et al. 2013). The temporal isotopic variability of DIN species in streamwater is the net result of changes in N sources with distinct $\delta^{15}N$ values reaching the stream from the watershed, as well as of isotopic fractionation processes associated with in-stream N cycling (i.e., nitrification, denitrification and N uptake; Kendall et al. 2007). Both the temporal variation of DIN inputs to streams and the relative proportions of different N sources from the watershed are highly subject to hydrological regimes, which in Mediterranean streams involve highly variable flows throughout the year, with stream floods overriding any seasonal trends (Bernal et al. 2013). In contrast, in-stream biological processes are expected to be influenced by local environmental conditions that vary seasonally, such as water temperature, and therefore should be themselves more subjected to seasonal variation. However, previous studies indicate that high flood events decrease the efficiency of in-stream N uptake (Martí and Sabater, 1996, Martí et al. 2004, Argerich et al. 2008), which implies that the influence of in-stream processing on $\delta^{15}N$- DIN should be higher at lower discharge. The fact that we found no significant autocorrelations in $\delta^{15}N$- DIN, even at the smallest time interval that our design permitted (one month), indicates no clear evidence of temporal patterns within the study period. Instead, we found that $\delta^{15}N$ of DIN species was strongly related to discharge and nutrient concentrations, and that these relationships were specific to each stream (Fig. SB.5). These findings suggest a dominant effect of factors operating at the catchment scale (i.e., land

uses, discharge, and N reaching the stream) compared to the effect of in-stream N processes.

Similar to $\delta^{15}N$ of DIN species, variation of $\delta^{15}N$- PUC did not follow any temporal pattern. The isotopic variability explained by non-linear GAM models at the annual timescale, while significant, was low (<40 % of deviance), which further emphasizes the relevance of other modes of variability besides seasonality for biotic compartments. Moreover, there was temporal asynchrony in $\delta^{15}N$- PUC patterns among streams despite the fact that they were all located within tens of kilometers in the same fluvial network, which suggests that local hydrological and nutrient conditions might be prevalent controllers of isotopic variability. Nevertheless, the relationship between concurrent measurements of $\delta^{15}N$- DIN and $\delta^{15}N$- PUC was weak at best when data from each stream were considered separately. Cross-correlations between $\delta^{15}N$- PUC and $\delta^{15}N$- DIN for time lags up to 5 months were not significant either. These findings suggest that $\delta^{15}N$- DIN may vary at time scales shorter than our temporal resolution (i.e., <one month), and that N turnover times of PUCs may also be shorter than one month. This is in agreement with past studies that have indicated that the variability in $\delta^{15}N$- DIN of streamwater is substantial high within a month or even within a day (Gammons et al. 2011), and can be quickly integrated by the $\delta^{15}N$ of PUCs (O'Reilly et al. 2002, Hill et al. 2012, Mulholland et al. 2000).

Alternatively, our inability to detect temporal trends could be due to high variability within a stream reach at each sampling occasion. We could test for

this using replicate samples for epilithon and biofilm-litter. Variance partitioning within and between dates showed that, at least for these two compartments, within date (i.e., spatial) variability in $\delta^{15}N$ was low compared to variability between dates in the two streams with higher mean $\delta^{15}N$, but was more substantial at the more pristine sites (FOR and HOR; Table SB.2). In addition, this variability was mostly associated to particular sampling dates (Table SB.3) and is not likely to occur throughout the year.

Temporal variability of $\delta^{15}N$ as a function of human impact

We found that $\delta^{15}N$ values of both DIN species and PUCs increased with nutrient concentrations among streams, which is consistent with previous findings Peipoch et al. 2012, Pastor et al. 2013). In addition, results showed that temporal variability of $\delta^{15}N$ values of DIN species and PUCs also increased with nutrient concentrations among streams, being the largest at the most urban stream. Urban streams are subjected to multiple impacts (e.g. changes in hydrology, diffuse and point pollution, etc.) that result in a variety of physical, chemical, and biological effects (Paul and Meyer 2001, Walsh et al. 2005, Bernhardt et al. 2008), which in turn may affect the dynamics of DIN as well as of its $\delta^{15}N$ values. In fact, remarkable daily cycles in the isotopic composition of DIN species have been reported in streams receiving treated municipal sewage (Gammons et al. 2011), due to their high productivity and nitrification rates compared to low-nutrient streams (Pellerin et al. 2009).

Streams draining catchments with human activity are likely to receive DIN from highly diverse N sources, which may contribute to increased isotopic variation over time compared to non-impacted streams. Our models showed that in the urban stream (URB), discharge contributed up to one third of the variability explained for $\delta^{15}N$ of DIN species, which supports the role of discharge as one of the main drivers explaining the high $\delta^{15}N$- DIN variability in impacted streams (Kaushal et al. 2011). Point sources from WWTP effluents are characterized by relatively large concentrations of DIN with enriched $\delta^{15}N$ values (Ribot et al. 2012). The negative relationship between discharge and both $\delta^{15}N$- NH_4^+ and $\delta^{15}N$- NO_3^- in the receiving stream (Table SB.1) indicates a dilution of the isotopically-enriched WWTP point source during high flows. Other N sources such as diffuse urban and non-urban runoff, which are characterized by lower $\delta^{15}N$ values compared to DIN from treated sewage (Holtgrieve et al. 2011, Kendall et al. 2007, Xue et al. 2009), can additionally contribute to dilute the isotopic values of DIN in the stream under high flows.

Consistently, PUCs in the stream receiving a WWTP effluent showed the highest temporal variability in their $\delta^{15}N$ values, possibly as a result of the higher isotopic variability of their DIN sources. Likewise, greater fluctuations of stream DIN concentrations could have affected isotope fractionation processes in PUCs, enlarging their $\delta^{15}N$ variability. Additionally, elevated concentration ratios of NH_4^+ to NO_3^-, which are typically found in streams affected by WWTPs (Martí et al. 2004), were on average eight times higher in URB than in FOR, the most pristine stream; and thus could have stimulated the

uptake rates of NH_4^+. Because $\delta^{15}N$- NH_4^+ was more variable than $\delta^{15}N$- NO_3^-, this might have eventually resulted in an increase of $\delta^{15}N$ variability of PUCs.

Overall, our results suggest that PUCs receiving large nutrient inputs from anthropogenic activity might undergo larger temporal changes in their $\delta^{15}N$ values than PUCs in pristine streams. Recent literature reviews at the global scale have found a similar pattern of increasing seasonal amplitude in the $\delta^{15}N$ values in lakes, both for basal compartments (Gu et al. 2009) and for primary consumers (Woodland et al. 2012b). Thus, the temporal variability of $\delta^{15}N$ of DIN and PUCs can be seen as indicative of anthropogenic pressure, mostly from urban activity, enlarging the list of symptoms consistent with the urban stream syndrome (Walsh et al. 2005).

Temporal variability of $\delta^{15}N$ among PUC types

We expected that $\delta^{15}N$ variability would differ among PUC types because their distinct biological traits, such as biomass and structural complexity, would result in differences in the dynamics of N demand and turnover time, ultimately affecting their $\delta^{15}N$ value. Based on this premise, we used the C:N ratios of each PUC type as a surrogate of N turnover rates (Dodds et al. 2000, Dodds et al. 2004) and expected higher $\delta^{15}N$ temporal variability in PUCs with lower C:N ratios (i.e., higher N turnover rates) because they can better trace the variability in $\delta^{15}N$- DIN values. However, our data only partially supported this expectation. Variability in $\delta^{15}N$ values tended to be higher in PUCs with lower C:N ratios, such as filamentous algae, and much lower in PUCs with higher C:N

ratios and more complex structure, such as macrophytes or alder leaves. This finding is in line with the negative relationship reported between body size of aquatic consumers and their temporal isotopic variability, which has also been attributed to higher turnover times in larger organisms (Cabana and Rasmussen, 1996, Woodland et al. 2012b). Nevertheless, our results should be interpreted with caution as the correlation was weak, suggesting that other factors besides C:N may contribute to the observed variability. For instance, macrophytes on the stream-riparian banks and alder trees may rely on DIN sources from the riparian phreatic zone, which may be subjected to different variability than streamwater DIN sources (Peipoch et al. 2014). In addition, nodules of endosymbiotic N-fixing bacteria were occasionally observed in alder roots (but not sampled), suggesting atmospheric N as an additional N source for these trees (Huss-Danell 1997, Millet et al. 2012). The isotopic signature of atmospheric N is considered to be temporally stable, and thus could explain the low temporal variability in $\delta^{15}N$ of alder leaves.

In conclusion, our results suggest that streams receiving high anthropogenic nutrient inputs are likely to have greater fluctuations in their stream chemical environment and large temporal variability in $\delta^{15}N$- DIN values, especially for $\delta^{15}N$- NH_4^+. This higher variability was also observed in $\delta^{15}N$- PUC, except for those PUCs located at the stream-riparian banks, which might be decoupled from the N isotopic variability in streamwater. In- stream PUCs characterized by fast N turnover rates, such as filamentous algae, were more responsive to variability in $\delta^{15}N$- DIN and thus showed the highest variability in

their $\delta^{15}N$ values. In contrast, PUCs with larger biomass and with the ability to obtain N from sources other than streamwater DIN showed less temporal variability in $\delta^{15}N$. Overall, results from this study have two main implications. First, researchers must be aware of the high temporal variability in $\delta^{15}N$ of DIN and PUCs, especially observed in urban impacted streams. Under these conditions, it is critical to properly assess $\delta^{15}N$ of basal resources to infer trophic interactions among consumers based on the use of stable isotopes. Second, the temporal variability in $\delta^{15}N$ associated with each PUC should be considered when applying isotopic techniques as ecological monitoring tools. Our results suggest that indicator PUCs can be selected to optimally provide information on anthropogenic pressures at the aimed temporal scale of resolution.

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3

Effects of successional stage and nutrient availability on nitrogen stable isotopes of stream epilithic biofilm

Ada Pastor, Joan Lluís Riera, Marc Peipoch, Joan Gomà, Lídia Cañas, Eugènia Martí, Francesc Sabater. Effects of successional stage and nutrient availability on nitrogen stable isotopes of stream epilithic biofilm. Manuscript in preparation.

ABSTRACT

Epilithic biofilms (i.e., microbial assemblages developed on stream cobbles) can substantially contribute to in-stream nitrogen (N) cycling, but how variations in biomass accrual influence this process remains unclear. To address this question, we explored the variability of natural abundance of N stable isotopes ($\delta^{15}N$) of epilithic biofilms at an early and late successional stage in four streams differing in nutrient availability. The use of $\delta^{15}N$ provides simple ecological tools to track N assimilation and mineralization because isotopic fractionation can result in changes of $\delta^{15}N$ values. We expected that early-stage biofilm would assimilate N from the water at rates exceeding those of N mineralization; and thus, under the same isotopic N sources, would result in $\delta^{15}N$ values lower than those of late-stage biofilm. We also predicted that differences between early- and late-stage biofilm would be more pronounced at high-nutrient streams, because fractionation associated to assimilation increases with nutrient availability. We used two approaches to examine the $\delta^{15}N$ variability of different epilithic biofilms. First, we conducted a monthly-based survey, where early-stage biofilm (colonizing artificial substrates) and late-stage biofilm (attached to stream cobbles) were sampled during one year in four streams differing in nutrient concentrations. Second, epilithic biofilm development was examined for a month under low and high nutrient concentrations. The study covered a wide range of biofilm biomass (0.1 to 36.5 g ash-free dry mass [AFDM]/m^2) and $\delta^{15}N$ values (-3.6 to 22.7‰). In all streams, early-stage biofilm had lower AFDM than late-stage biofilm. $\delta^{15}N$ of biofilm was positively related to AFDM, and hence to successional stage, except in the stream with the lowest nutrient concentration. During biofilm colonization, $\delta^{15}N$ increased with AFDM, and changes were more pronounced at the high-nutrient stream. Overall, these results suggest successional stage as a relevant factor controlling $\delta^{15}N$ variability of epilithic biofilm at the local scale.

3.1. INTRODUCTION

Nitrogen (N) removal by fluvial networks has been estimated to account for more than half of the total inputs arriving from the watershed, as a consequence of N processing by benthic biota, which regulates N export to downstream ecosystems (Peterson et al. 2001, Seitzinger et al. 2002). Epilithic biofilms (i.e., microbial assemblages developed on stream cobbles) substantially contribute to stream nutrient dynamics (Mulholland 1996, Pusch et al. 1998, Dodds et al. 2000, Dodds 2003, Battin et al. 2003b), and are basic resources for aquatic consumers (Cummins and Klug 1979, Lamberti 1996). Factors influencing N biotic uptake at stream-reach scale, such as discharge and N concentrations in streamwater, have been extensively studied (Peterson et al. 2001, Webster et al. 2003, Hall et al. 2009, Mulholland and Webster 2010). However, less information is available about how underlying biological factors operate at a small scale. In this direction, recent studies have shown how epilithic biofilm growth can effect organic matter processing (Battin et al. 2003b) and N function of epilithic biofilm, which might be even better explained by biofilm biomass than N concentrations in stream (Teissier et al. 2007).

Development of epilithic biofilm starts with bacterial microcolonies, and then, algal cells, mostly diatoms, accrue from the basal layer forming the biofilm canopy (Battin et al. 2003a). Assimilation of N rises with epilithic biomass accumulation, but can be offset by dissimilation processes (i.e.

mineralization and nitrification) in thick mature biofilm (Teissier et al. 2007). Moreover, high N recycling rates within the biofilm matrix are expected in mature biofilm, because of the tight coupling between autotrophic and heterotrophic activity, but also by constraints to solute diffusion into thicker biofilms (Stewart 2003, Battin et al. 2003b). Epilithic biofilm development can be reset by multiple factors, but especially by hydrological disturbances in fluvial ecosystems (Boulêtreau et al. 2006, 2010, Graba et al. 2014), and patterns of biofilm biomass are reported to be highly variable, both temporally and spatially (Elósegui and Pozo 1998, Godwin and Carrick 2008, Merbt et al. 2011). Thus, biofilm biomass variability might drive changes in N processes and have consequences on the N concentrations delivered by streams (Stevenson and Glover 1993, Teissier et al. 2007, Arnon et al. 2007).

The use of natural abundance N stable isotopes ($\delta^{15}N$, in ‰) of epilithic biofilm provide simple ecological tools to track N interactions because processes often result in recognizable changes in isotopic ratios (Kendall et al. 2007, Ribot et al. 2012). $\delta^{15}N$ have been widely studied in ecological research during the last decades (Peipoch et al. 2012), especially to infer food web relationships (e.g. Fry 1991, Ishikawa et al. 2012). However, the high $\delta^{15}N$ variability, usually found for basal compartments, can limit their applicability in these studies (e.g. Cabana and Rasmussen 1996). Recently, $\delta^{15}N$ of epilithic biofilm has been shown to be dependent on $\delta^{15}N$ of dissolved inorganic nitrogen (DIN, mostly nitrate and ammonium), across strong gradients of human influence (Pastor et al. 2013). Biomass accrual is also likely to influence

$\delta^{15}N$ variability of epilithic biofilm, because changes in the main N processes involved in biofilm growth might result in modifications of $\delta^{15}N$. Changes in stable isotopes ratios of carbon ($\delta^{13}C$) have been observed during biofilm development (Hill et al. 2008, Hill and Middleton 2006), but information on N stable isotopes of N is still lacking.

In this study, we aimed to fill some of these gaps by exploring $\delta^{15}N$ variability of epilithic biofilm in different stages of development under contrasting stream nutrient concentrations. Because microbial N isotopic composition is determined by the balance between N assimilation and dissimilation (Dijkstra et al. 2008), we hypothesized that fractionation effects of N assimilation would be counterbalanced or overridden by dissimilation processes during biofilm growth. Thus, we predicted that early-stage biofilm would be ^{15}N-depleted compared to late-stage biofilm. Furthermore, because isotopic fractionation associated to assimilation increases with N availability (Hoch et al. 1992, Pennock et al. 1996, Waser et al. 1998), we further predicted that the isotopic effects by biofilm biomass could be expected to be more pronounced at high-nutrient streams.

We used two approaches to examine the $\delta^{15}N$ variability of different epilithic biofilms. First, we conducted a monthly-based survey, where early-stage biofilm (colonizing artificial substrates) and late-stage biofilm (attached to stream cobbles) was sampled during one year in four streams differing in nutrient concentrations. Second, biofilm development was examined for a

month under low- and high- nutrient concentrations. Collectively, these two approaches allowed us to test N isotopic differences among biofilm at different development stages, and on contrasting temporal scales and nutrient concentration conditions. Understanding the controls of biofilm $\delta^{15}N$ might provide insights into the basic N dynamics of biofilm improve the precision of the isotopic baselines and, consequently, increase the accuracy of food web analyses.

3.2 MATERIAL AND METHODS

Study site

The four sampled reaches were located in La Tordera catchment (868.5 km^2), which is situated approximately 50 km northeast of Barcelona (NE Iberian Peninsula) and characterized by siliceous lithology. They differed in their land use type adjacent to the stream, and thus had contrasting nutrient concentrations (Table 3.1; Pastor et al. in review). *Font del Regàs* is a forested stream and had relatively low concentrations of N and phosphorus (hereafter referred to as: "low-nutrient stream"). There were two agricultural sites, *Santa Coloma de Farners*, which was influenced by horticultural production (i.e. irrigated agriculture), and *Sant Celoni,* which was surrounded by extensive cropping (i.e. non-irrigated agriculture). Both these sites were characterized by intermediate nutrient concentrations and hereafter referred to as: "low/mid-nutrient stream" and "high/mid-nutrient stream", respectively. Finally, *Santa Maria de Palautordera* site was located at 800 m downstream of a municipal

wastewater treatment plant (WWTP) outfall (Table 3.1) and had the highest nutrient concentrations (hereafter referred as “high-nutrient stream”). With the exception of the high-nutrient stream, the three other sampling reaches had a well-developed deciduous riparian forest.

Table 3.1 Average and standard deviation of physical and chemical characteristics of sampled streams over sampling period. Data from: Pastor et al. in review.

	Low-nutrient	Low/mid-nutrient	High/mid-nutrient	High-nutrient
Longitude (UTM 31N)	454275	469369	455165	455763
Latitude (UTM 31N)	4630617	4635715	4618071	4614587
Altitude (m a.s.l)	528	163	240	154
Discharge (L/s)	65±40	64±51	40±30	311±337
Temperature (ºC)	10.8±3.9	13.7±4.8	12.3±4.8	15.8±5.3
SRP (µg P/L)	7.4±3.1	16.7±5.6	27.3±20.5	481.2±605.8
DIN (µg N/L)	249±83	676±257	700±336	2549±1091

Monthly-based survey in four streams

From July 2010 to July 2011, early- and late-stage epilithic biofilm was monthly sampled at the four reaches. Each month, early-stage biofilm was obtained from tiles incubated in the stream during one month and late-stage biofilm was sampled from cobbles found in the same reach. Previously to the start of the monthly-based survey (April 2010), differences in ash-free dry mass (AFDM) and diatom communities were tested between early- and late-stage biofilm at low-nutrient, mid/low-nutrient and high-nutrient streams. Biofilm biomass differed between early- and late-stage biofilm (Kruskal-Wallis test, $p < 0.05$). Dominant diatom species were similar between substrates at each stream, but differed highly among locations (Table 3.1).

Each month, tiles were sealed with silicon glue to three cement blocks and anchored with rebars to the streambed at thalweg sites in fast flowing areas (0.2-0.5 m/s). After four weeks, blocks were collected and replaced for the next sampling. Collected tiles on blocks were placed in plastic pots with streamwater for AFDM and elemental and isotopic N analyses, and wrapped in aluminum foil for chlorophyll *a* analyses (*chl a*). All tile samples were stored at -20ºC for further analysis in the laboratory.

Also at each sampling time, late-stage biofilm samples were obtained by randomly collecting three cobbles from thalweg stream areas with rapidly flowing water (0.2-0.5 m/s). Biofilm samples were also analyzed for AFDM, *chl a*, elemental and isotopic N analyses. Light-exposed sides of the cobbles were scrapped using a soft metal brush, excluding filamentous algae or bryophytes on them. Sludge of each cobble was collected onto ignited, pre-weighted glass fiber filter. For each cobble, the estimation of the surface scraped was conducted by aluminum foil cover followed by a weight-to-area relationship. Filters for *chl a* analysis were wrapped in aluminum foil and frozen at -20 ºC, whereas the remaining filters were oven-dried at 60ºC.

Colonization experiment

In May 2011, tiles glued on blocks were deployed at two sites upstream (UP) and downstream (DW) of the WWTP outfall of *Santa Maria de Palautordera* to evaluate the development of the biofilm under two contrasting nutrient environments. After 2, 8, 16 and 36 days of deployment, biofilm growing on

tiles and reference biofilm on stream reach cobbles were collected as described above. Concurrently, water samples for concentrations and isotopic characterization of ammonium (NH_4^+) and nitrate (NO_3^-) were obtained. Concentrations of NH_4^+ were lower at UP than at DW, but similar concentrations of NO_3^- were found for both sites (Peipoch et al. in review). Similarly, the $\delta^{15}N$-NH_4^+ values were lower at UP than DW, but did not differ between sites for $\delta^{15}N$-NO_3^- (Peipoch et al. in review).

Laboratory analysis

To determine AFDM, the difference of mass between dry and combusted filters after four hours at 450 ºC was calculated and reported relatively to cobble surface (units in g/m^2). For determination of *Chl a* content (units in mg/cm^2), samples were extracted in 90% acetone for 24h and analyzed by spectrophotometry (Steinman et al. 2006).

A smaller section of biofilm filters were clipped, usually a 1 cm^2-diameter circle or half of the filter, weighed and encapsulated in small tins for elemental and isotopic N content. Analyses were carried out by University of California Stable Isotope Facility (Davis, California, USA) using a continuous-flow isotope-ratio mass spectrometry (20-20 mass spectrometer; PDZ Europa, Northwich, UK) after sample combustion in an on-line elemental analyzer (PDZ Europa ANCA-GSL, Sercon Ltd., Cheshire, UK). The ^{15}N natural abundance was expressed in standard notation ($\delta^{15}N$ in ‰) relative to a standard (i.e. atmospheric N_2), where $\delta^{15}N = 1000^* [(R_{sample}/R_{standard})-1]$, and R is the $^{15}N/^{14}N$

molar ratio. The analytical precision on repeated measures of our leaf alder standard was ±0.24 ‰ (SD; n = 18). To avoid negative ratios (i.e.< 0), the ratio of ^{15}N of early-stage biofilm to late-stage biofilm was calculated in units of atom%, where atom% = 100 × [Rsample/(1 + Rsample)].

Statistical analysis

Relationships between biofilm AFDM and temperature, discharge and *chl a* were evaluated using Spearman rank nonparametric correlations at each stream. Differences in biofilm AFDM among streams and between the two periods of contrasting canopy cover of the riparian trees (leaf in/leaf out) at each stream were tested using Kruskal-Wallis test. Because we did not observed AFDM dependency on time-associated variables (i.e. temperature nor canopy cover), we used samples of each month for each stream as replicates. Differences between the characteristics of early- and late-stage biofilm were tested using Wilcoxon matched pair test for each stream separately (average data for each harvest). The relationship between the ratio of $\delta^{15}N$ of early-stage biofilm to late-stage biofilm and stream nutrient concentrations were tested using Spearman rank correlations. To estimate the relative importance of temporal vs stream-reach factors in examining the variability of $\delta^{15}N$ of early- and late-stage biofilm, for each separately stream, a mixed model was conducted using “time” as random variable and the proportion of the total variance estimated was computed. Finally, relationships between $\delta^{15}N$ and AFDM were also tested using Spearman correlations. All data analyses were

carried out using free R software, version 2.15.1 (R Development Core Team 2012).

3.3 RESULTS

Epilithic biofilm characterization

There was a wide variation in biofilm biomass (i.e. measured as AFDM), which ranged from 0.1 to 36.5 g/m^2, including early- and late-stage biofilm. No temporal patterns in biofilm biomass were found at any of the streams, neither related to streamwater temperature (Spearman correlation, $p > 0.05$) nor between growing seasons of the riparian trees (i.e. leaf in/leaf out; Kurskal-Wallis test, $p > 0.05$; except at the high-nutrient stream). Discharge was negatively related to AFDM at mid/low-nutrient stream (Spearman correlation; $r = -0.28$, $p < 0.05$) and high-nutrient stream sites (Spearman correlation; $r = -0.31$, $p < 0.05$). The content of *chl a* was significantly related to AFDM at the four streams (coefficients of Spearman correlation were 0.49, 0.78, 0.48 and 0.52 from low- to high-nutrient stream, respectively; $p < 0.001$. Biofilm biomass was higher at the high-nutrient stream compared to the low-nutrient and low/mid-nutrient stream (Kruskal-Wallis test, $p < 0.01$; Table 3.2).

For all streams, AFDM was markedly higher for late- than early- stage biofilm (Wilcoxon matched pair test $p < 0.05$) with the smallest differences found at the high-nutrient stream (Table 3.2). *Chl a* content did not show differences between early- and late-stage biofilm, except at mid/low-stream,

where late-stage biofilm was nearly two times higher than early-stage biofilm (Wilcoxon matched pair test, $p < 0.05$, Table 3.2). Thus, on average, the ratio *chl a* to AFDM was higher in early-stage biofilm than in late-stage biofilm for all sites. N content (as % of dry mass) was higher for early- than late-stage biofilm (Wilcoxon matched test $p < 0.05$), except at the mid/low-nutrient stream where no differences were found (Table 3.2).

Table 3.2 Average and standard deviation of epilithic-biofilm characteristics of sampled streams over the sampling period for early- and late-stage epilithic-biofilm.

	Low-nutrient stream		Low/mid-nutrient stream		High/mid-nutrient stream		High-nutrient stream	
	Early	Late	Early	Late	Early	Late	Early	Late
AFDM (g/m^2)	0.8±0.5	3.5±6.8	0.6±0.4	3.8±3.0	0.9±0.8	4.0±2.7	1.5±0.8	4.1±3.2
Chl a (µg/cm2)	1.2±0.9	1.1±1.0	1.0±0.8	1.8±1.8	2.9±3.8	3.2±2.9	2.9±2.9	2.8±3.2
N (%)	2.9±0.8	1.5±1.3	2.3±0.9	2.0±1.2	2.6±0.8	1.2±0.7	2.9±0.8	1.9±1.2
Dominant algal species[a]	GPEL, RSIN, ADMI		ADBI, ADMI, CPLE		n.a.		NDIS, SSEM, NIFR	

[a]Code species are the following: GPEL: *Gomphonema pumilum* var. *elegans* Reichardt & Lange-Bertalot; RSIN: *Reimeria sinuata* (Gregory) Kociolek & Stoermer; ADMI: *Achnanthidium minutissimum* (Kutz.) Czarnecki; ADBI: *Achnanthidium biasolettianum* (Grunow in Cl. & Grun.) Lange-Bertalot; CPLE: *Cocconeis placentula* Ehrenberg var.*euglypta* (Ehr.) Grunow; NDIS: *Nitzschia dissipata* (Kutzing)Grunow var.*dissipata*; SSEM: *Sellaphora seminulum* (Grunow) D.G. Mann; NIFR: *Nitzschia frustulum* (Kutzing) Grunow var.*frustulum*.

Variability in $\delta^{15}N$ epilithic biofilm at an early and late successional stage

During the sampled year, $\delta^{15}N$ values of biofilm presented a wide range of variation, from -3.6 to 22.7‰. This variability corresponded to the complete range for late-stage biofilm; in contrast, early-stage biofilm had a narrower range of $\delta^{15}N$ values, from -0.1 to 15.1‰ (Fig. 3.1). Among locations, temporal variability was the lowest at low-nutrient stream, intermediate variability at

mid-nutrient streams and the highest at high-nutrient stream, and was wider for late-stage biofilm thanearly-stage biofilm (Fig. 3.1).

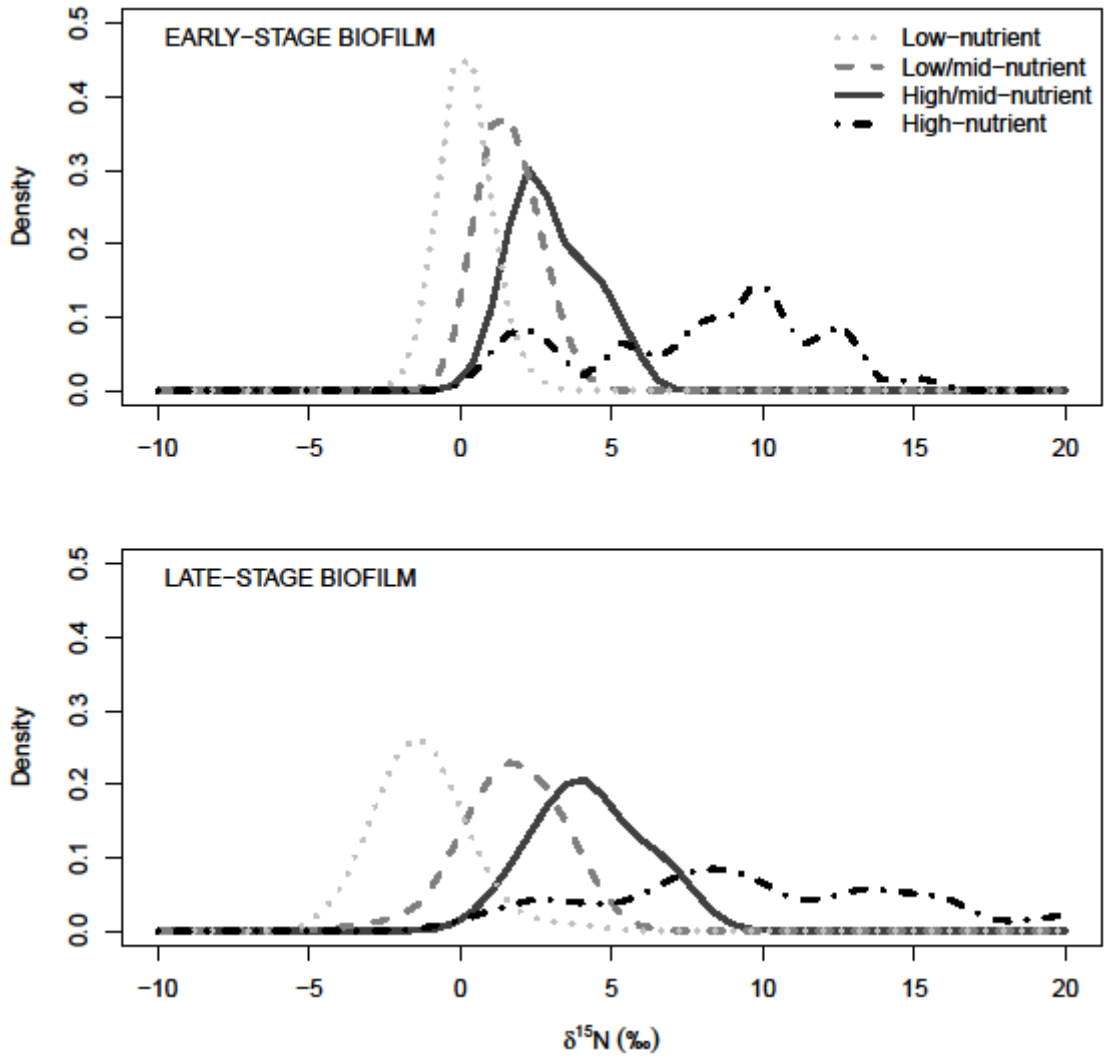

Figure 3.1 Frequency of nitrogen stable ratios ($\delta^{15}N$) of early- (n = 146) and late-stage biofilm (n = 147) at the four sampled streams during one-month basis survey.

Spatial variation within sampled reaches, measured as the amplitude of values of single sampling date, was also high and ranged from 0.5 to 17.3‰

considering biofilm at both successional stages and growing on all sites. For late-stage biofilm, variability explained among “dates”, versus “within stream-reach”, accounted for more than 80% at high/mid-nutrient and high-nutrient streams, but only around 35% at the other two streams. For early- stage biofilm a similar pattern was reported. The variability explained among “dates”, was the highest at high/mid-nutrient and high-nutrient streams (around 70%), and less at the low-nutrient (63%) and low/mid-nutrient stream (49%).

Contrasting patterns of $\delta^{15}N$ values were found between early- and late-stage biofilm depending on the location. At the low-nutrient stream, $\delta^{15}N$ values of early-stage biofilm was on average 1.4‰ higher than those reported for late stage biofilm (Wilcoxon matched pair test $p < 0.05$). The opposite pattern was found at mid/high-nutrient and high-nutrient streams; where respectively, early-stage biofilm was 1.2‰ and 2.5‰ lower, on average, than late-stage biofilm (Wilcoxon matched pair test $p < 0.05$). Not significant differences were found at mid/low nutrient stream ($p > 0.05$).

Considering all sites, the ratio of ^{15}N of early stage biofilm to late stage biofilm was negatively related to the DIN (Spearman correlation, r = -0.48, $p < 0.01$) and solute reactive phosphorous (SRP) streamwater concentrations (Spearman correlation, $r = -0.76$, $p < 0.01$) and was closer to one at low nutrient concentrations (Fig. 3.2). Thus, in low nutrient conditions, $\delta^{15}N$ tended to be slightly higher in early-stage biofilm than in late-stage biofilm, but at higher

nutrient conditions, $\delta^{15}N$ was much lower in early-stage biofilm than in late-stage biofilm.

The comparison between the yearly ranges of $\delta^{15}N$ and AFDM values of biofilm, considering both successional stages, further indicated a positively relationship at mid/low-nutrient stream (r = 0.23, p < 0.05), at mid/high-nutrient stream (r = 0.45, p <0.01) and high-nutrient stream (r = 0.40, P<0.01). However, the direction of the relationships was inversed at the low-nutrient stream (Spearman correlation; r = 0.65, p < 0.01).

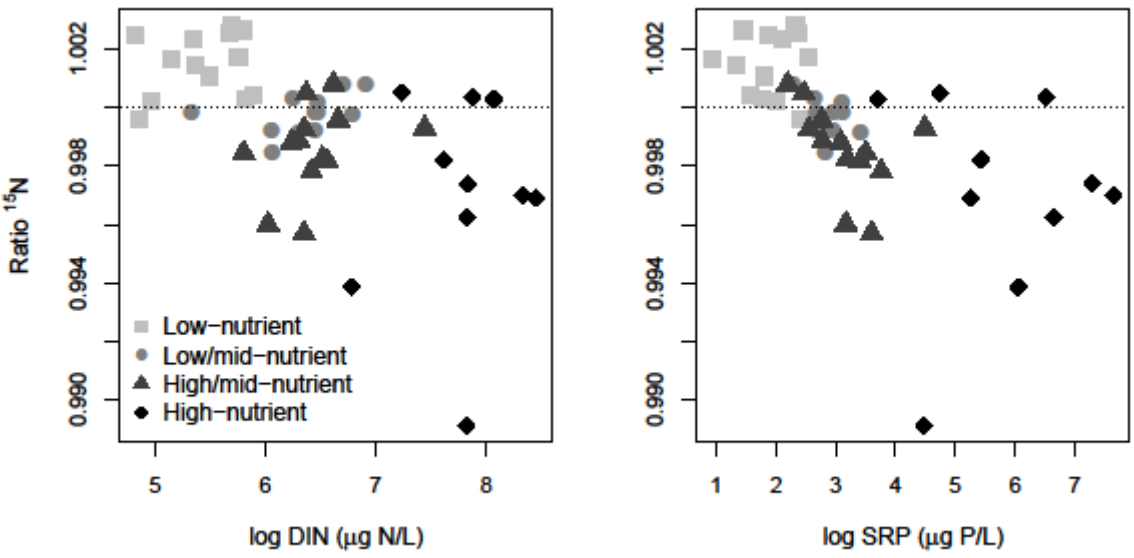

Figure 3.2 Isotopic ratio (^{15}N %) of early-stage biofilm to late-stage biofilm in relation to nutrient concentrations during monthly survey for the four sampled streams. Correlation coefficients from Spearman's nonparametric correlation analysis were for DIN concentration: r = -0.48, p < 0.01, SRP concentration: r = -0.76, p < 0.01. Dot-line represents ratio=1, when N isotopic values are the same between early- and late-stage biofilm.

$\delta^{15}N$ during epilithic biofilm development

Contrasting patterns of $\delta^{15}N$-biofilm development were found upstream (UP) and downstream (DW) the WWTP (Fig. 3.3). At UP, $\delta^{15}N$ values remained unaltered during the incubation period, with values slightly lower than $\delta^{15}N$ of reference biofilm growing on cobbles (Fig. 3.3). In contrast at DW, $\delta^{15}N$ of early-stage biofilm started off with similar values than at UP site, which highly differed from reference biofilm on cobbles at DW, and increased sharply through time. Changes in $\delta^{15}N$ of DIN species did not match changes on $\delta^{15}N$ of biofilm. At the UP site, $\delta^{15}N\text{-}NH_4^+$ increased over time (from -5.3 to -1.0‰), and $\delta^{15}N\text{-}NO_3^+$ decreased (from 10.7 to 2.8‰), whereas at the DW site $\delta^{15}N\text{-}NH_4^+$ (range from 19.5 to 25.4‰) and $\delta^{15}N\text{-}NO_3^+$ (range from 9.5 to 9.9‰) did not present any remarkable temporal trend (Data from Peipoch et al. in review). Scatter plots of $\delta^{15}N$ and AFDM confirmed the positive association between biofilm growth and ^{15}N enrichment, which was most pronounced at the DW site (Fig. 3.4).

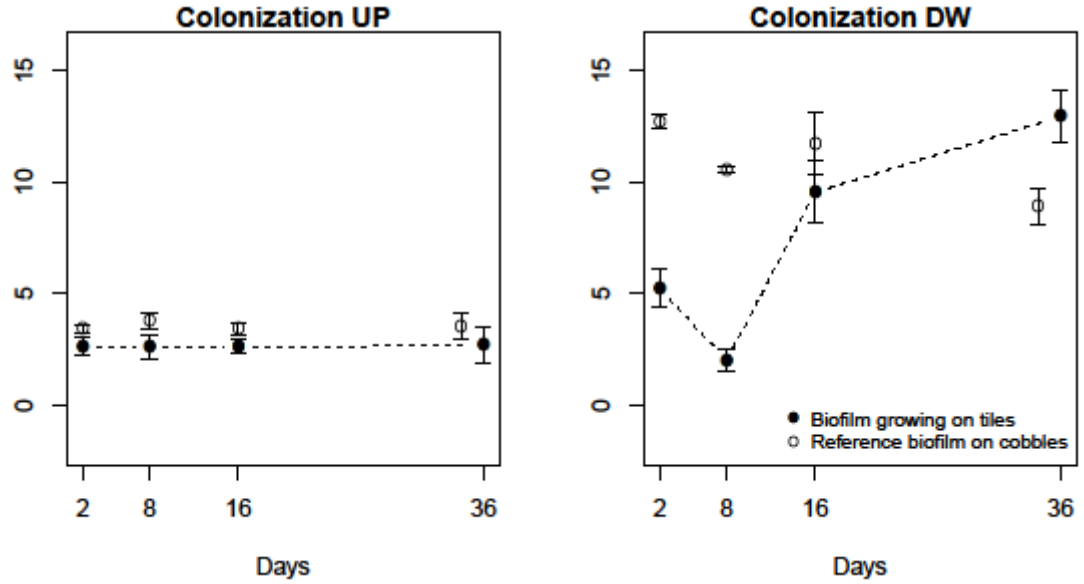

Figure 3.3 $\delta^{15}N$ of biofilm colonizing tiles (filled dots) and of biofilm on reference cobbles (white dots) upstream (UP) and downstream (DW) a WWTP. Each data point represents the mean of 3 observations; error bars represent SE. During the colonization time, $\delta^{15}N\text{-}NH_4^+$ species values were lower at UP than DW, but did not differ between sites for $\delta^{15}N\text{-}NO_3^-$. Data for $\delta^{15}N$ of DIN and $\delta^{15}N$ of reference biofilm on cobbles was obtained from Peipoch et al. in review.

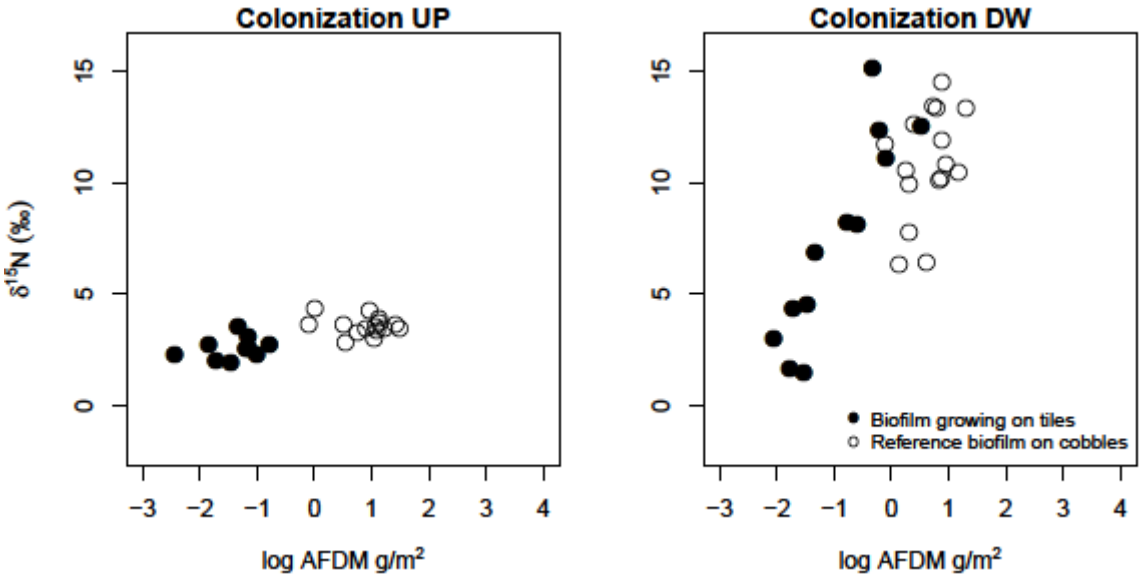

Figure 3.4 Relationships between $\delta^{15}N$ with AFDM of biofilm during the colonization experiment upstream (UP) and downstream (DW) a WWTP. Filled dots corresponded to biofilm growing on tiles for one month (sampled after 2, 8, 16 and 36 days) and white dots correspond to reference biofilm on cobble sampled concurrently. Correlation coefficients from Spearman's nonparametric correlation analysis were at UP: $r = 0.64$, $p < 0.01$; and at DW: $r = 0.62$, $p < 0.01$. Data for reference biofilm on cobbles ($\delta^{15}N$ with AFDM) was obtained from Peipoch et al. in review.

3.4 DISCUSSION

Variability in $\delta^{15}N$ epilithic biofilm at an early and late successional stage

Our study reported high $\delta^{15}N$ variability of epilithic biofilm, both within stream and over one year, which was relevant compared to the trophic enrichment of 3.4‰ usually assumed in food-web studies (Peterson and Fry 1987, Cabana and Rasmussen 1994, Post 2002). Contrasting patterns of variability for biofilm under different development stages emerged, and higher variability of $\delta^{15}N$ was associated to late-stage biofilm, compared to early-stage biofilm. Early-stage biofilm was restricted to one-month old, in contrast to late-stage biofilm which might have comprised a higher variety of life-spans, including the potential occurrence of spates or other events that might have reset their development. The falling and rising of the biofilm biomass can result in changes in $\delta^{13}C$ of biofilm (Hill and Middleton 2006), which can be integrated over a certain period of time (Singer et al. 2005). Thus, different past carryovers are expected to increase the isotopic variability of biofilm in late-stage biofilm. Our results also appear to support this hypothesis for $\delta^{15}N$ variability. Alternatively, compared with cobble substrates, flat and spatially homogenous surfaces of tiles could have had reduced microhabitat heterogeneity, diminishing the variability of $\delta^{15}N$ in early-stage biofilm (Trudeau and Rasmussen 2003).

Among streams, the highest variability was associated to the high-nutrient stream receiving a WWTP effluent. High $\delta^{15}N$ variability in several basal compartments has been reported at stream reaches influenced by WWTP,

where it has been associated to the high variability of $\delta^{15}N$ of DIN species (Pastor et al. in review). Furthermore, sludge particles can increase C isotopic spatial heterogeneity in biofilm downstream effluents from WWTP (Singer et al. 2005). The deposition of these particles, which are usually $\delta^{15}N$ enriched (Kendall et al. 2001, Ulseth and Ershey 2005), and their subsequent incorporation into biofilm (Battin et al. 2003b), would have also increased the patch-heterogeneity of $\delta^{15}N$ values at the high-nutrient stream.

Epilithic biofilm N dynamics: insights from $\delta^{15}N$ values

We hypothesized that the stage of development of biofilm would result in changes in N dynamics with predictable results on their $\delta^{15}N$ values. Young and actively growing biofilm accumulate net biomass and assimilate N from the water at rates exceeding of N dissimilation. In contrast, in late-stage biofilm, N assimilation rates can be counterbalanced by N dissimilation rates or even exceeding them (Teissier et al. 2007). Thus, we predicted that early-stage biofilm would be depleted in ^{15}N compared to late-stage biofilm at the same stream, because assimilation would predominate over dissimilation fluxes and result with higher net ^{15}N discrimination from their DIN source. The enrichment of $\delta^{15}N$-biofilm observed during the colonization experiment and the positive relationships between biomass and $\delta^{15}N$ of biofilm in three out of four streams during the monthly sampling sustained this hypothesis.

Among locations, we further expected that this relationship would be more pronounced at high nutrient concentration because the isotopic fractionation

effects would be enhanced under high nutrient availability (Hoch et al. 1992, Pennock et al. 1996, Waser et al. 1998). The negative relationship found between the ratio of ^{15}N in early-stage biofilm to ^{15}N in late-stage biofilm with nutrient concentrations, with values farther from one at high nutrient concentrations, over the annual sampling (Fig. 3.2), supported this hypothesis. Moreover, the results for the colonization experiment contributed to explain the patterns observed during the annual sampling. As predicted, the relationship between $\delta^{15}N$ and AFDM during biofilm development was also more pronounced at the high-nutrient stream, compared to the low-nutrient stream.

Surprisingly, and contrary to our expectations, $\delta^{15}N$ values in late-stage biofilm were lower than in early-stage biofilm at the low-nutrient stream. The sampled streams were not likely to be nutrient limited according to a previous experiment using nutrient diffusing substrata in similar streams within the same watershed (von Schiller et al. 2007). However, the role of the biofilm as a buffer against sporadic episodes of limited nutrient external supply might be more prevalent at the low-nutrient stream. The polysaccharide matrix of biofilm can function as a storage site for nutrients, which are entrapped by ion exchange from streamwater, and these reserves can buffer against external nutritional supply changes (Freeman and Lock 1995, Romaní and Sabater 2001). At low nutritional external supplies, these reserves might be especially relevant to fulfill nutrient requirements of microorganisms living in the biofilm, especially in biofilm with high biomass. Thus, larger nutrient pool

availability in late-stage biofilm might have resulted in high isotopic fractionation from N streamwater source, resulting in the lower $\delta^{15}N$ values for late-stage biofilm, compared to the early-stage biofilm.

During biofilm development, values of $\delta^{15}N$ in biofilm were more closely related to AFDM (i.e. higher *r*) compared to the month-basis sampling, regardless of the nutrient environment in the stream. This fact might suggest that the temporal scale which is relevant for ^{15}N incorporation into microbial biofilm should be lower than one-month resolution. This is concordance with recent studies which showed that changes of $\delta^{15}N$-DIN are quickly integrated by biofilm compartment, compared to other stream compartments such as primary consumers (e.g. Jardine et al. 2012). Our data support the notion that not only rapid changes in $\delta^{15}N$-DIN are integrated by biofilm as previously shown, but also biofilm biomass changes can quickly modify $\delta^{15}N$ values of biofilm.

Other factors not considered in this study might have also driven changes in $\delta^{15}N$ of epilithic biofilm. Here, biofilm was considered as a single functional group, while in reality biofilm is composed by an amalgam of diverse microorganisms within a polysaccharide matrix. Although there was no evidence of differences in the dominant diatom species between successional stages, heterotrophic communities are likely to be more important on late-stage biofilm than on early-stage biofilm as suggested by differences in AFDM to *chl a* ratios between them. Thus, differences in the community composition,

but also the microarquitecture of the biofilm, could have resulted in changes in nutrient diffusion (Battin et al. 2003b). Additionally, differences in solutes diffusivity could also clarify some of the unexplained N isotopic variability. For example, streams influenced by WWTP are typically characterized by elevated concentrations of NH_4^+, in relation to NO_3^- (Martí et al. 2004), with also higher $\delta^{15}N$ values for NH_4^+ (Pastor et al. in review). The higher potential diffusivity of NH_4^+ into biofilms, in relation to NO_3^- (Stewart 1998), might also contribute to explain higher $\delta^{15}N$ values in thicker biofilms under high-nutrient conditions. Although our approach does not allow us to quantify the effects of these mechanisms and their interactions, we cannot dismiss their potential effects on the $\delta^{15}N$ of biofilms. Further studies should investigate the mechanisms underlying these observed patterns of variability. However, our data supported the hypothesis that successional stage of epilithic biofilm has a relevant effect on the N isotope fractionation and can explain a significant part of the variability observed in $\delta^{15}N$ values of epilithic biofilm.

Our study suggests that $\delta^{15}N$ values can provide insights into biofilm N dynamics and indicate that the stage of biofilm development should be considered as a local-scale factor controlling N transformations in streams with further consequences on streamwater N concentrations. Moreover, biofilm biomass should be considered as another potential variable to explain the high variation in natural occurring isotope ratios of epilithic biofilm when applied as an ecological tool. This might be especially relevant during temporal monitoring after high-flow events, which can reset biomass development or at

different microhabitats that allow differences in biofilm development. Moreover, isotopic differences related to biomass can be magnified at high nutrient conditions. The consideration of biofilm biomass should improve isotopic models relying on the isotopic ratios of biofilm.

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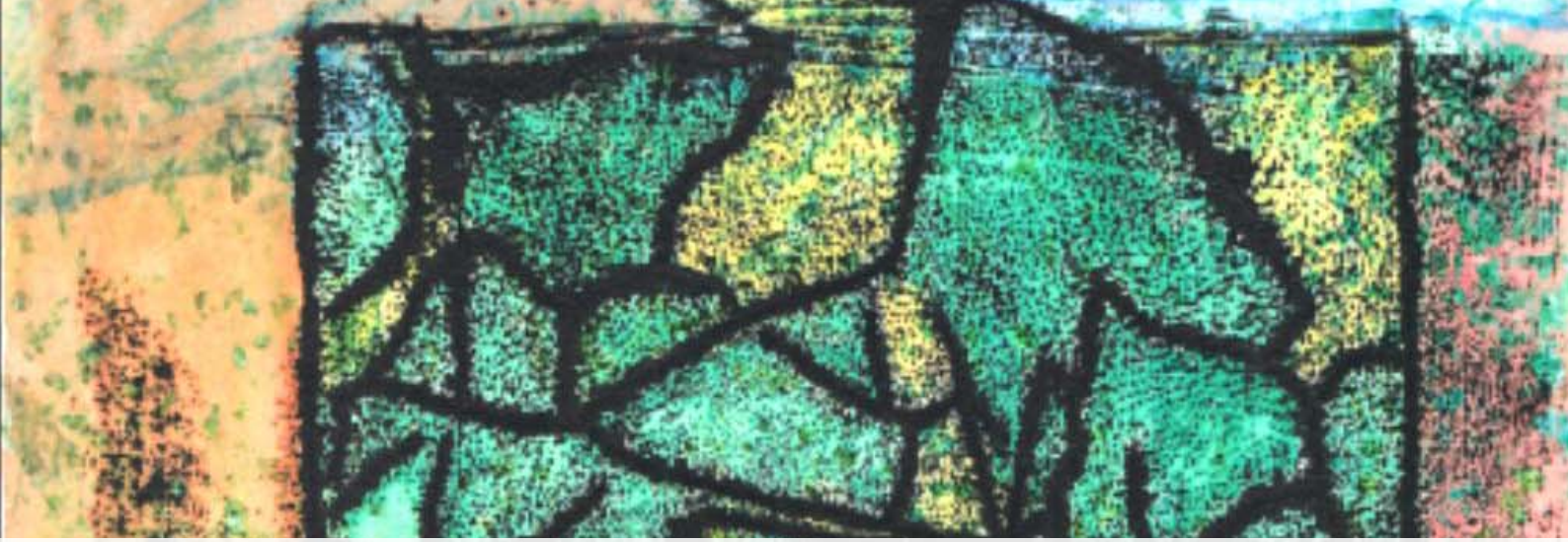

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Stream carbon and nitrogen supplements during leaf litter decomposition: contrasting patterns for two foundation species

Ada Pastor, Zacchaeus G. Compson, Paul Dijkstra, Joan L. Riera, Eugènia Martí, Francesc Sabater, Bruce A. Hungate, and Jane C. Marks. Stream carbon and nitrogen supplements during leaf litter decomposition: contrasting patterns for two foundation species, Oecologia, submitted for publication.

ABSTRACT

Leaf litter decomposition plays a major role in nutrient dynamics in forested streams. The chemical composition of litter affects its processing by microorganisms, which obtain nutrients from litter, but also use nutrients flowing downstream to supplement their nutrient demand. However, little information exists about this biogeochemical interaction with streamwater. We examined carbon (C) and nitrogen (N) flow from streamwater to microbial biofilms on litter throughout decomposition. We used isotopic enriched leaves (^{13}C and ^{15}N) from two riparian foundation species: fast-decomposing *Populus fremontii* and slow-decomposing *P. angustifolia,* which differed in their concentration of recalcitrant compounds. We used an adaptation of the isotope pool dilution method to estimate gross elemental fluxes into litter microbes over decomposition time. Three key findings emerged. (1) Litter type strongly affected biomass and stoichiometry of microbial assemblages growing on litter. (2) The proportion of C and N in microorganisms derived from the streamwater, as opposed to the litter, did not differ between litter types, but increased throughout decomposition. (3) Gross immobilization of N from the streamwater was higher for *P. fremontii* litter compared to *P. angustifolia* litter, probably as a consequence of the higher microbial biomass on *P. fremontii*. In contrast, gross immobilization of C from the streamwater was higher for *P. angustifolia* litter, suggesting that streamwater C is used as an additional energy source by microbial assemblages growing on slow-decomposing litter. These results indicate that biofilms on decomposing litter have specific element requirements driven by litter characteristics, which might have implications to the whole-stream nutrient retention.

4.1 INTRODUCTION

Leaf litter inputs are important resources for forested headwater streams (Vannote et al. 1980), strongly affecting dissolved organic carbon dynamics (Meyer et al. 1998), stream metabolism (Fisher and Likens 1973), in-stream nutrient uptake (Mulholland et al. 1985; Webster et al. 2000; Argerich et al. 2008), and stream food webs (Wallace et al. 1997). Rates of detrital mass loss are positively correlated with nutrient content and negatively correlated with concentration of recalcitrant compounds in the litter (Melillo et al. 1984, Taylor et al. 1989). Stream nutrient concentrations can also accelerate detrital mass loss rates (Meyer and Johnson 1983, Suberkropp and Chauvet 1995, Gulis and Suberkropp 2003), although this effect can be reversed at high nutrient concentrations (Carreiro et al. 2000, Woodward et al. 2012). Variation in relationships among decomposition rates, leaf characteristics (litter quality), and stream nutrient concentrations have been partially explained by different responses in biomass accrual or activity of microbial assemblages (hereafter referred to as biofilms) on leaf litter (Gessner and Chauvet 1994, Gessner 1997, Gulis and Suberkropp 2003, Stelzer et al. 2003).

Litter decomposition in streams is usually measured as net loss of litter mass and net changes in its element content over time (Tank et al. 2010). However, changes in element content are the result of simultaneous gross fluxes of elements released from and retained in the litter. Processes driving litter mass loss include chemical leaching, microbial mineralization of organic

matter, physical fragmentation, and breakdown by stream consumers. Additionally, biofilms growing on litter assimilate organic carbon (C) and nitrogen (N) from the leaf and/or from streamwater, which can be further lost from the biofilm-litter system through respiration, deamination, and mineralization. Concurrently, C and N gross fluxes from streamwater into the biofilm-litter system take place due to silt deposition and abiotic adsorption, (Bott et al. 1984, Webster and Benfield 1986).

Several lines of evidence are provided to explain biofilm immobilization of dissolved organic C (DOC) and dissolved inorganic and organic N from streamwater. First, C and N stoichiometry of litter frequently does not fulfill the elemental requirements of biofilms because microorganisms have lower C to N ratios than the litter substrate (Sterner and Elser 2002, Parton et al. 2007). Second, recalcitrant compounds in litter are less readily available resources for biofilms (Gessner and Chauvet 1994) and might enhance biofilm DOC and N uptake from the water column. Third, DOC and N in the water could occur in readily available forms and are thus easily assimilated (Wiegner et al. 2005, Kaplan et al. 2008). Therefore, differences in litter characteristics may influence nutrient immobilization from streamwater during decomposition. Finally, biofilms may vary in biomass and composition depending on litter types (Wymore et al. 2013, Frossard et al. 2013). The accumulation of microbial biofilm on the decomposing leaf increases the capacity to assimilate elements from the surrounding environment by presenting a higher assimilating surface area to the surrounding water column. Furthermore, the nature of this

assimilating surface could influence the stoichiometry of microbial biosynthesis and the need to import elements from streamwater into the leaf-biofilm complex.

The aim of this study was to understand the biogeochemical interaction between the biofilm-litter system and the streamwater during litter decomposition. In particular, we quantified the relative importance of the C and N fluxes from streamwater into biofilm on litter. We used ^{13}C and ^{15}N enriched leaf litter and applied a variation of the isotope pool dilution method (Kirkham and Bartholomew 1954), which has been widely used in soil biogeochemistry to study nutrient dynamics during decomposition in soils (Murphy et al. 2003). This method consists of tracing the rate at which the isotopic value of an artificially enriched element pool declines due to the mass fluxes from an un-labeled pool (Kirkham and Bartholomew 1954).

We used ^{13}C and ^{15}N labeled litter from two foundation riparian tree species, *Populus fremontii* and *Populus angustifolia*. Phytochemical differences (i.e. tannins, lignin; Table 4.2) between these species, especially for tannins content, have been documented to drive changes in their decomposition rates with implications for adjacent terrestrial and aquatic ecosystems (Driebe and Whitham 2000, LeRoy et al. 2006, Whitham et al. 2006, Holeski et al. 2012). Here, we expected that microbes growing on litter with higher content of recalcitrant compounds would show a relatively greater reliance on C and N from streamwater than those growing on leaves with lower content of

recalcitrant compounds because these compounds are less accessible resource for heterotrophic microbes. Understanding the relative importance of C and N sources for biofilms on litter and how it varies during decomposition and among litter types will yield insights on the mechanisms driving litter decomposition, how decomposition controls the flux of C and N to the microbial food web, and the basic microbial and chemical controls on stream biogeochemical cycling.

4.2 MATERIAL AND METHODS

Study Site

This study was conducted in upper Oak Creek (1600 m a.s.l) on the southern edge of the Colorado Plateau (35˚02´N, 111˚43´W; Arizona, USA). Oak Creek is a first-order stream, which drains a 77,450 km^2 catchment extensively covered by ponderosa pine (*Pinus ponderosa*). This area is characterized by steep topography and limestone and sandstone bedrock (LeRoy et al. 2006; Wymore et al. 2013). The riparian vegetation is predominately deciduous, including Fremont cottonwood (*P. fremontii*), narrowleaf cottonwood (*P. angustifolia*), Arizona alder (*Alnus oblongifolia* Torr.), Arizona sycamore (*Platanus wrightii* S. Wats.), coyote willow (*Salix exigua* Nutt.), and Goodding's willow (*Salix gooddingii* Ball; LeRoy et al. 2006).

This experiment was conducted from November to December 2011. During this time, discharge, streamwater temperature, pH, oxygen concentration and

specific conductivity were relatively constant and concentrations of stream nutrients and isotopic values of dissolved N and DOC were low (Table 4.1).

Table 4.1 Physical and chemical parameters measured at Oak Creek during the experimental period. Range of values or SE between brackets.

Parameter	Mean (range or SE)
Discharge ($m^3 s^{-1}$)	1.0 (0.9 - 1.7)
Temperature (ºC)	11.4 (11.3 - 11.5
pH	7.1 (7.0 - 7.3)
SpC ($\mu S\ cm^{-1}$)	295.7 (294.4 - 297.8)
DO ($mg\ L^{-1}$)	8.6 (8.3 - 8.8)
DO(%)	94.2 (91.6 - 95.1)
NH_4 ($mg\ N\ L^{-1}$)	0.05 (±0.00)
NO_3 ($mg\ N\ L^{-1}$)	0.06 (±0.00)
DOC ($mg\ C\ L^{-1}$)	0.52 (±0.03)
^{13}C-DOC (atom %)	1.08 (±0.00)
$^{15}N\text{-}NO_3^-$ (atom %)	0.37 (±0.00)

SpC=Specific Conductivity; DO=Dissolved Oxygen;

Field experiment with labeled leaf litter

Tree cuttings of *P. fremontii* and *P. angustifolia,* from the Ogden Nature Center common garden (Ogden, Utah, USA) were grown at the NAU Arboretum Research Greenhouse. Plants were grown in a hydroponic nutrient solution with $(^{15}NH_4)_2SO_4$ and pulsed with 99 atom% $^{13}CO_2$ for four hours twice a week for four months (Compson et al. in review). Naturally senesced leaf litter was collected, air-dried and stored. For each genotype, litter was mixed and three

composite samples were analyzed for initial C and N content and isotope composition using a Carlo Erba NC 2100 Elemental Analyzer (CE Instruments, Milan, Italy) interfaced with a Thermo-Finnigan Delta Plus XL (Thermo-Electron Corp., Bremen, Germany) isotope ratio mass spectrometer (IRMS) at the Colorado Plateau Stable Isotope Laboratory (CPSIL; http://www.isotope.nau.edu). *P. angustifolia* litter (high-tannin litter), had higher % C values than *P. fremontii* litter (low-tannin litter), but % N and C:N did not statistically differ (Table 4.2).

Table 4.2 Initial litter characteristics and decomposition dynamics for *Populus fremontii* and *P. angustifolia* (mean and SE).

	P.fremontii **(low-tannin litter)**	***P. angustifolia*** **(high-tannin litter)**	**Statistical significance**
Leaf litter label			
^{13}C (atom %)	2.20±0.98	2.02±0.64	
^{15}N (atom %)	3.57±1.60	3.13±0.99	
Initial leaf litter characteristics			
Soluble condensed tannin (%)[a]	0.11±0.06	1.94±0.49	t_8= 3.69; P<0.01
Bound condensed tannin (%)[a]	0.17±0.02	2.91±0.34	t_8= -6.53; P<0.01
Lignin (%)[a]	9.58±0.18	23.05±1.39	t_8= -7.72; P< 0.001
% C	38.0±0.6	41.2±0.5	t_{13}=-3.77; P<0.005
% N	3.3±0.5	3.0±0.2	*n.s.*
C:N	12.6±1.7	15.0±1.0	*n.s.*
Decomposition dynamics			
Decompositon rate constant (k; day^{-1})	0.063±0.002	0.037±0.004	t_{13}=4.70, P<0.001

n.s. stands for not statistically significant at alpha = 0.05; [a] Data from Wymore et al. 2013

Litter was incubated in the stream using fine mesh litterbags (10.5 x 10.5 cm^2, 0.5 mm mesh), which were deployed in the river zip-tied to rebar on 10-Nov-2011. Each litterbag contained 1 g of leaf litter. After 6, 13, 20 and 27 days of the experiment, 45 litterbags were collected from the stream (only *P. angustiolia* litterbags were collected for the final harvest). Upon harvest, litterbags were placed into zip-lock bags, and transported on ice to the laboratory where they were processed within 24 hours.

For each harvest, dissolved oxygen (DO), conductivity, pH and water temperature were determined using a Hydrolab Minisonde (Hydrolab-Hach Corporation, Loveland, CO, USA) in a 5-point transect along the experimental reach. Stream discharge data were obtained from the United States Geological Survey (USGS) Oak Creek weather station. Three replicate water samples (~4 L each) were collected upstream from the experimental reach on day 13 of litterbag incubation and analyzed for nitrate (NO_3^-) and organic carbon (DOC) concentration and isotope composition. Water was filtered through a 0.2 µm Acrodisk filters and analyzed colorimetrically for ammonium and nitrate concentration using an autoanalyzer (Lachat Quickchem FIA+8000, Lachat Instruments, Milwaukee, WI, USA). DOC concentration was analyzed by the persulfate oxidation method with an OI Analytical Model 1010 Total Carbon Analyzer connected to a Delta Plus Advantage IRMS. The $\delta^{15}N$ of NO_3^- was determined by reduction to N_2O followed by coupled gas chromatography (Thermofinnigan Precon and Delta Advantage IRMS), using the denitrifier method (Casciotti et al. 2002) at CPSIL.

Laboratory analysis

Litter was removed from the litterbags, rinsed with deionized water, and wet mass was recorded. At each harvest date, litter content from the three replicate bags were pooled together and homogenized, resulting in five composite samples for *P. fremonti* and ten for *P. angustifolia*. Each composite sample was subsequently split into two subsamples, one for bulk litter elemental and isotope analysis (~1 g wet weight), and the rest for determination of microbial biomass.

Percent moisture of litter samples was determined by weighing bulk-litter before and after oven-drying at 60ºC for 24 h. Dried litter was ground with mortar and pestle to a fine powder and a subsample was analyzed for C and N content and isotopic composition at CPSIL, as described above. Subsamples for microbial biomass determination were processed using an adaptation of the chloroform fumigation-extraction technique, originally developed for soils (Brookes et al. 1985; Vance et al. 1987) and later modified for stream detritus (Mulholland et al. 2000; Sanzone et al. 2001; Cheever et al. 2013). Litter was extracted with 50 mL of 0.05 M K_2SO_4, stored on ice overnight, shaken for one hour, and centrifuged at 9,800 *g* for 10 minutes, after which the supernatant was poured off and discarded. Litter samples were then placed in glass beakers in a desiccator and fumigated with alcohol-free chloroform. The desiccator was evacuated until chloroform boiled. Samples were vented three times, and then sealed under vacuum and kept in the dark for 24 hours. Fumigated samples

were then removed from the desiccator, extracted with 50 mL of 0.05 M K_2SO_4, shaken for one hour, and centrifuged at 9,800 *g* for 10 minutes. The supernatant was filtered through 1.2 µm filters (*Supor®* Membrane, PALL Live Sciences, NY, USA) and placed in a ventilated oven (60ºC) for 48 hours. Dried K_2SO_4 salt with extracted C and N from the microbial biomass was ground with a mortar and pestle to a fine powder, weighed, and analyzed for C and N elemental and isotope composition as described above. To calculate immobilization rates, ^{15}N and ^{13}C isotopic values were expressed in atom percent excess (at% excess); that is, ^{13}C atom (at% excess) = $100 \times (^{13}C/(^{13}C+^{12}C))$-1.0111% and ^{15}N atom (at% excess) = $100 \times (^{15}N/(^{15}N+^{14}N))$ - 0.3663%. For salt samples, the precision of the international standard NIST 2711 MT soil was $\pm 5.8 \times 10^{-7}$ at% excess for ^{13}C and $\pm 7.3 \times 10^{-7}$ at% excess for ^{15}N (standard deviation of 6 replicate samples). The precision of the NIST peach leaves standard, when bulk litter samples were run, was $\pm 6.2 \times 10^{-6}$ at% excess for ^{13}C and $\pm 2.6 \times 10^{-4}$ at% excess for ^{15}N (standard deviation of 32 replicate samples).

Parameters calculations

To characterize decomposition rates for litter types, we calculated the leaf litter decomposition rate constant (*k,* in units of day^{-1}) as the slope of the log-transformed percentage of remaining mass over time (Benfield 2006). Microbial biomass, in terms of C (MB_C) and N (MB_N), was estimated using the C and N content in the fumigated litter samples for each date. MB_C and MB_N were expressed per unit of litter mass (i.e., mg C or N g $litter^{-1}$) to compare results

among harvest dates and between the two litter types. The change in isotopic and N isotope composition in the fumigated litter samples was used in a two-end member mixing model to quantify the relative contribution of leaf litter and water column C and N to microbial biomass (Phillips and Gregg 2003; Boecklen et al. 2011). For each harvest date, the percentage of C or N in microbial biomass derived from streamwater was calculated using the following equations:

$$(1)\ \%\ C\ streamwater\ source = \frac{^{13}C\ microorganisms - {}^{13}C\ leaf}{^{13}C\ DOC - {}^{13}C\ leaf} \times 100$$

$$(2)\ \%\ N\ streamwater\ source = \frac{^{15}N\ microorganisms - {}^{15}N\ leaf}{^{15}N\ NO_3^- - {}^{15}N\ leaf} \times 100$$

where ^{13}C and ^{15}N in microorganisms are the isotopic values (in atom %) of the chloroform-extracted fraction, ^{13}C and ^{15}N in leaf are the initial isotope values (in atom %) measured in the leaves, and ^{13}C-DOC and ^{15}N-NO_3^- are the isotope values (in atom %) measured in the streamwater samples (Table 4.1). Thus, the percentage of C and N in microbial biomass derived from leaf source are the remaining percentage, that is % C leaf source = 100 - % C streamwater source and % N leaf source = 100 - % N streamwater source.

To quantify C and N fluxes from the water column into the biofilm-litter system on each harvest date, gross immobilization rates of C and N (GI_C and GI_N, respectively; in mg C or N g litter^{-1}day^{-1}) were calculated using the isotope pool dilution method (Kirkham and Bartholomew 1954, Hart et al. 1994):

$$(3)\ GI_{(\Delta t)} = \frac{M_i - M_f}{t_f - t_i} \times \frac{\log \frac{M_{i\ at\%\ excess}}{M_{f\ at\%\ excess}}}{\log \frac{M_i}{M_f}}$$

where M is the mass of C or N in the biofilm-litter system at the initial (t_i) and final (t_f) time (in mg C or N) and $M_{i\ at\%\ excess}$ and $M_{f\ at\%\ excess}$ are the ^{15}N or ^{13}C at% excess of the biofilm-litter system for the same interval. Therefore, the measurement of GI is based on the dilution of the isotope composition of the biofilm-litter system over time as a reflection of the import of C and N from the un-labeled water column pools. In order to integrate the isotopic data from all harvests, we generated linear models of decay of ^{15}N and ^{13}C isotope for each replicate, and then used the measured isotopic values of the litter (at% excess) for the initial and final pool, for the interval of time studied, as input into equation 3. Finally, GI rates of C and N were standardized by MB_C and MB_N, respectively, as a measurement of microbial efficiency for GI of these elements, which can be compared between litter types and among sampling dates.

Data analysis

Two-group Student's t-tests were used to compare values of k between litter types. Analyses of covariance (ANCOVA) were used to analyze the effect of litter type, with harvest days as covariate, on MB_C and MB_N, % of microbial mass derived from the leaf source for both C and N, and GI_C and GI_N. To test for differences between litter types in the GI_C: GI_N, we bootstrapped the difference of these ratios between litter types (1000 iterations for each time point and

species), determined the 95% confidence intervals for this difference, and determined whether it overlapped with zero. The ResampleStat add-in for Excel software was used for the bootstrapping procedure (http://www.resample.com/excel/). All other statistical analyses were conducted using R, version 2.15.1 (R Development Core Team 2012).

4.3 RESULTS

Decomposition rates and microbial biomass

As expected, leaf litter decomposition rate was significantly higher for low-tannin litter (*P. fremontii*) than high-tannin leaf litter (*P. angustifolia;* Table 4.2). For the two litter types, microbial biomass, either measured as C or N biomass, increased until day 13, but then leveled off. Microbial C:N showed the opposite pattern, decreasing sharply at the second harvest, then leveled off. Microbial biomass (both as C and N) per g of litter was higher in litter with low-tannin content litter than in high-tannin litter (ANCOVA: MB_C: $F_{1,42}$= 32.09, p < 0.0001; MB_N: $F_{1,42}$= 38.38, p < 0.0001; Fig. 4.1A, 4.1B). In contrast, microbial C:N was higher in *P. angustifolia* litter than in *P. fremontii* (ANCOVA, $F_{1,42}$= 12.39, p < 0.01, Fig. 4.1C). Microbial C accounted for 2.7 to 8.4% of the total litter C pool in *P. fremontii* litter and between 1.1 to 5.3 % for *P. angustifolia* litter. Microbial N represented between 4.7 and 18.5 % for *P. fremontii* litter and between 2.1 and 15.0% for *P. angustifolia* litter.

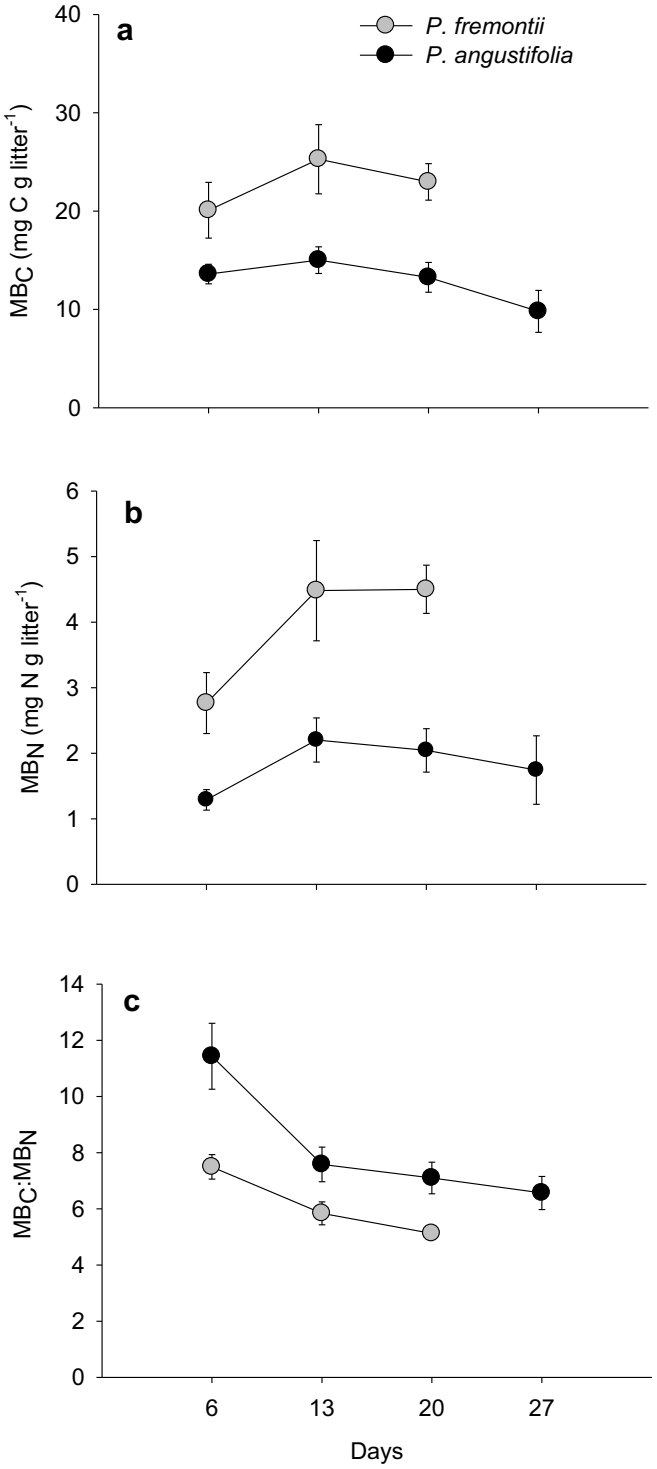

Figure 4.1 Temporal variation of microbial biomass carbon (a), nitrogen (b) and C:N mass ratio (c) during the leaf litter decomposition period for *P. fremontii* (grey circles, n = 5) and *P. angustifolia* (black circles, n = 10). Data points are means and vertical bars represent standard errors

Relative contribution of microbial C and N from the streamwater

The proportion of C and N derived from streamwater increased during the incubation, and by the end accounted for 32% for C and 38% for N (average values) of the microbial biomass (Fig. 4.2). For both litter types, leaf litter was the major source of C and N for the growth of microbial assemblages (average over the two litter types: 89±2% for C, 81±3% for N; $t_{52} = 3.59$, $p < 0.001$; Fig. 4.2). We did not find significant differences between leaf types in the percentages of C and N in microbial mass that were derived from the streamwater (ANCOVA: $p > 0.05$).

Immobilization rates of C and N from streamwater into the biofilm-litter system

The gross immobilization rate of C was on average almost two times higher for *P. angustifolia* litter ($GI_C = 3.79 \pm 0.41$ mg C g leaf^{-1} day^{-1}) than for *P. fremontii* litter ($GI_C = 1.94 \pm 0.59$ mg N g leaf^{-1} day^{-1}; Fig. 4.3A, ANCOVA: $F_{1,42} = 5.55$, $p < 0.05$). The pattern reversed for gross microbial N immobilization, which was, on average, two times higher for *P. fremontii* litter ($GI_N = 0.08 \pm 0.02$ mg N g leaf^{-1} day^{-1}) compared to *P. angustifolia* litter ($GI_N = 0.16 \pm 0.02$ mg C g leaf^{-1} day^{-1}; Fig. 4.3B, ANCOVA: $F_{1,42} = 6.82$, $p < 0.05$). The ratio between GI_C and GI_N was significantly higher for *P. angustifolia* litter (on average: 35.2) than for *P. fremontii* litter (on average: 13.9; the 95% confidence interval for the difference, 6.5-59.6, Fig. 4.3C). GI_C standardized by the microbial C content was nearly three times higher for *P. angustifolia* litter (mean: 0.40 ± 0.06 mg C mg

$MB_C^{-1}day^{-1}$) than for *P. fremontii* litter (mean: 0.12 ± 0.05 mg C mg $MB_C^{-1}day^{-1}$; ANCOVA: $F_{1,42} = 7.09$, $p < 0.05$). In contrast, there was no difference between litter types for GI_N standardized by the microbial N content (average for both species: 0.07 ± 0.01 mg N mg $MB_N^{-1}day^{-1}$; ANCOVA: $p > 0.05$).

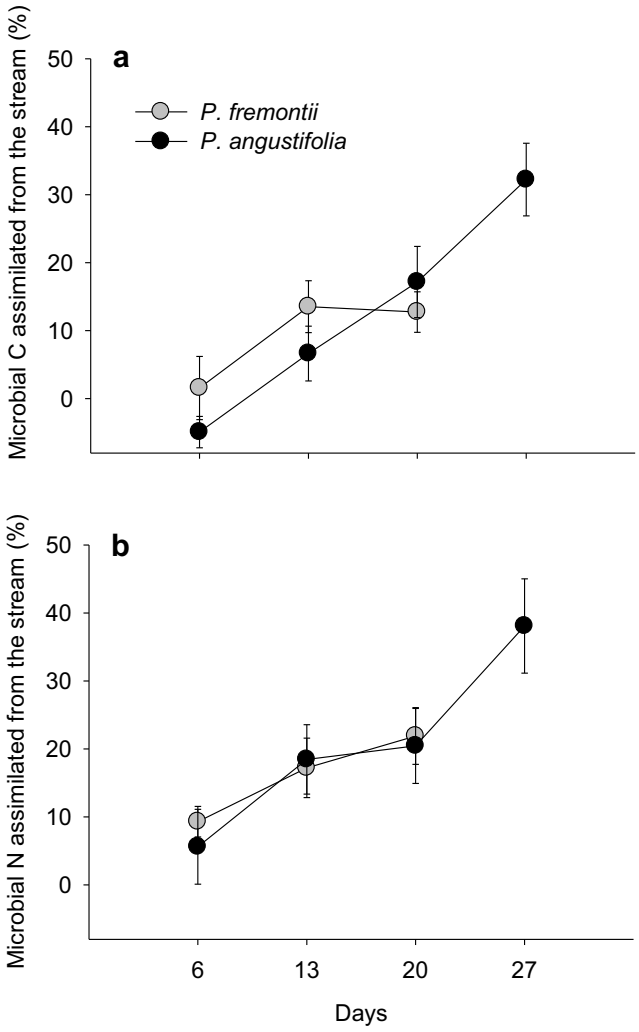

Figure 4.2 Temporal variation of the percentage of carbon (a) and nitrogen (b) in the microbial assemblage that is derived from the streamwater for *P. fremontii* (grey circles, $n = 5$) and *P. angustifolia* (black circles, $n = 10$). Due to method error, some percentages are lower than 0%. Data points are means and vertical bars represent standard errors.

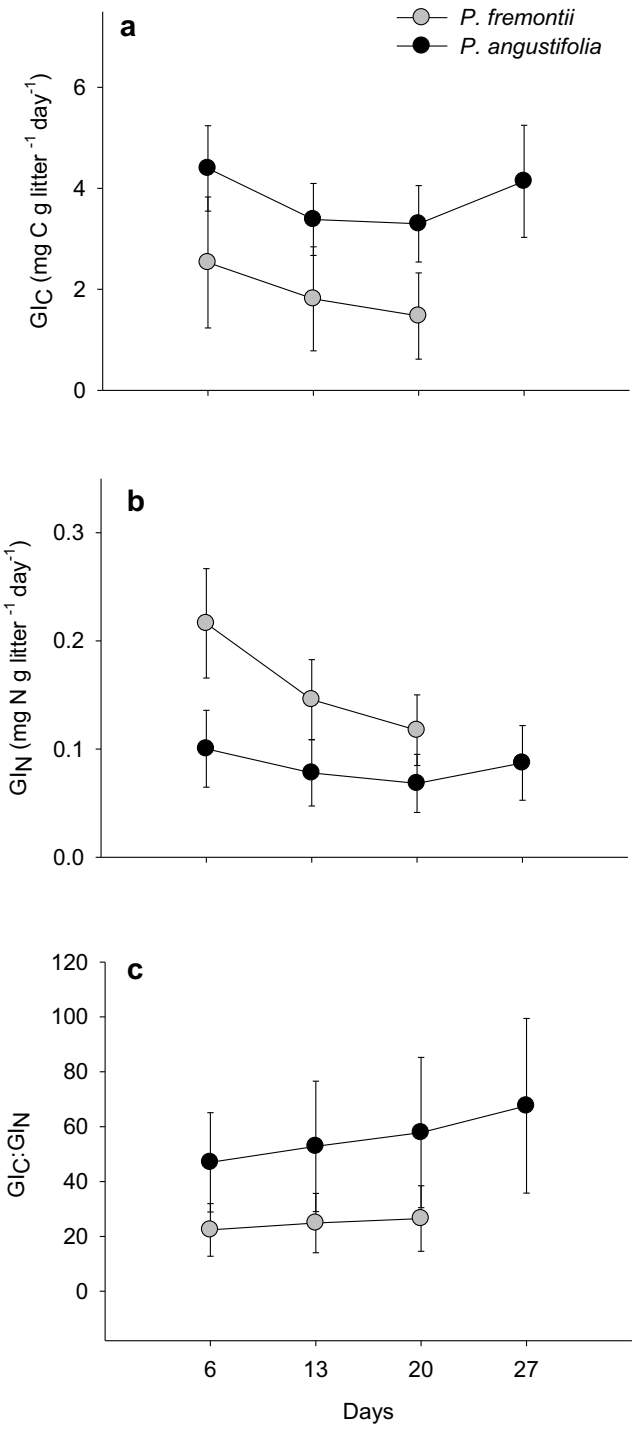

Figure 4.3 Temporal variation of gross immobilization rates for carbon (a) nitrogen (b) and their stoichiometric relationship (c) for *P. fremontii* (grey circles, n = 5) and *P. angustifolia* (black circles, n = 10). Data points are means and vertical bars represent standard errors.

4.4 DISCUSSION

The main goal of this study was to explore the capacity of the biofilm-litter system to immobilize nutrients from the streamwater during the decomposition process. Our results indicated that litter phytochemical characteristics had strong effect on biomass and stoichiometry of microorganisms growing on litter. Immobilization of C and N from streamwater into biofilm-litter compartment also presented differences between both litter types (Fig. 4.3), which suggested these microbial assemblages might have different C and N demand from streamwater driven by leaf characteristics.

Litter decomposition and microbial biomass

Decomposition rates differed among the two cottonwood species, as previously shown (Driebe and Whitham 2000, LeRoy et al. 2006, Holeski et al. 2012). Leaf litter with lower recalcitrant compounds content (*P. fremontii*) accrued more microbial biomass compared to litter with higher recalcitrant compounds content (*P. angustifolia)*. This finding is in agreement with results from previous studies where recalcitrant litter types showed low microbial biomass accrual (Gulis and Suberkropp 2003, Talbot and Treseder, 2012, but see LeRoy et al. 2007). In addition, elemental stoichiometry of biofilms differed between litter types, with higher C:N values for high-tannin litter. Differences in C:N ratio among microbial biofilms on litter might be explained by

differences in the composition of the microbial assemblage, as filamentous fungi often have higher C to N ratios than bacteria (Sterner & Elser, 2002; Strickland & Rousk, 2010). Our results suggest that the microbial biofilm colonizing high-tannin litter might have a relatively higher abundance of fungi than biofilms on low-tannin litter. This is further supported by a related study on decomposing cottonwood litter in the same stream reach where qPCR results revealed a higher fungi:bacteria gene abundance ratio for *P. angustifolia* than for *P. fremontii* (Wymore et al. 2013). Fungi may be better competitors in more recalcitrant leaves due to their hyphal networks and enzymatic capabilities to break down recalcitrant materials compared to bacteria (Kohlmeier et al. 2005; Boer et al. 2005; Moorhead and Sinsabaugh 2006; Romaní et al. 2006).

The relative contribution of C and N from streamwater during decomposition

The reliance on elemental resources from streamwater by biofilms was low at the beginning of the decomposition process and increased with time for both litter types, probably as labile compounds were used up by microbes or leached out of the leaf. Previous studies reported similar patterns. For example, Cheever et al. (2013) showed that microorganisms colonizing decomposing leaves acquired more N from the streamwater during late decomposition stages compared to early stages, deriving up to 80-90% of N from the water column by the end of the decomposition experiment (i.e. 12-15 weeks). In other systems, such as a N-rich estuary, microbial assimilation of

DIN into particulate organic material also increased with time, reaching nearly 70% by the end of the experiment (Caraco et al. 1998). Information of microbial dependency from streamwater for C is mostly limited to microorganisms in sediments, where DOC has been estimated to support up to half of their metabolism (Findlay et al. 1993; Fischer et al. 2002; Sobczak and Findlay 2002; Wiegner et al. 2005). Considering C and N together, our data suggest that N derived from streamwater is a more important supplement for microbial growth than C. This was expected based on the stoichiometric constraints faced by microorganisms growing on litter (Sterner and Elser 2002).

Immobilization of C and N into biofilm-litter system: contrasting patterns between litter types

Leaf species differed in the stoichiometry of C and N fluxes from the streamwater to the biofilm-litter system. Gross immobilization rates of N were higher on low-tannin litter compared to litter with high-tannin content, contrary to our expectations, probably because higher content of recalcitrant compounds in the latter slowed down microbial growth and consequently reduced N demand from the streamwater. This is supported by specific rates of N immobilization (per unit microbial biomass) which did not differ between litter types.

In contrast, gross immobilization rates of C were higher in high-tannin litter, even when standardized by microbial biomass, indicating higher import of C from streamwater into the biofilm-litter compartment in high-tannin litter.

Because tannin compounds are associated with phenolic molecules, it is reasonable to think that C in the high-tannin litter is a less accessible resource, such that microorganisms obtain C more efficiently from the water column. Thus, observations for C immobilization rate support to our hypothesis that concentration of recalcitrant compounds in litter would increase the dependence on streamwater by microbial biofilms.

Our immobilization estimates might have included other inputs of C and N besides active uptake by microbes, such as abiotic chemical adsorption and deposition, but the isotopic dilution observed over time suggested the relevance of the biotic uptake over these other processes. Thus, the application of the isotope pool dilution method with labeled litter proved successful and enabled us to discern contrasting patterns in element immobilization fluxes during the decomposition stages of different leaf litter.

Ecological implications

Terrestrial litter inputs are one of the most important resources in forested headwater streams, providing nutrients and energy to aquatic ecosystems (Vannote et al. 1980). Cottonwoods are dominant in riparian zones of the western United States, providing more than 80% of the litter to these streams (Driebe and Whitham 2000). They are often considered foundation species due to their large effects on ecosystem structure and function (Whitham et al. 2006); specifically, the influence of recalcitrant compounds of *Populus* litter has significant impacts on C and N dynamics within terrestrial ecosystems

(Schweitzer et al. 2004, Schweitzer et al. 2008). Our findings extend these ideas, demonstrating that the *Populus* system modulates elemental fluxes in streamwater during decomposition, with initial litter characteristics likely driving nutrient cycling during decomposition (Parton et al. 2007).

Forested headwater streams are usually considered subsidiary ecosystems because they are energetically dependent on detrital inputs arriving from their adjacent terrestrial ecosystem (Fisher and Likens 1973). Terrestrial inputs, however, are often not readily available resources for aquatic ecosystems, therefore requiring biogeochemical interactions with streamwater to supplement deficiencies in carbon and nutrients, especially when the resource is relatively recalcitrant. Understanding streamwater biogeochemical interactions with litter should provide insights into nutrient retention in streams, which are responsible for the breakdown, nutrient transfer, and transport of this resource. This is especially relevant in forested headwater streams, which are considered key sites for nutrient retention and transformation along the stream continuum where inorganic nitrogen uptake rates often account to be more than half of the total input arriving from the watershed (Alexander et al. 2000; Peterson et al. 2001). Overall, our results indicate that litter characteristics of two cottonwood species drove specific streamwater element requirements of biofilms and suggest that changes in the proportion of inputs arriving into the streams of these two cottonwood species can have strong control on stream cycling and export downstream.

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General discussion and conclusions

GENERAL DISCUSSION

This dissertation aimed to study the N biogeochemical interactions between streamwater and the most representative PUC types in stream ecosystems, and to elucidate some of the main environmental and biological factors controlling them, by using N stable isotopes. The first section of this general discussion is focused on the spatial and temporal patterns observed for natural abundance of ^{15}N in DIN species and PUCs within La Tordera fluvial network. The second section analyzes contrasting patterns of interaction with streamwater found among and within PUC types. The third section discusses the implications of this work and briefly addresses some new avenues of investigation which are left open in this work.

D.1 Patterns of ^{15}N natural abundance variability across a strong anthropogenic gradient

Higher and more variable: the effects of humans on $\delta^{15}N$ in streams

La Tordera catchment constitutes a heterogeneous watershed with a rich land use mosaic. This translates into a large variability in the amount of nutrient concentrations among stream reaches along the fluvial network (von Schiller et al. 2008, Caille et al. 2011). In *Chapter one*, we showed that the wide range of nutrient concentrations along La Tordera watershed covaried with the large variability in $\delta^{15}N$ of DIN species. In particular, ranges for $\delta^{15}N$ of DIN species, especially for NH_4^+, exceeded those found in a recent worldwide meta-analysis of fluvial ecosystems (Peipoch et al. 2012). The highest values of nutrients and

$\delta^{15}N$ of DIN were found at mainstem sites where urban impacts are the strongest, and where most of WWTPs are located. WWTPs have a large influence on streamwater nutrient concentrations (Martí et al. 2004, Merseburger et al. 2005) and DIN species are commonly enriched in ^{15}N, and therefore result in high $\delta^{15}N$ values of DIN in the receiving stream (Kendall et al. 2007, Merbt et al. 2011, Ribot et al. 2012). Our data further supported the large effects of point sources in stream chemistry, which might be amplified in streams from the Mediterranean region, such as La Tordera, because of their reduced dilution capacity, especially during summer low flow (Martí et al. 2010).

Over time, the effects of urban point sources are likely to result in a greater fluctuation of stream chemistry downstream the WWTP due to the highest variability of primary production and nitrification rates, and runoff changes (Gammons et al. 2011, Kaushal et al. 2011). Our data in *Chapter two* also supported this pattern. The highest temporal variability of $\delta^{15}N$ of DIN species was found at the reach influenced by the WWTP effluent, and was partly driven by stream discharge. Chemical characteristics of WWTP effluent are not likely to vary over a year, but the isotopic dilution effect driven by the spiky hydrological regime, typical in Mediterranean streams (Bernal et al. 2012), can result in abrupt changes in elemental and isotopic composition of N in streamwater.

The variability of $\delta^{15}N$ of PUCs was sensitive to the strong anthropogenic gradient in La Tordera catchment previously revealed by nutrient concentrations and their isotopic values. The results from *Chapter one* showed that $\delta^{15}N$ of PUCs was mostly explained by their location, as opposed to PUC types, and was associated to $\delta^{15}N$ of DIN species across this strong anthropogenic gradient. Hence, mainstem reaches characterized by high $\delta^{15}N$-DIN values, also presented the highest isotopic values for PUCs. This is in agreement with previous studies, which have shown isotopic linkages between single PUC types and NO_3^- in rivers across anthropogenic gradients, for example for macrophytes (Kohzu et al. 2008), particulate organic matter (Kendall et al. 2001), or algae (Kaushal et al. 2006). Our work further confirmed these isotopic linkages for the majority of PUC types and with both DIN species. While $\delta^{15}N$-PUC was more strongly related to $\delta^{15}N$-NH_4^+, the $\delta^{15}N$ of PUCs was more similar to $\delta^{15}N$-NO_3^-. This fact, together with the results of mixing model analyses, showed that most of the N obtained by PUCs was derived from NO_3^-, which is the principal DIN species across the fluvial network.

Human pressures not only influenced the enrichment of $\delta^{15}N$-PUC, but, as observed for $\delta^{15}N$-DIN, also amplified variability of $\delta^{15}N$-PUC over time (*Chapter two*). These results suggested that PUCs developed in streams affected by point sources are more likely to undergo temporal changes in their $\delta^{15}N$ values, because their $\delta^{15}N$-DIN sources are more variable, especially for $\delta^{15}N$ of NH_4^+. Additionally, $\delta^{15}N$-PUC can also vary substantially within the same reach scale (*Chapter three*). For example, the highest isotopic variability of epilithic biofilm

within a stream reach was found at the high-nutrient stream, probably associated with the heterogeneity in the spatial deposition of sludge particles from the WWTP (Singer et al. 2005) and the incorporation of these particles into the biofilm (Battin et al. 2003b).

Across the fluvial network, δ^{15}N-PUC was not only responsive to δ^{15}N-DIN species but also to the whole stream nutrient environment, including nutrient concentrations and stoichiometry. One plausible explanation is that nutrient concentrations covariate with δ^{15}N-DIN species across the watershed, resulting in significant relationships between δ^{15}N-PUC and nutrient concentrations. Another, more intriguing possibility is that δ^{15}N reflected the availability of other essential elements to PUCs. PUC stoichiometric demand from DIN pool can increase at higher DOC (e.g. Bernhardt and Likens 2002) and at higher SRP concentrations relative to DIN availability (e.g. Camarero and Catalan 2012), thus affecting the isotopic values in δ^{15}N-PUC due to changes in fractionation (Mckee et al. 2002, Dijkstra et al. 2008, Wanek and Zotz 2011). Unfortunately, our approach did not allow to explicitly testing stoichiometry effects on δ^{15}N-PUC because of the high covariation between nutrients. An experimental approach would be needed to discern among nutrient effects.

PUCs are what PUCs assimilate…well, almost

The isotopic composition of an organism is strongly determined by that of its elemental source, as reflected by the common adage “you are what you eat”. This conjecture is the cornerstone of the application of stable isotopes

analyses in trophic ecology. The $\delta^{15}N$, $\delta^{13}C$, and hydrogen ($\delta^{2}H$) isotope ratios of consumers are commonly strongly correlated with their dietary inputs (e.g. Post 2002, Ehleringer et al. 2008, Boecklen et al. 2011). Small isotopic differences between organism and diet arise due to fractionation effects, which are usually smaller than 1‰ for $\delta^{13}C$ and $\delta^{2}H$, but can be considerably higher for $\delta^{15}N$, with 3.4‰ often taken as representative value (Minagawa and Wada 1984, Vander Zanden and Rasmussen 1999, Post 2002), although fractionation factors can be highly variable (Martínez del Rio et al. 2009). Understanding the magnitude and causes of these variations is crucial for the application of stable isotope analyses because it provides the basis for further inferences (Boecklen et al. 2011).

At the base of stream food webs, the assumptions that $\delta^{15}N$ of source nitrogen is preserved during N acquisition and that the $\delta^{15}N$-PUC reflects that of the N streamwater sources are important. Our results show that the high variability of $\delta^{15}N$-PUCs across a strong anthropogenic gradient was mostly explained by the location of PUCs within the fluvial network and was related to the variability of $\delta^{15}N$ of DIN species (*Chapter one*). The absence of distinct N isotopic values among specific PUC types has been also previously reported in other ecosystems such as estuaries (Cloern et al. 2002), lakes (Jones et al. 2004) or wetlands (Jones et al. 2004). Moreover, our data suggested fast interaction between PUCs and streamwater since our temporal survey did not yield isotopic relationships between these two compartments within the same stream reach (*Chapter two*). This is in agreement with recent studies which

have indicated that the temporal variation in δ^{15}N-DIN of streamwater can be quickly integrated by PUCs (Hill et al. 2012, Jardine et al. 2014).

However, it is clear that the assumption that δ^{15}N-PUC strictly reflects that of the streamwater N sources needs to be qualified. PUCs physiological factors, such as different N acquisition and dissimilation pathways, or N recycling PUCs, can result in changes of δ^{15}N among PUC types (Evans 2001). These effects may in fact be more relevant at the base of food webs. Indeed, δ^{15}N have been reported to be more variable among PUCs than among consumers (Cabana and Rasmussen 1996), and a wide range of fractionation factors have been estimated for the former (Evans 2001, York et al. 2007). Although we found strong isotopic relationships between PUCs and DIN across the fluvial network, when looking at the data separately for each stream (*Chapter two*), these relationships vanished, which suggest that other modes of variability may become more relevant in the absence of strong environmental and anthropogenic gradients. The next section discusses the factors underpinning variations in δ^{15}N, including access to other N sources, physiological transformations of N, and N cycling turnover, which can affect the ^{15}N biogeochemical relationships between PUCs and streamwater and result in differences among and within PUC types. This information provides insights into N dynamics in PUCs, which can have effects on N stream cycling.

D.2 Biogeochemical relationships between DIN and PUCs: patterns among and within PUC types

It is not only streamwater DIN that matters

This dissertation comprised the study of the most representative PUCs in stream ecosystems, which include multiple types of organisms distributed within the stream channel across a gradient of water exposure. Differences in PUC's preferential habitat can result into differences in the strength of their interaction with streamwater and in their dependence on DIN streamwater. In *Chapter one*, we observed that PUCs growing on the stream banks (i.e. stream-bank macrophytes and riparian trees) showed weaker relationships with δ^{15}N-DIN species, than those living in the stream channel. In particular, macrophytes, depending on their characteristics, occupy a wide range of habitats (Riis et al. 2001, Bowden et al. 2007), and interspecific differences in N biogeochemical relationships with streamwater were also observed (*Chapter one)*. The natural abundance of ^{15}N in the two most frequent macrophyte species in the watershed occupying different habitats within the stream reach, presented contrasting patterns in their relationships with δ^{15}N-DIN species. The δ^{15}N of *Apium nodiflorum*, mostly found at the margins of the wetted stream channel, was related to δ^{15}N-NH_4^+, whereas that of *Carex pendula,* which grows on the stream banks, was related neither to δ^{15}N-NH_4^+ nor to δ^{15}N-NO_3^-. Thus, these results suggested that PUCs located farther from the stream channel are likely to rely more on N sources other than streamwater DIN, including N in soil or groundwater. Moreover, other N sources can also be important. The δ^{15}N

of alder trees (*Alnus glutinosa*), particularly in leaf tissues, was largely decoupled from streamwater both when considering spatial (*Chapter one*), and temporal variability (*Chapter two*). These observations together with the low values of $\delta^{15}N$ found across all the sampling sites, suggested an additional N supply from the atmospheric pool through the endosymbiotic relationships established with bacteria in root nodules.

Submerged in the streamwater, heterotrophic organisms colonizing organic matter may also rely on other N sources besides streamwater DIN, because they can obtain N from their organic substrate. In *Chapter four*, the reliance on streamwater resources versus their organic substrate by the microbial community was evaluated during leaf litter decomposition. The relative proportion of N assimilated from streamwater by the microbial community increased with time during decomposition, probably as labile compounds in leaf litter were used up by microbes or leached out of the leaf. In contrast, N immobilization fluxes were positively associated to microbial biomass accrual on litter. These results are in concordance with the isotopic spatial patterns found for detritus in *Chapter one*, where $\delta^{15}N$ variability was related to $\delta^{15}N$ of streamwater DIN species. Detritus samples corresponded to the small fraction of leaf litter accumulated on the stream (CBOM) and deposited organic matter on the sediment (FBOM), which is likely to belong to late stages of decomposition where N interaction with streamwater is the highest, regardless of N concentrations in the stream (Cheever et al. 2013).

In contrast to biofilm on litter, epilithic biofilm did have access to an extra source of nutrients from the organic substrate at the beginning of the colonization. Despite that, as epilithic community develops and biomass accrues, late-stage biofilm can increasingly rely, to some degree, on its own nutrient resources. In *Chapter three*, we explained some of the observed patterns in $\delta^{15}N$ variability between successional stages of biofilm arguably due to differences in nutrient recycling within the biofilm. First, the biofilm matrix can retain and store DOC from streamwater, which can later be catabolized to maintain microbial metabolism (Freeman and Lock 1995). Second, a closer coupling between the autotrophic and heterotrophic organisms is likely to develop as the biofilm progresses (Battin et al. 2003a). In particular, the high quality of algae exudates in the late stage biofilms can support an important part of the heterotrophic community (Kühl et al. 1996, Sabater and Romaní 1996, Romaní and Sabater 1999). Therefore, nutrient recycling must be enhanced in late-stage biofilms where microorganisms living in them can take advantage of these internal resources, thus becoming a more closed system (Jackson 2003).

The temporal dimension: N turnover time

PUCs comprise a wide range of body sizes which include contrasting biological traits, from simple and metabolically active cells in microorganisms to complex biological tissues in vascular plants (such as rhizomes and wood). Whereas consumers are more narrowly constrained, C to N ratios of PUCs are

more variable because of changes in structural C and responses to nutrient limitation (Sterner and Elser 2002). These differences are also likely to influence biogeochemical interactions with streamwater, because changes in the dynamics of N demand and turnover time would ultimately affect $\delta^{15}N$ of PUCs. We approached this question in *Chapter two* by using the C to N ratio as a proxy of N turnover time of the PUC types (Dodds et al. 2000, 2004). We expected higher $\delta^{15}N$ temporal variability in PUCs with lower C:N ratios (i.e., higher N turnover rates) because they can better trace the variability in $\delta^{15}N$-DIN values. Although results have to be interpreted with caution because of the weak relationship found, $\delta^{15}N$-PUC variability tended to decline with lower turnover rates. In particular, our data pointed out filamentous algae as the type of PUC holding the highest temporal variability. Although microorganisms are likely to have the simplest and most metabolically active cells, in biofilms (both epilithic biofilm and biofilm on litter) they form associations that provide effectively buffer against environmental variability (Freeman and Lock 1995). Thus, biofilms might have been able to damper $\delta^{15}N$ temporal variability in relation to filamentous algae, which are more exposed to environment variability.

At higher trophic levels, positive relationships between N turnover of the organisms and the temporal variability of $\delta^{15}N$ have also been suggested. The turnover rate of an element scales with body mass (Hildrew et al. 2007), with large organisms such as fishes integrating N over longer time spans, as compared to small consumers. Negative relationships between the variability

of $\delta^{15}N$ and body size have been observed in different studies (Cabana and Rasmussen 1996, Post 2002, Woodland et al. 2012b). In general terms, our results would extend the negative relationship between N turnover $\delta^{15}N$ variability found for aquatic consumers to the broader category of basal compartments. Understanding the dynamics of isotopic N turnover may allow researchers to detect temporal changes in $\delta^{15}N$ of DIN sources by selecting indicator organisms which optimally provide information at the temporal scale of interest, including seasonal and sporadic changes.

Fractionation holds the stage

We have already showed the important effects of N source in the $\delta^{15}N$ values of an organism, but at a lower degree, isotopic fractionation can also influence $\delta^{15}N$ of PUCs. In *Chapter one*, our estimates of fractionation factors ranged on average from 2 to 5‰ in contrast to the high variability found for $\delta^{15}N$ of PUCs. Thus, isotopic differences due to fractionation processes are likely to be negligible across strong gradients of $\delta^{15}N$ sources. Minor isotopic fractionation effects have also been shown for phytoplankton across a gradient of nutrient inputs in lakes (Leavitt et al. 2006, Jankowski et al. 2012). However, when considering PUCs which have been growing under the same DIN isotopic sources, fractionation effects can have a relatively significant role.

The $\delta^{15}N$ of an organism has been hypothesized to reflect the balance between fractionation vectors associated to assimilation and dissimilation in animals (Olive et al. 2003, Martínez del Rio and Wolf 2005) and heterotrophic

bacteria (Dijkstra et al. 2008). Thus, assimilation of N results in a decrease of δ^{15}N-PUC, whereas dissimilation fluxes increase δ^{15}N-PUC, because fractionation processes select against ^{15}N isotope. This is in relation to the common ^{15}N enrichment between consumer and its diet which has been attributed to the observation that materials excreted by the animals tend to be isotopically lighter than tissues (Martínez del Rio et al. 2009). In contrast, autotrophs isotopic fractionation associated to N assimilation often results in lower δ^{15}N values (Evans 2001). Moreover, the relatively importance of N assimilation and dissimilation is also likely to vary within the same organisms due to changes in resource quality in relation to their N demand or N cycling within the organism (Dijkstra et al. 2008, Martínez del Rio et al. 2009). Understanding N assimilation, cycling within the organisms and dissimilation fluxes is important because these processes give us insights into net N retention in streams and N regulation export to downstream ecosystems.

In *Chapter three* we suggested that changes in δ^{15}N values reported for epilithic biofilm at different successional stages within the same stream reach were related to contrasting patterns of interaction with DIN streamwater. Because biofilm at early stages of development is under biomass expansion, assimilation rates are likely to exceed those of mineralization. In contrast, uptake may be offset by mineralization in late-stage biofilm. Our results were consistent with our hypothesis except at the low- nutrient stream, where other factors must have been more relevant. Moreover, differences between successional stages of biofilm were more pronounced under high nutrient

concentrations. Overall, these results suggested that successional stage of biofilm can be an important factor explaining the small scale spatial variability of $\delta^{15}N$.

D.3 Implications of variations in N stable isotope ratios in PUCs

Insights for ecological processes and future directions

Advances in isotopic techniques during the last decades have conveyed exciting progresses in ecological and environmental research in aquatic systems. The high variability and flexibility of natural abundance of $\delta^{15}N$ gives the basis to trace N processes and origins by using techniques relying on natural abundance of N isotope ratios. Indeed, not only $\delta^{15}N$ values but also the range of the variability of $\delta^{15}N$ gives a good deal of information. The understanding of $\delta^{15}N$ changes can give insights into the coupling with other major element cycles, such as carbon (Dijkstra et al. 2008, Roussel et al. 2014) or phosphorous (Mckee et al. 2002, Wanek and Zotz 2011). Also, the isotopic fractionation effects on $\delta^{15}N$ values of PUCs can provide simple ecological tools to track N interactions. However, the use of fractionation values is limited only when other controlling factors of $\delta^{15}N$ natural abundance are well known. Probably, more laboratory experiments are needed to disentangle confounding variables, and the magnitude and direction of their effects. The comprehension of the primary processes controlling $\delta^{15}N$ natural abundance can be used to further develop robust predictive models (i.e. 'isoscapes') of spatial isotopic variation, which have been successfully developed for isotopic ratios of

hydrogen, oxygen and carbon, whereas for nitrogen more information is still required (Bowen 2010).

^{15}N labelling techniques have proved enormously useful to quantify simultaneously occurring N processes in fluvial ecosystems, and have been especially successful and widely applied to trace additions of ammonium and nitrate in streams (Peterson et al. 2001, Hall et al. 2009). The use of ^{15}N enriched compounds is expensive and time consuming but can give insights into N stream cycling without altering ambient concentrations and effectively tracing into inorganic and organic compartments. However, the application of enriched material has still a long way to advance in stream ecosystems. For example, the use of ^{15}N enriched organic material is just now starting to emerge in fluvial research (e.g. see: Cheever et al. 2013, Atkinson et al. 2014). Learning from other ecological disciplines will also allow using ^{15}N labelling techniques in other imaginative ways, which will surely improve our understanding of stream ecosystems.

Research from this dissertation maintains open some other interesting ecological research questions, including the following:

- *What are the relevant temporal and spatial scales for $\delta^{15}N$ variations?*

Our study showed that $\delta^{15}N$ variation can be substantial at both temporal and small-spatial scales. Further studies should try to specify the relevant scales in which $\delta^{15}N$ PUCs change are. This would improve the accuracy of

applications of N stable isotopes by delimiting when and where sampling should be conducted.

- *How important is streamwater DIN source relative to other N sources for PUCs?*

So far as the lateral dimension of the stream is concerned, from the water channel to the banks of the stream, our results pointed out that $\delta^{15}N$ variability of PUCs farther from the streamwater, such as macrophytes and riparian trees, was weakly associated to that of $\delta^{15}N$ of DIN species in streamwater. To completely understand the interaction with DIN streamwater by PUCs, it would be necessary to include the other N isotopic sources in the analyses. With regards to the vertical dimension of the stream, from the surface to the hyporheo, we did not include the latter, which is considered to play an important role in nutrient and DOC retention (Findlay et al. 1993, Boulton et al. 1998). It would be interesting to study how the variability of $\delta^{15}N$ of these not so aquatic PUCs, respond to the variability of N sources which are likely to be used by these compartments, such as ground and soil water. Adding multiple sources to the mixing models would require the use of other isotopes or complementary bioindicators to successfully evaluate the biogeochemical relationships with the streamwater pool.

- *How important are the taxonomic effects relative to the compartmental approach taken here?*

In this study, PUCs are considered as black boxes. We acknowledge that although the approach is useful, it is necessary to further investigate biological characteristics of each PUC in order to fully understand some of the patterns observed in this dissertation. For example, biofilms are amalgams of microorganisms which change over time in their composition. Analyses of the biofilm community and its architecture, for example by means of confocal microscopy and molecular techniques, are necessary to fully understand the role of these communities.

Shaking the isotopic baseline: pitfalls and little recommendations for food web studies

The use of stable isotope analysis is a relevant tool in trophic ecology, and as a consequence it has rapidly proliferated during the last decade (Boecklen et al. 2011, Layman et al. 2012). The two elements most commonly employed in a food web context are N and C, although sulphur, oxygen, and deuterium can also be applied. Ratios of N isotopes are useful to estimate trophic position of organisms because consumers usually exhibit stepwise ^{15}N enrichment with trophic transfers. The determination of the trophic position of an organism in a food web by using $\delta^{15}N$ provides a continuous measure of its trophic position, which supposed a major advance compared to assignments of discrete trophic levels based on stomach content and natural-history observations. However, the interpretation of $\delta^{15}N$ information can be challenging because the high variability in organisms at the base of the food webs make it more difficult to determine the isotopic baseline from where to infer organisms trophic level

and the discrimination factors in each trophic step (Boecklen et al. 2011, Layman et al. 2012).

The $\delta^{15}N$ variability of aquatic organisms can be especially high in running waters due to the dynamic nature of these systems (e.g. hydrological variability, organic matter inputs, and terrestrial connectivity; Jardine et al. 2012). In particular, as shown by this study (*Chapter two*), streams affected by human impacts are likely to hold the highest temporal variation. In lakes, one of the most commonly solutions to address isotopic baseline variation is the use of long-lived primary consumers, with well-documented trophic strategies, such as bivalves and gastropods, to infer an isotopic baseline (Vander Zanden and Rasmussen 1999, Post 2002). Large and long-lived organisms are considered to be able to smooth out temporal variability of N sources and provide $\delta^{15}N$ time-integrated values because of their longer N turnover times (Cabana and Rasmussen 1996). However, these types of organisms can be uncommon and/or patchy in some systems such as rivers and streams, and we have little previous trophic information about them (Jardine et al. 2014), which makes it necessary to sample smaller and more obvious consumers. These smaller organisms are also likely to be subjected to substantial temporal variability (Woodland et al. 2012b), restricting the accuracy of the isotopic estimations.

Overall, our results and data from the literature pointed out that $\delta^{15}N$ variability can be high, especially in fluctuating streams such as Mediterranean

ones affected by human pressures and for organisms with fast turnover times. This high variability can suppose an important drawback for the application of N stable isotopes in food webs. Nonetheless, some considerations can help to improve the reliability of the food web analyses. First, on the basis of these high isotope variability at the base of the food webs, multiple dates samplings has been recommended, whenever feasible, to obtain a representative isotopic baseline (Sabo et al. 2010, Jardine et al. 2014, Walters and Post 2014). This should be more intensive with PUCs which are likely to vary the most (such as filamentous algae) and in streams with a high fluctuant ambient. Second, the use of simultaneous multiple tracers (i.e. other isotopes and bioaccumulation of metals) can also supplement and complete the information provided by $\delta^{15}N$ (Soto et al. 2013, Jardine et al. 2014) and the use of the compound-specific isotope analyses, such as amino acids and fatty acids, might improve the accuracy over bulk $\delta^{15}N$ measures (Boecklen et al. 2011, Ishikawa et al. 2014). Finally, understanding the temporal variability of the isotopic baseline can be useful to incorporate isotopic variations into models which would take baseline variability into account (Woodland et al. 2012a, Dethier et al. 2013).

On the uses of $\delta^{15}N$ as indicator of human pressure

The alteration of the N cycle as a consequence of human processes has resulted in enormous amounts of reactive N reaching the environment, adding a number of gases to the atmosphere and polluting aquatic systems (Vitousek et al. 1997, Galloway et al. 2003, Erisman et al. 2008, Rockström et al. 2009).

Understanding the spatial and temporal extension of these human impacts is challenging because of the many sources and pathways where N is involved. In this sense, the composition of N stable isotopes can help to determine the temporal and geographical extension of the human impacts.

At a global level, a decline in $\delta^{15}N$ values has been recorded in nitrate from ice and sediment cores from remote zones in the Northern Hemisphere, starting at the beginning of the 20th century, and consistently accelerating with the widespread use of fossil fuels and N industrial production (Hastings et al. 2009, Holtgrieve et al. 2011). These studies showed, by means of N stable isotopes, the extent of the effects of N human disturbances both at an large spatial scale and a fast temporal scale (Elser 2011).

In fluvial ecosystems, N isotopes can also help to determine the extent of the human pressures that these ecosystems receive both at the watershed scale and at the reach scale. Some freshwater studies have observed patterns of increasing $\delta^{15}N$ values in organisms with increasing human pressures in the watershed (Vander Zanden et al. 2005, Kohzu et al. 2008, Clapcott et al. 2010, Clapcott et al. 2012). Human disturbances in the watershed can be hierarchically transferred to reach and microhabitats in stream (Allan 2004, Burcher et al. 2007), and subsequently result in changes in stream community (Vitousek et al. 1997, Allan 2004). However, the mechanisms that lead to changes in N stable isotopes are not clear yet. Understanding these mechanisms is important not only because $\delta^{15}N$ provides information of the

temporal and spatial extent of N disturbances, but also because it can be used as the basis for further application of $\delta^{15}N$ as indicator of human pressure by environmental managers.

Our results in *Chapter one* further supported the advantages of the use of N isotopic ratios as a monitoring tool to evaluate the state of stream nutrient environments. We found that $\delta^{15}N$ variability was mostly explained by the location where the PUC was growing rather than the PUC type considered, with the highest values at the mainstem stations where human activity was mostly located. In addition, $\delta^{15}N$ of PUC reflected not only $\delta^{15}N$-DIN, but also the whole nutrient stream environment suggesting $\delta^{15}N$-PUC as a potentially good integrator of the nutrient state of the ecosystem. The fact that PUCs can integrate isotopic changes over time and are easily sampled, less time-consuming and cheaper in monetary terms, would make $\delta^{15}N$ of PUCs a more suitable indicator of stream health than $\delta^{15}N$ values of DIN. Moreover, it is worth noting that each biotic type would respond to changes to anthropogenic impacts at different temporal resolutions, and the selection of one type over another should fit the temporal scale which the researcher aims to consider (e.g. filamentous algae would respond faster to changes than macrophytes; *Chapter two*).

A further step would be to develop watershed models which would allow the analyses of pressures across watersheds holding different agricultural practices and urban uses. Sources of organic matter from animal waste versus

human waste rarely can be differentiated using $\delta^{15}N$ alone, because $\delta^{15}N$ values usually overlap (Kendall et al. 2007). However, $\delta^{15}N$ can be used as a proxy of "human-intensity" in the watershed, including both agricultural and urban uses. The determination of the spatial and temporal scales at which human impacts can be integrated by $\delta^{15}N$ values will be crucial to effectively apply $\delta^{15}N$ as an ecological monitoring tool. Probably the use of ancillary indicators, for example by using a multi-isotope approach, would be necessary to have a more comprehensive understanding of the pressures that fluvial ecosystems withstand. The effective communication of these results to local stakeholders is necessary to develop operative environmental responses to reduce N emissions and mitigate anthropogenic N impacts. Isotopic techniques have been argued to be easy to communicate by means of strong graphical supports, such as "isoscapes" (Kendall et al. 2010), which can help to raise awareness of environmental impacts of anthropogenic N.

In sum, the research presented in this thesis suggests that there are solid grounds for exploring further the uses of nitrogen stable isotopes. $\delta^{15}N$ can help to disentangle the hierarchy that connects catchment-scale N sources, mobilization and emissions with in-stream processes and within-PUC physiological processes. A better understanding of the major factors driving $\delta^{15}N$ variability in stream-riparian PUCs will provide the basis to use $\delta^{15}N$ as indicator of human pressures.

CONCLUSIONS

The main conclusions of this dissertation are the following:

Chapter one: "Nitrogen stable isotopes in primary uptake compartments across streams differing in nutrient availability"

1. The spatial variability of δ^{15}N-PUC was mostly explained by location within the fluvial network; with the highest values at the mainstem reaches where human activity in the watershed is most intense (i.e. agricultural and urban uses).
2. Along a strong anthropogenic gradient, values of δ^{15}N-PUC were strongly related to the δ^{15}N of DIN species, especially of NH_4^+, and PUCs living within the stream channel and using streamwater as the main N source.
3. Stream nutrient concentrations and stoichiometry improved the predictive power for δ^{15}N-PUCs, compared to models including only δ^{15}N of DIN species, indicating that δ^{15}N of PUCs are a function of the stream nutrient environment in which PUCs grow.

Chapter two: "Temporal variability of nitrogen stable isotopes in primary uptake compartments in four streams differing in human impacts"

4. Our results showed no evidence of isotopic temporal patterns, neither for δ^{15}N of DIN species or δ^{15}N-PUCs, and suggested that other factors, such as hydrological regimes, should be more important in Mediterranean streams.

5. The highest temporal isotopic variability was found in the urban stream indicating that the effects of biological rates and runoff might be more important than in more pristine sites.
6. Among compartments, PUCs characterized by fast turnover rates, such as filamentous algae, tended to have the highest temporal variability in their $\delta^{15}N$ values.

Chapter three: "Effects of successional stage and nutrient availability on nitrogen stable isotopes of stream epilithic biofilm"

7. The $\delta^{15}N$ variability of early-stage biofilm was lower than late-stage biofilm, indicating that carryover effects occurred before the month previous the sampling might be integrated by $\delta^{15}N$ values of biofilm.
8. Differences in $\delta^{15}N$ values between early- and late-stage epilithic biofilm were found and might be associated to changes of the net balance of assimilation and mineralization fluxes during biofilm development.
9. During biofilm biomass development, there was a ^{15}N-enrichment in biofilm, which was partially decoupled from $\delta^{15}N$ of DIN species, and was more pronounced at the high-nutrient stream.

Chapter four: "Stream carbon and nitrogen supplements during leaf litter decomposition: contrasting patterns for two foundation species."

10. Litter type strongly affected biomass and stoichiometry of microbial assemblages growing on litter.

11. The proportion of C and N in microorganisms derived from the streamwater, as opposed to the litter, did not differ between litter types, but increased throughout decomposition.
12. Gross immobilization of N from the streamwater was the highest for the low-tannin litter, probably as a consequence of the highest microbial biomass, contrasting to C fluxes which were the highest the high-tannin litter suggesting C limitation for this substrate.

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Supporting information

APPENDIX A

Chapter one: "Nitrogen stable isotopes in primary uptake compartments across streams differing in nutrient availability"

Appendix A comprises 12 pages, 1 figure and 7 tables.

TABLE OF CONTENTS

Supporting information is available at the supporting information of this dissertation (Appendix A). It includes information on the characteristics of the stream reaches, isotopic relationships between PUCs and DIN species, mixing model analyses and multiple linear regressions; Figure SA.1 and Tables SA.1-SA.7. This information is also available free of charge via the Internet at http://pubs.acs.org.

1. Characteristics of streams reaches

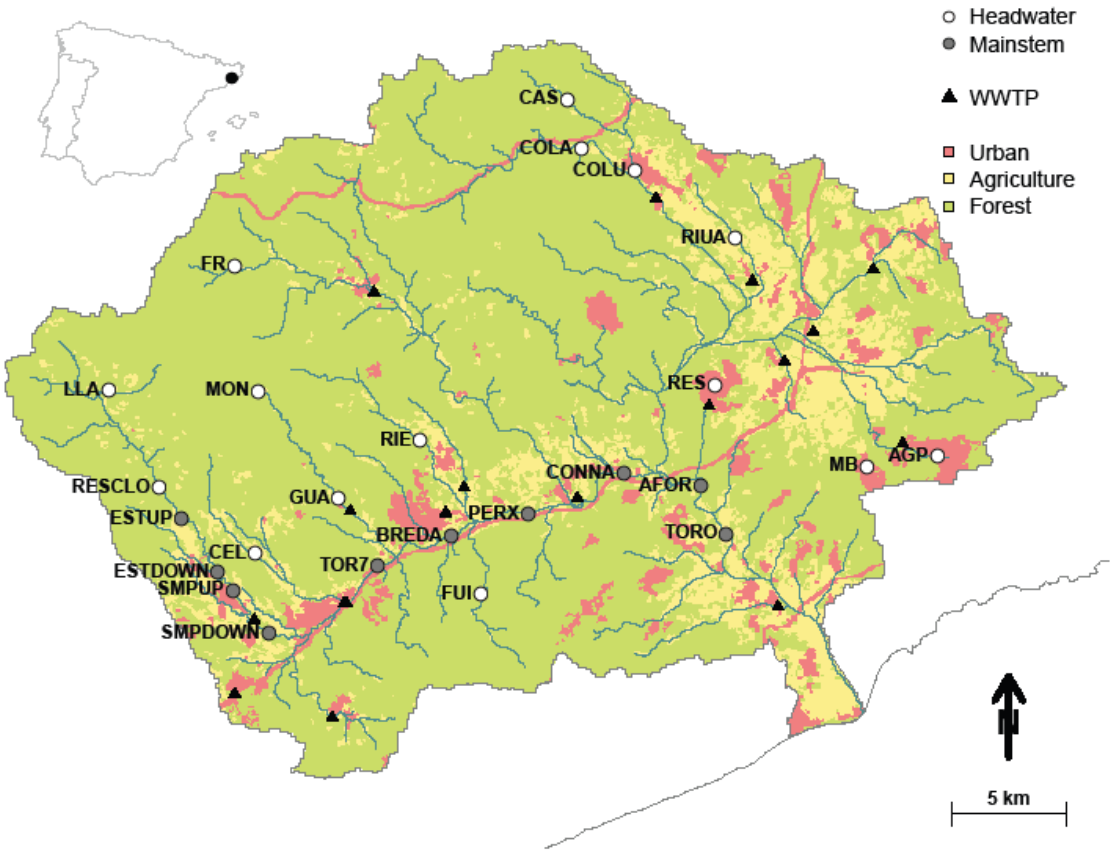

Figure SA.1 Location of La Tordera catchment in the Iberian Peninsula and of the study streams within the catchment. The type of reach (headwaters or mainstem), is highlighted. Land uses are grouped into urban (including towns, residential areas, industrial and commercial zones, and roads; in red), agricultural (including irrigated and dry land crops; in orange) and forested (area covered by trees or shrubs and not for agricultural purposes; in green). Triangles indicate the locations of the wastewater treatment plants (WWTP).

Table SA.1 Physical and chemical characteristics of sampled streams. Values within brackets represents the range of values for headwater and mainstem.

Stream	Discharge (L/s)	SRP[1] (µg P/L)	NH_4^+-N (µg N/L)	NO_3^-N (µg N/L)	DON[2] (µg N/L)	TN[3] (µg N/L)	$\delta^{15}N$-NH_4^+ (‰)	$\delta^{15}N$-NO_3^- (‰)
Headwaters	**(0.3-211.0)**	**(1.6-42.4)**	**(9.0-188.6)**	**(65.2-1156.0)**	**(41.0-366.0)**	**(153.5-1542.3)**	**(-3.3-14.8)**	**(1.9-15.9)**
CAS	19.8	4.2	12.0	209.1	82.5	303.6	6.6	4.0
COLA	73.7	4.7	14.6	608.3	90.3	713.2	8.3	7.8
GUA	9.3	1.6	17.7	150.7	58.8	227.2	13.5	1.9
RIE	73.9	7.8	9.0	211.8	114.2	334.9	9.9	4.2
CEL	65.5	9.4	17.2	422.9	82.1	522.2	14.8	4.7
COLU	n.a.	10.9	18.5	708.2	146.7	873.3	1.5	8.1
FUI	15.7	6.3	14.2	65.2	110.9	190.3	3.7	6.7
FR	88.3	5.2	13.3	135.0	51.0	199.4	-3.3	3.9
MON	28.8	11.9	13.3	75.4	64.8	153.5	-1.3	6.2
RIUA	12.5	38.3	20.3	1156.0	366.0	1542.3	10.1	12.3
RES	n.a.	42.4	55.5	926.0	218.5	1200.0	9.0	6.8
MB	0.3	3.2	188.6	132.6	189.4	510.5	2.3	8.5
AGP	0.3	13.5	21.6	80.2	196.3	298.1	3.2	2.1
LLA	105.8	6.3	9.0	229.0	41.0	279.0	*n.a.*	4.2
RESCLO	211.0	16.1	16.4	590.1	69.5	675.9	11.5	15.9
Mainstem	**(41.0-580.0)**	**(12.5-227.6)**	**(12.4-746.9)**	**(345.3-1012.8)**	**(112.6-801.7)**	**(665.8-2247.4)**	**(7.4-36.6)**	**(2.0-15.4)**
ESTUP	52.8	17.1	12.4	512.21	141.1	665.8	7.4	7.9
ESTDOWN	n.a.	12.5	16.4	536.3	331.3	883.9	7.4	2.0
SMPUP	138.8	16.6	13.7	345.3	552.3	911.4	8.7	5.0
SMPDOWN	122.5	71.3	118.1	936.7	221.2	1275.9	29.5	7.8
TOR7	41.0	227.6	746.9	1012.8	487.7	2247.4	36.6	7.0
BREDA	99.6	179.1	213.3	370.6	801.7	1385.7	17.6	9.2
PERX	364.0	173.4	204.7	635.4	112.6	952.6	24.9	15.4
CONNA	580.0	150.2	22.0	701.9	231.9	955.8	16.9	15.3
AFOR	271.4	110.0	25.5	369.7	344.9	740.0	15.0	6.3
TORO	546.2	89.3	19.4	426.8	314.9	761.1	10.6	4.9

n.a. stands for not available data; [1]*SRP* stands for Soluble Reactive Phosphorus, [2]DON for Dissolved Organic Nitrogen and [3]TN for Total Nitrogen.

Table SA.2 Correlation matrix for stream nutrient concentrations.

	NH_4^+	NO_3^-	DON	TN
SRP	0.71	0.45	0.59	0.72
NH_4^+		n.s.	0.43	0.70
NO_3^-			n.s.	0.85
DON				0.61

n.s. stands for not significant correlations ($p > 0.05$)

2. Isotopic relationships between PUCs and DIN species.

Table SA.3 Number of samples (*n*), mean and standard error (SE) of $\delta^{15}N$ of PUCs, and r^2 of their relation with $\delta^{15}N\text{-}NH_4^+$ and $\delta^{15}N\text{-}NO_3$. In bold, average values by functional types.

PUC	*n*	$\delta^{15}N$-PUC mean and and SE	r^2 with $\delta^{15}N\text{-}NH_4^+$	r^2 with $\delta^{15}N\text{-}NO_3^-$
Detritus	**47**	**4.64 ± 0.65**	**0.55*** **	**0.22*** **
CBOM	23	4.65 ± 0.92	0.63***	0.32**
FBOM	24	4.62 ± 0.94	0.48***	n.s.
Epilithon	**19**	**7.34 ± 1.45**	**0.65*** **	**0.25***
Algae	**20**	**6.84 ± 1.45**	**0.65*** **	**0.32** **
Cladophora sp.	12	7.97 ± 1.92	0.56**	n.s.
Lemanea sp.	8	5.13 ± 2.20	0.82**	n.s.
Bryophyte	**26**	**2.98 ± 0.94**	**0.71*** **	**n.s.**
Fontinalis antipyretica	4	0.33 ± 1.15	n.s.	n.s.
Hepatica	3	0.43 ± 1.26	-	-
Rhynchostegium riparioides	19	3.95 ± 1.19	0.74***	n.s.
Aquatic macrophyte	**77**	**9.47± 0.76**	**0.45*** **	**0.13*** **
Alisma plantago-aquatica var. *lanceolatum*	5	14.04 ± 1.34	n.s.	n.s.
Apium nodiflorum	20	6.44 ± 1.66	0.67**	n.s.
Equisetum sp.	4	3.35 ± 1.14	n.s.	n.s.
Polygonum amphibium	8	13.91 ± 2.47	n.s.	n.s.
Ranunculus sp.	9	4.01 ± 2.04	0.67***	n.s.
Rorippa nasturtium-aquaticum	8	13.09± 1.18	n.s.	n.s.
Rumex sp.	3	10.25 ± 5.44	-	-
Typha latifolia	4	11.52 ±1.72	n.s.	n.s.
Veronica anagallis-aquatica	8	11.00 ± 1.05	0.48***	n.s.
Veronica beccabunga	2	5.26 ± 0.58	-	-
Callitriche stagnalis	6	14.94 ± 0.83	n.s.	n.s.

Stream-bank macrophyte	**44**	**6.7 ± 0.98**	**0.68*** **	**n.s.**
Arundo donax	2	9.33 ± 5.92	-	-
Athyrium filix-femina	4	-0.37 ± 0.76	n.s.	n.s.
Carex pendula	17	4.13 ± 0.98	n.s.	n.s.
Carex remota	5	3.49 ± 3.62	0.94*	n.s.
Cyperus longus	8	10.83 ± 1.80	0.52*	n.s.
Mentha sp.	2	9.27 ± 7.02	-	-
Phalaris arundinacea	6	14.11 ± 1.82	0.93**	n.s.
Alder	**36**	**0.77 ± 0.68**	**0.24****	**n.s.**
Alder roots	18	2.44 ± 0.69	0.64***	n.s.
Alder leaves	18	-0.90 ± 0.38	n.s.	n.s.
Total	**269**	**6.04 ± 0.39**		

r^2 are the adjusted coefficients of determination for linear regressions between PUC $\delta^{15}N$ and $\delta^{15}N$ values of NH_4^+ and NO_3^-. Asterisks indicate p-values: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$; n.s. means not significant.

Table SA.4 Linear regression equations between $\delta^{15}N$ of PUC and $\delta^{15}N$ of DIN species. It is also included the percentages of variance explained by each regression (measured as adjusted r-square). Only equations for PUCs with significant relations ($p < 0.05$) are included.

PUC	$\delta^{15}N$ -NH_4^+	$\delta^{15}N$ -NO_3^-
Detritus	$\delta^{15}N = 1.08 + 0.35\ \delta^{15}N$ -NH_4^+	$\delta^{15}N = 0.82 + 0.54\ \delta^{15}N$ -NO_3^-
	$r^2 = 0.54$	$r^2 = 0.21$
Epilithon	$\delta^{15}N = 1.44 + 0.50\ \delta^{15}N$ -NH_4^+	$\delta^{15}N = 2.12 + 0.71\ \delta^{15}N$ -NO_3^-
	$r^2 = 0.63$	$r^2 = 0.21$
Algae	$\delta^{15}N = 0.76 + 0.52\ \delta^{15}N$ -NH_4^+	$\delta^{15}N = 0.14 + 0.88\ \delta^{15}N$ -NO_3^-
	$r^2 = 0.63$	$r^2 = 0.28$
Bryophyte	$\delta^{15}N = -0.76 + 0.43\ \delta^{15}N$ -NH_4^+	n.s.
	$r^2 = 0.70$	
Aquatic macrophyte	$\delta^{15}N = 2.92 + 0.41\ \delta^{15}N$ -NH_4^+	$\delta^{15}N = 5.03 + 0.54\ \delta^{15}N$ -NO_3^-
	$r^2 = 0.45$	$r^2 = 0.13$
Stream-bank macrophyte	$\delta^{15}N=0.66 + 0.52\ \delta^{15}N$ -NH_4^+	n.s.
	$r^2=0.67$	
Alder root	$\delta^{15}N = -0.24 + 0.33\ \delta^{15}N$ -NH_4^+	n.s.
	$r^2 = 0.61$	
Alder leaf	n.s.	n.s.

3. Mixing model analyses

Table SA.5 Candidate mixing models fitted by maximum likelihood.

Candidate models	Equations
Model 1: no fractionation	$\delta^{15}N\_PUC = pNH4^{+} \times \delta^{15}N\_NH_4^{+} + (1 - pNH_4^{+}) \times \delta^{15}N\text{-}NO_3^{-}$
Model 2: single fractionation term for NH_4^{+} and NO_3^{-}	$\delta^{15}N\_PUC = pNH_4^{+} \times (\delta^{15}N\_NH_4^{+} - f) + (1 - pNH_4^{+}) \times (\delta^{15}N\_NO_3^{-} - f)$
Model 3: separate fractionation term NH_4^{+} and NO_3^{-}	$\delta^{15}N\_PUC = pNH_4^{+} (\delta^{15}N\_NH_4^{+} - fNH_4^{+}) + (1 - pNH_4^{+}) (\delta^{15}N\_NO_3^{-} - fNO_3^{-})$
Model 4: fractionation depends linearly on concentration	$\delta^{15}N\_PUC = pNH_4^{+} (\delta^{15}N\_NH_4^{+} - fNH_4^{+} \times NH_4^{+}) + (1 - pNH_4^{+}) (\delta^{15}N\_NO_3^{-} - fNO_3^{-} \times NO_3^{-})$
Model 5: fractionation depends on the logarithm of the concentration	$\delta^{15}N\_PUC = pNH_4^{+} (\delta^{15}N\_NH_4^{+} - fNH_4^{+} \times \log(NH_4^{+})) + (1 - pNH_4^{+}) (\delta^{15}N\_NO_3^{-} - fNO_3^{-} \times \log(NO_3^{-}))$
Model 6: fractionation depends on the concentration with a Monod saturating function	$\delta^{15}N\_PUC = pNH_4^{+} \{\delta^{15}N\_NH_4^{+} - [(fNH_{4\,max}^{+} \times NH_4^{+})/ (K \times fNH_4^{+} + NH_4^{+})]\} + (1 - pNH_4^{+}) \{\delta^{15}N\_NO_3^{-} - [(fNO_{3\,max}^{-} \times NO_3^{-})/ (K \times fNO_3^{-} + NO_3^{-})]\}$

pNH_4^{+} stands for the proportion of N in PUC derived from NH_4^{+}; f is the isotopic fractionation factor for NH_4^{+} (fNH_4^{+}) or NO_3^{-} (fNO_3^{-}); K is the half-velocity constant

Table SA.6 Best-performing models for predicting N of PUC derived from NH_4^+ and NO_3^-. Only models with AICc less than 2 units above the minimum AICc are selected. AICc is the Akaike Information Criterion corrected for small sample size. Goodness of fit is measured as r-square observed vs fitted values.

Best-performing models	AICc[a]	Weight	r^2	pNH_4^+ Estimate (±95 % IC)
Detritus				
Model 5	237.81	0.48	0.57	0.36 (0.25-0.46)
Model 2	238.30	0.37	0.57	0.33 (0.22-0.44)
Epilithon				
Model 2	104.94	0.39	0.67	0.46 (0.27-0.66)
Model 4	106.03	0.23	0.70	0.64 (0.36-0.94)
Model 1	106.14	0.21	0.64	0.37 (0.18-0.56)
Algae				
Model 2	104.89	0.67	0.75	0.46 (0.30-0.61)
Bryophyte				
Model 2	116.32	0.72	0.73	0.42 (0.30-0.54)
Aquatic macrophytes				
Model 2	468.35	0.46	0.45	0.40 (0.29-0.52)
Model 5	469.27	0.29	0.45	0.47 (0.31-0.61)
Stream-bank macrophytes				
Model 2	239.39	0.57	0.62	0.55 (0.42-0.68)
Alder root				
Model 2	90.04	0.53	0.51	0.44 (0.25-0.63)
Model 5	90.76	0.37	0.51	0.46 (0.29-0.64)

4. Multiple linear regressions

Table SA.7 Best-performing multiple linear regression models for predicting $\delta^{15}N$ of PUC. Only models with AICc less than 2 units above the minimum AICc are selected. AICc is the Akaike Information Criterion corrected for small sample size.

Best-performing models	AICc[a]	Weight	Adjusted-r^2	RMSE[b]
Detritus				
$\delta^{15}N = -15.24 + 6.38 \log(SRP) + 2.88 \log(DIN:SRP) - 0.96 \log(SRP) \times \log(DIN:SRP)$	172.71	1.00	0.90	1.43
Epilithon				
$\delta^{15}N = -9.16 + 0.74\ \delta^{15}N\_NH_4^+ + 4.33 \log(SRP) - 0.13 \log(SRP) \times \delta^{15}N\_NH_4^+$	79.14	0.79	0.91	1.87
Algae				
$\delta^{15}N = -0.24 + 0.45\ \delta^{15}N\_NH_4^+ - 2.66\ \log(NH_4^+) + 3.37 \log(SRP)$	84.60	0.55	0.91	1.94
$\delta^{15}N = -10.30 + 0.76\ \delta^{15}N\_NH_4^+ + 4.47 \log(SRP) - 0.13 \log(SRP) \times \delta^{15}N\_NH_4^+$	85.25	0.39	0.91	1.97
Bryophyte				
$\delta^{15}N = -13.12 + 0.33\ \delta^{15}N\_NH_4^+ + 0.34\ \delta^{15}N\_NO_3^- + 2.27 \log(DON)$	107.05	0.21	0.80	2.19
$\delta^{15}N = -12.08 + 0.25\ \delta^{15}N\_NH_4^+ + 2.92 \log(TN) - 1.19 \log(DIN:SRP)$	108.50	0.10	0.79	2.27
$\delta^{15}N = -9.75 + 0.28\ \delta^{15}N\_NH_4^+ + 1.22 \log(SRP) + 1.55 \log(DON)$	108.82	0.09	0.79	2.281
Aquatic macrophyte				
$\delta^{15}N = -11.36 + 0.75\ \delta^{15}N\_NH_4^+ + 5.11 \log(SRP) - 0.15 \log(SRP) \times \delta^{15}N\_NH_4^+$	400.32	0.98	0.76	3.24
Stream-bank macrophyte				
$\delta^{15}N = -10.37 + 0.30\ \delta^{15}N\_NH_4^+ + 1.95 \log(SRP) - 1.41 \log(DON)$	209.60	0.21	0.79	2.91
$\delta^{15}N = -3.74 + 0.29\ \delta^{15}N\_NH_4^+ - 0.20\ \delta^{15}N\_NO_3^- + 2.67 \log(SRP)$	210.38	0.14	0.79	2.93

$\delta^{15}N = -13.59 + 0.25\ \delta^{15}N\_NH_4^+ + 2.10\ \log(SRP) + 1.64\ \log(TN)$	210.66	0.12	0.79	2.94
$\delta^{15}N = -6.61 + 0.21\ \delta^{15}N\_NH_4^+ + 1.02\ \log(NH_4^+) + 2.37\ \log(SRP)$	210.67	0.12	0.79	2.94
$\delta^{15}N = -13.66 + 0.28\ \delta^{15}N\_NH_4^+ + 3.70\ \log(TN) - 1.88\ \log(DIN:SRP)$	210.79	0.14	0.79	2.95
$\delta^{15}N = -4.44 + 0.30\ \delta^{15}N\_NH_4^+ + 2.43\ \log(SRP)$	211.23	0.09	0.78	3.00
Alder root				
$\delta^{15}N = -5.69 + 0.21\ \delta^{15}N\_NH_4^+ + 1.19\ \log(NH_4^+) + 1.19\ \log(SRP)$	57.21	0.67	0.86	1.11

Interactions terms are expressed using a multiplication term, "×". [a]AICc is the Akaike Information Criterion corrected for small sample size and; [b]RMSE is the root mean-square error.

APPENDIX B

Chapter two: "Temporal variability of nitrogen stable isotopes in primary uptake compartments in four streams differing in human impacts"

Appendix B comprises 15 pages, 9 figure and 4 tables.

TABLE OF CONTENTS

Supporting information is available at the supporting information of this dissertation (Appendix B). It includes information on Information on the temporal correlation analyses, relationships between stream environmental variables and δ^{15}N-DIN species, temporal versus with-in reach variability, isotopic relationships between DIN species and PUCs, and cross-correlations between δ^{15}N-PUC and δ^{15}N-DIN species; Figures SB.1-SB.9 and Tables SB.1-SB.4.

1. Temporal correlation analyses

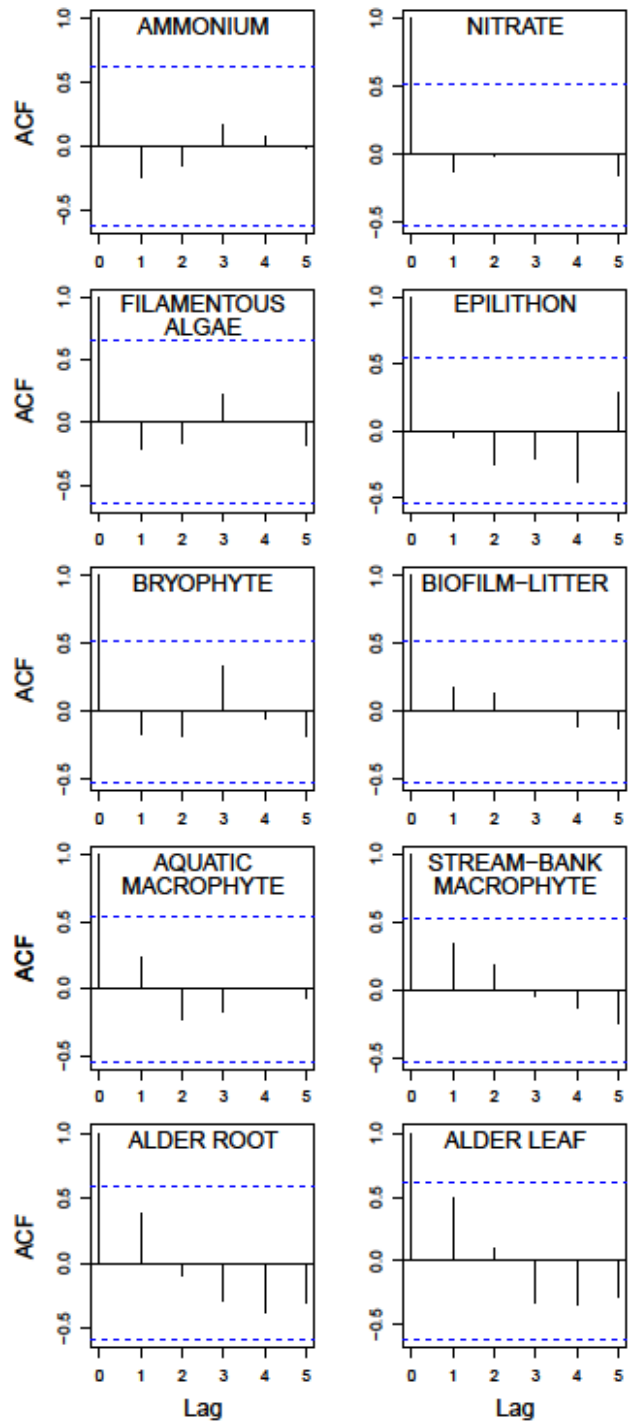

Figure SB.1 Temporal autocorrelation of $\delta^{15}N$ values for DIN species and PUC types at FOR stream. Blue dashed lines correspond to 95% confidence interval for an uncorrelated series.

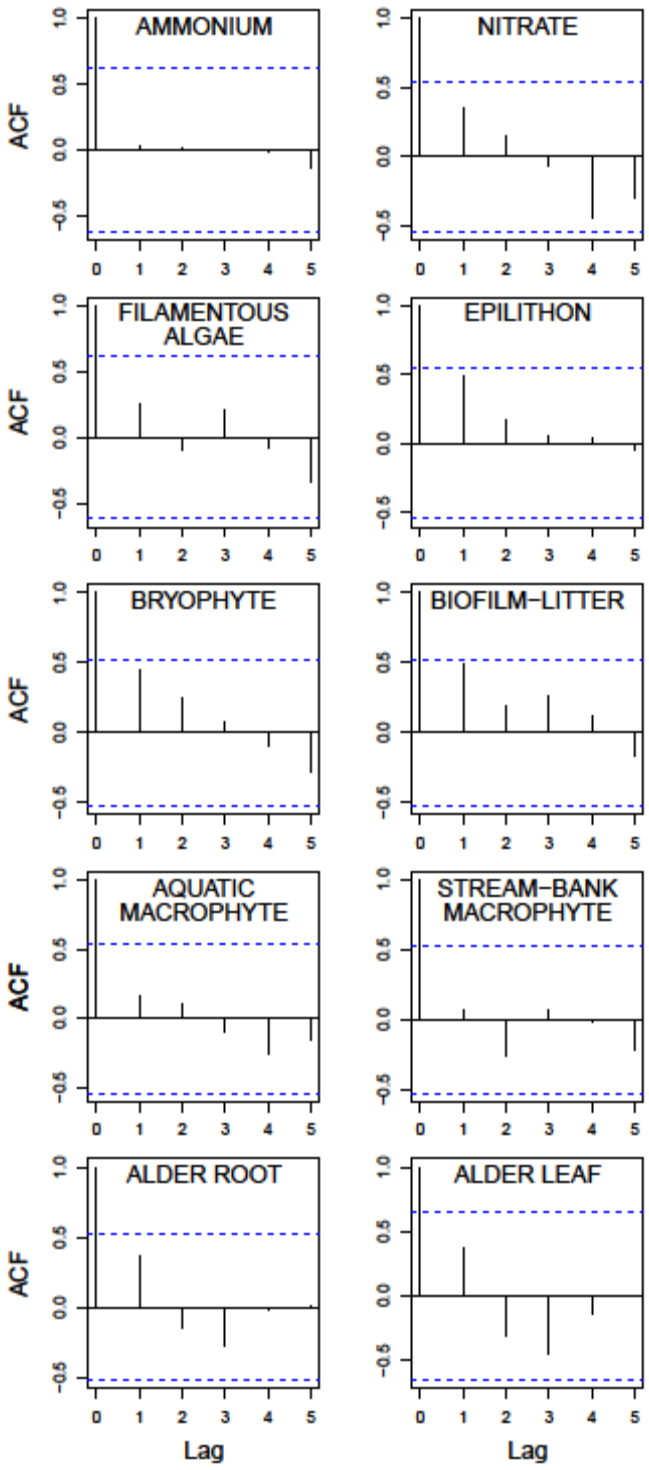

Figure SB.2 Temporal autocorrelation of $\delta^{15}N$ values for DIN species and PUC types at HOR stream. Blue dashed lines correspond to 95% confidence interval for an uncorrelated series.

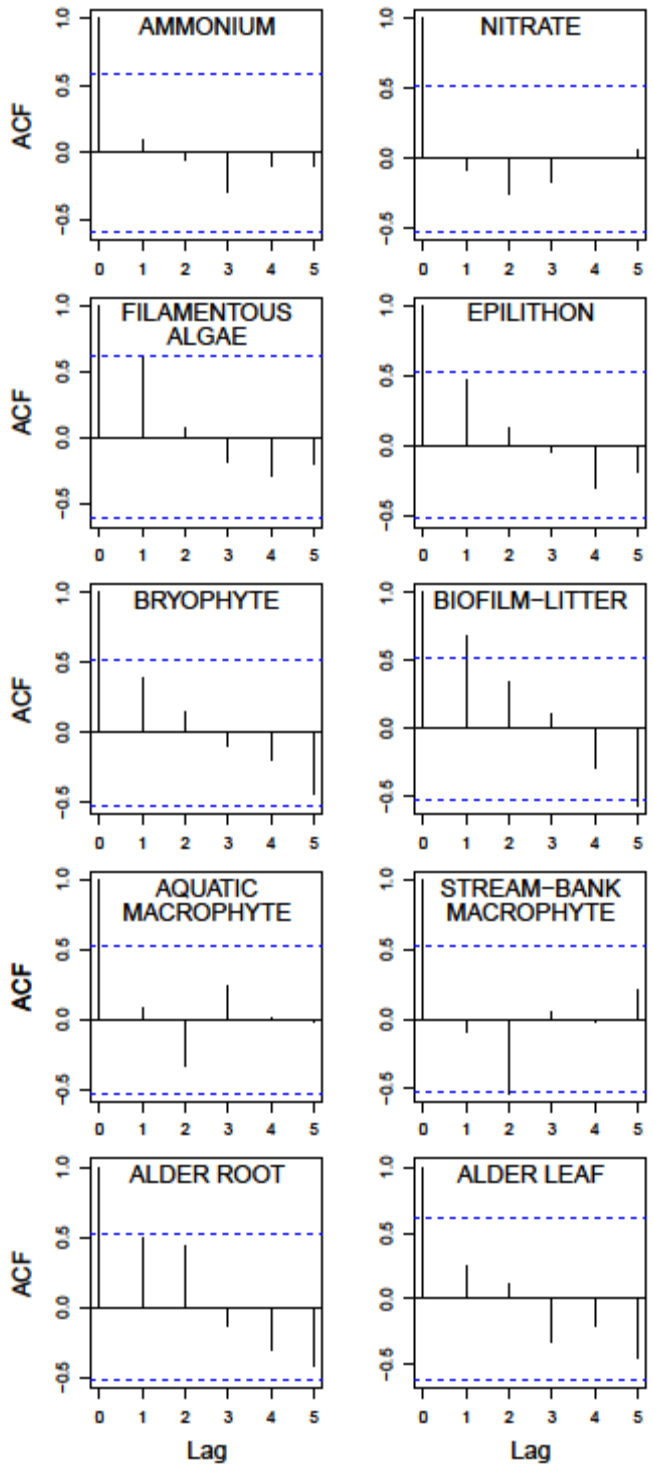

Figure SB.3 Temporal autocorrelation of $\delta^{15}N$ values for DIN species and PUC types at AGR stream. Blue dashed lines correspond to 95% confidence interval for an uncorrelated series.

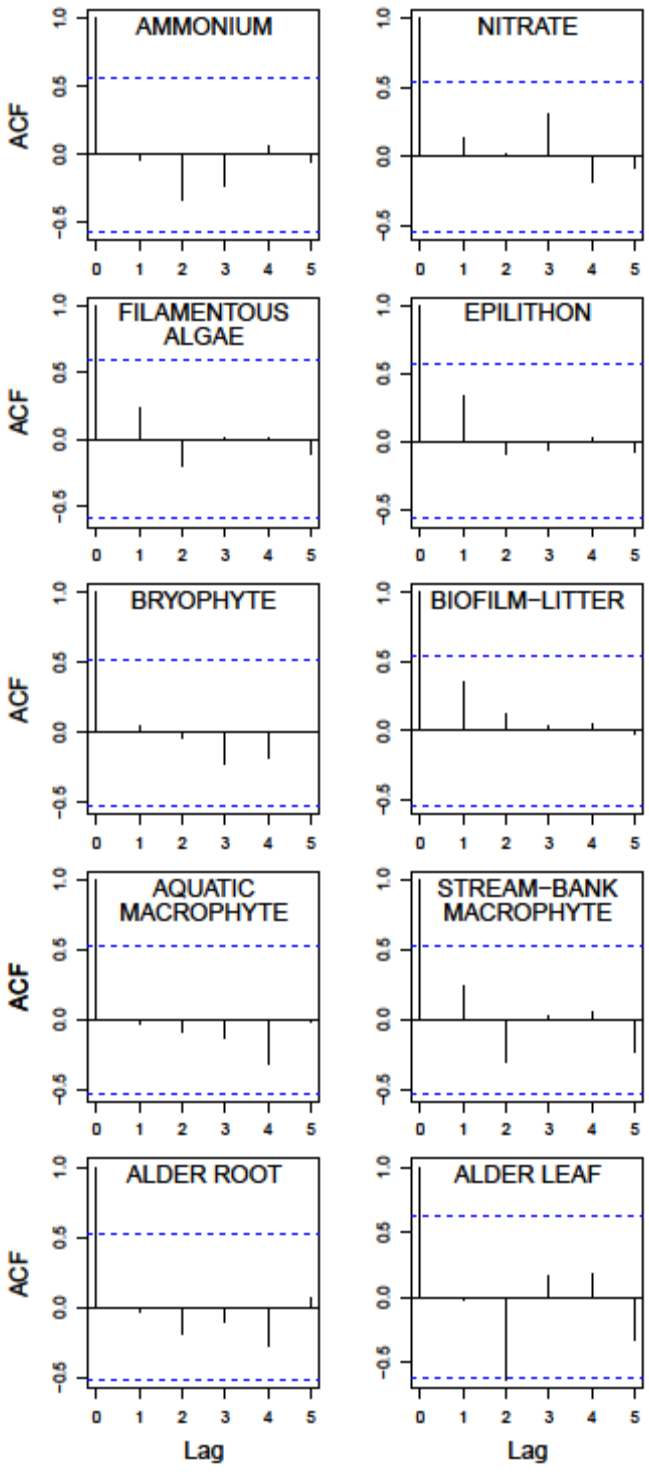

Figure SB.4 Temporal autocorrelation of $\delta^{15}N$ values for DIN species and PUC types at URB stream. Blue dashed lines correspond to 95% confidence interval for an uncorrelated series.

2. Relationships between stream environmental variables and $\delta^{15}N$-DIN species.

Table SB.1 Best-performing multiple linear regression models for predicting $\delta^{15}N$ of DIN species from stream environmental parameters (discharge, and NH_4^+, NO_3^-, SRP and DOC concentrations). The selected models differed less than two units from the minimum AICc and were significant at a $p < 0.05$. AICc is the Akaike Information Criterion corrected for small sample size.

Stream	Selected models	n	AICc	Akaike weight	r^2	RMSE
$\delta^{15}N$-NH_4^+						
FOR	$\delta^{15}N = 10.2 - 1.32NO_3^-$	13	27.05	0.32	0.49	0.60
	$\delta^{15}N = 3.11$	13	28.05	0.16	-	0.84
	$\delta^{15}N = 13.67 - 1.44NO_3^- - 1.35SRP$	13	28.43	0.16	0.73	0.43
HOR	$\delta^{15}N = 3.7$	13	24.86	0.39	-	0.69
AGR	$\delta^{15}N = -8.22 + 6.05NH_4^+$	14	54.99	0.57	0.74	1.91
	$\delta^{15}N = -8.84 + 6.10NH_4^+ + 1.27DOC$	14	56.58	0.25	0.78	1.92
URB	$\delta^{15}N = 118.73 - 23.14Q + 5.92NH_4^+$	12	69.33	0.60	0.82	4.86
$\delta^{15}N$-NO_3^-						
FOR	$\delta^{15}N = 4.55 - 0.83NO_3^-$	13	21.59	0.39	0.41	0.39
	$\delta^{15}N = 5.29 - 0.93NO_3^- + 0.94DOC$	13	23.46	0.15	0.54	0.36
HOR	$\delta^{15}N = 1.86 + 1.29SRP$	13	28.11	0.28	0.38	0.54
AGR	$\delta^{15}N = 67.41 - 9.73\ NO_3^- - 221.52DOC + 34.11\ NO_3^- \times DOC$	14	55.88	0.76	0.73	1.02
URB	$\delta^{15}N = 31.64 - 3.62Q - 2.69DOC$	13	42.24	0.77	0.70	0.90

Interactions terms are expressed using a multiplication sign "×". "Q" stands for discharge. All variables were log-transformed before the analyses.

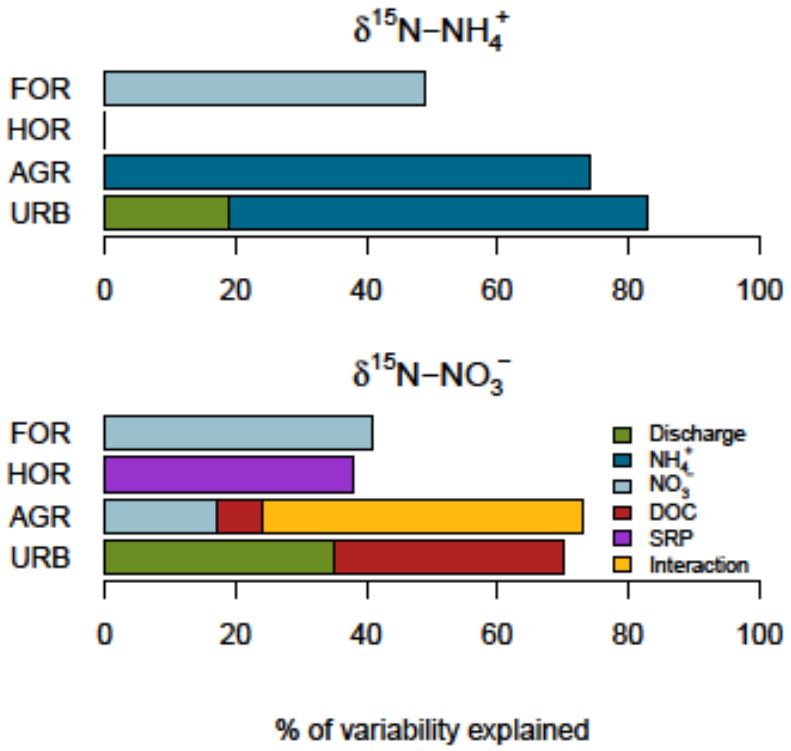

Figure SB.5 Contribution of stream environmental parameters (discharge, and NH_4^+, NO_3^-, SRP and DOC concentrations) to variance of $\delta^{15}N\text{-}NH_4^+$ and $\delta^{15}N\text{-}NO_3^-$, based on the results of the best regression model for each study site (lowest AICc; see Table SB.1 above).

3. Temporal versus with-in reach variability

Table SB.2 Estimated variances and the relative proportion of variance explained by among and within sampling dates for δ^{15}N-epilithon and δ^{15}N- biofilm-litter (replicates: $n = 3$).

	Among sampling dates		Within sampling dates	
	Variance	% explained	Variance	% explained
Epilithon				
FOR	0.54	34	1.04	66
HOR	0.74	38	1.22	62
AGR	2.37	85	0.41	15
URB	28.8	87	4.33	13
Biofilm-litter				
FOR	0.07	29	0.17	71
HOR	0.65	66	0.34	34
AGR	2.61	84	0.50	16
URB	9.18	90	1.05	10

Table SB.3 Standard deviation of epilithon and biofilm-litter replicates (n = 3) taken within the same sampling date for each stream. The highest SD for each time-series is denoted in *italics*.

	Epilithon				Biofilm-litter			
Date	FOR	HOR	AGR	URB	FOR	HOR	AGR	URB
Jul-10	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Aug-10	0.72	0.27	0.52	1.10	0.42	0.14	0.62	*2.81*
Sep-10	0.49	0.61	0.53	3.18	0.26	0.68	0.35	1.15
Sep-10 (2)	0.48	0.67	0.35	0.89	0.13	*1.06*	0.15	0.62
Oct-10	*2.30*	0.77	*1.42*	2.93	0.50	0.57	*1.26*	1.22
Nov-10	2.07	0.31	0.27	2.37	0.30	0.70	0.43	0.61
Dec-10	1.07	0.33	0.10	0.93	0.54	0.74	1.01	0.34
Jan-11	0.96	0.63	0.67	0.80	0.26	0.62	1.06	0.55
Feb-11	0.55	*3.45*	0.24	*3.65*	0.31	0.30	0.79	0.29
Mar-11	n.a.	n.a.	1.15	1.14	0.43	0.45	0.37	0.33
Apr-11	0.50	0.36	0.45	1.04	*0.75*	0.41	0.57	0.23
May-11	0.85	0.64	0.54	n.a.	0.40	0.31	0.28	1.04
Jun-11	0.31	0.04	0.34	1.74	0.19	0.40	0.79	0.61
Jul-11	0.39	0.45	0.76	1.98	0.25	0.52	0.35	0.11

4. Isotopic relationships between DIN species and PUCs

Table SB.4 Pearson correlation coefficients between $\delta^{15}N$ of DIN species (i.e. NH_4^+ and NO_3^+) and $\delta^{15}N$ of PUC types pooling the data for all streams ($p < 0.01$).

PUC types	$\delta^{15}N$-NH_4^+	$\delta^{15}N$-NO_3^+
Filamentous algae	n.s.	0.54
Bryophyte	0.72	0.80
Epilithon	0.63	0.76
Biofilm-litter	0.71	0.80
Leaf	0.51	0.60
Root	0.81	0.80
Stream-bank macrophyte	0.67	0.82
Aquatic macrophyte	0.80	0.79

n.s. stands for not significant correlations ($p > 0.01$). For each stream separately, correlations were not significant for any PUC type at any stream ($p > 0.01$).

5. Cross-correlations between δ^{15}N-PUC and δ^{15}N of DIN species

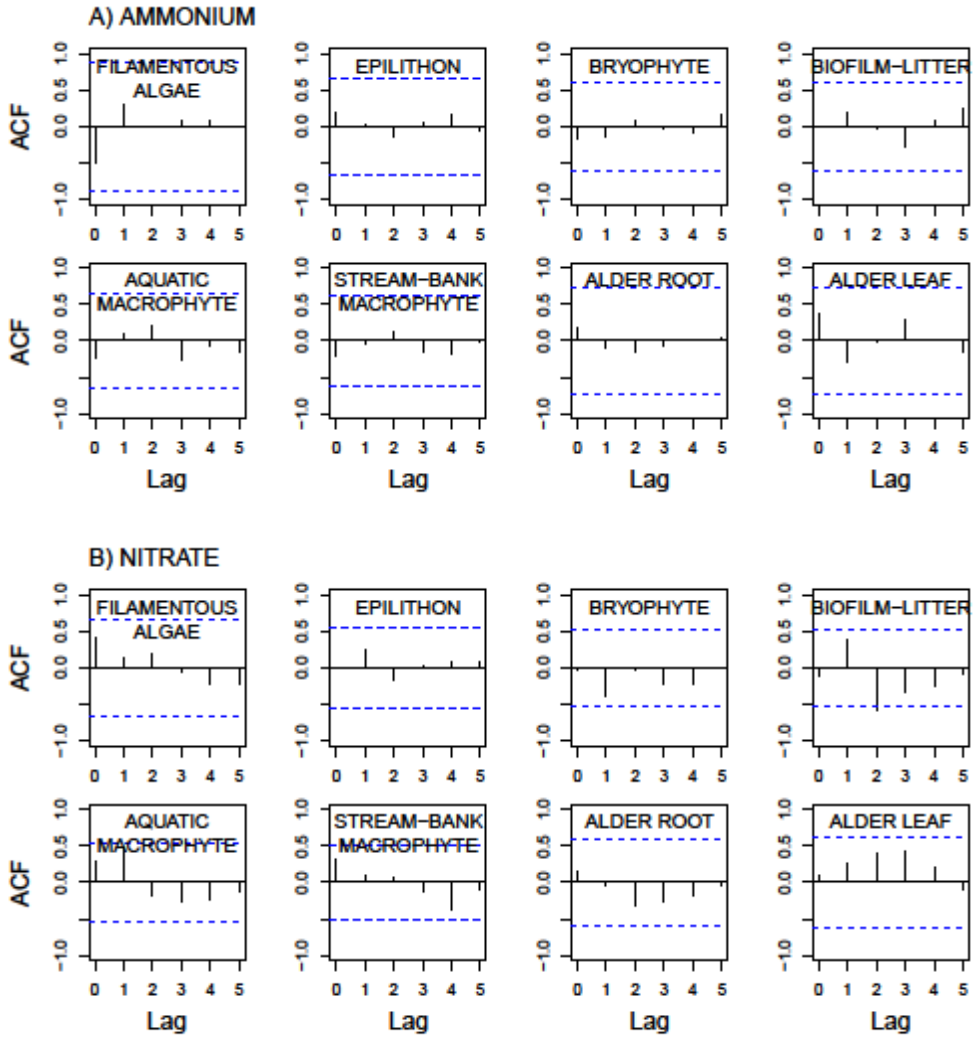

Figure SB.6 Temporal cross-correlations between δ^{15}N of each PUC type and δ^{15}N-NH_4^+ (A) and δ^{15}N-NO_3^- (B) for FOR stream. Blue dashed lines correspond to 95% confidence interval for an uncorrelated series.

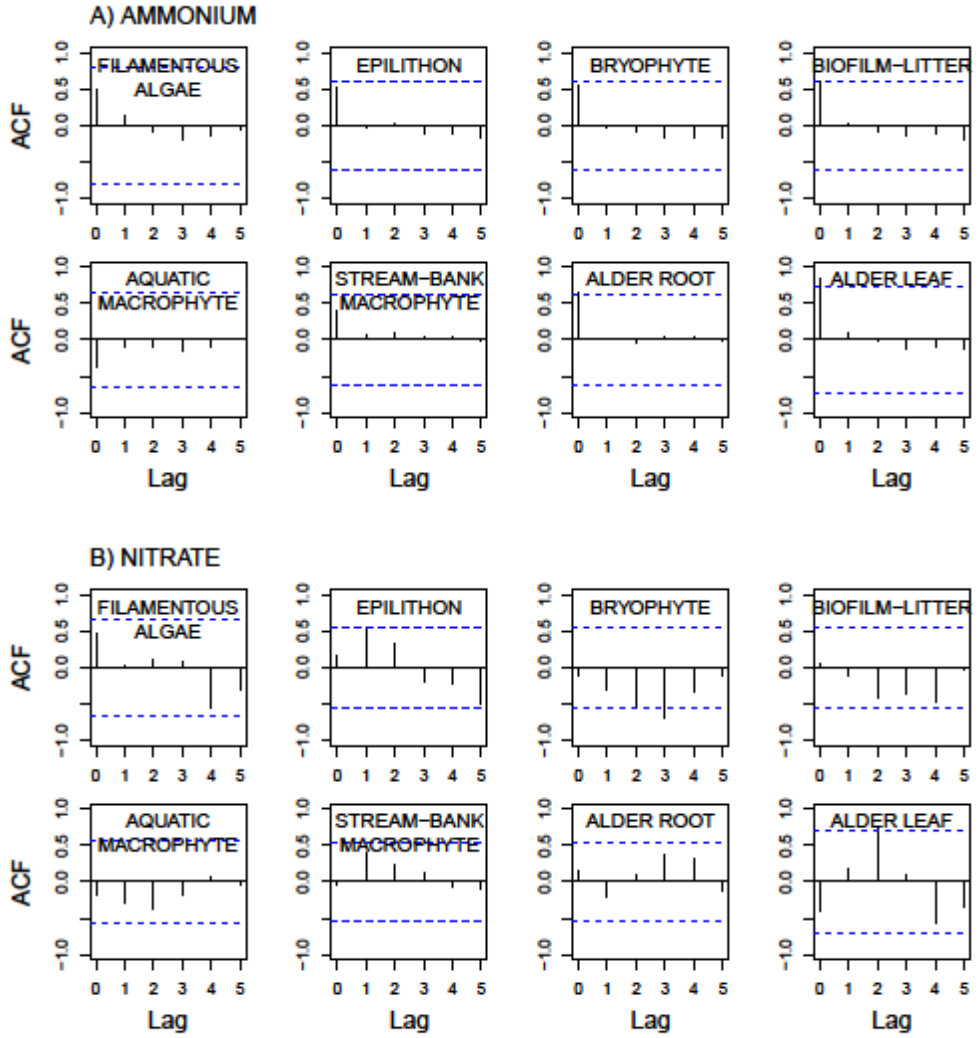

Figure SB.7 Temporal cross-correlations between $\delta^{15}N$ of each PUC type and $\delta^{15}N\text{-}NH_4^+$ (A) and $\delta^{15}N\text{-}NO_3^-$ (B) for HOR stream. Blue dashed lines correspond to 95% confidence interval for an uncorrelated series.

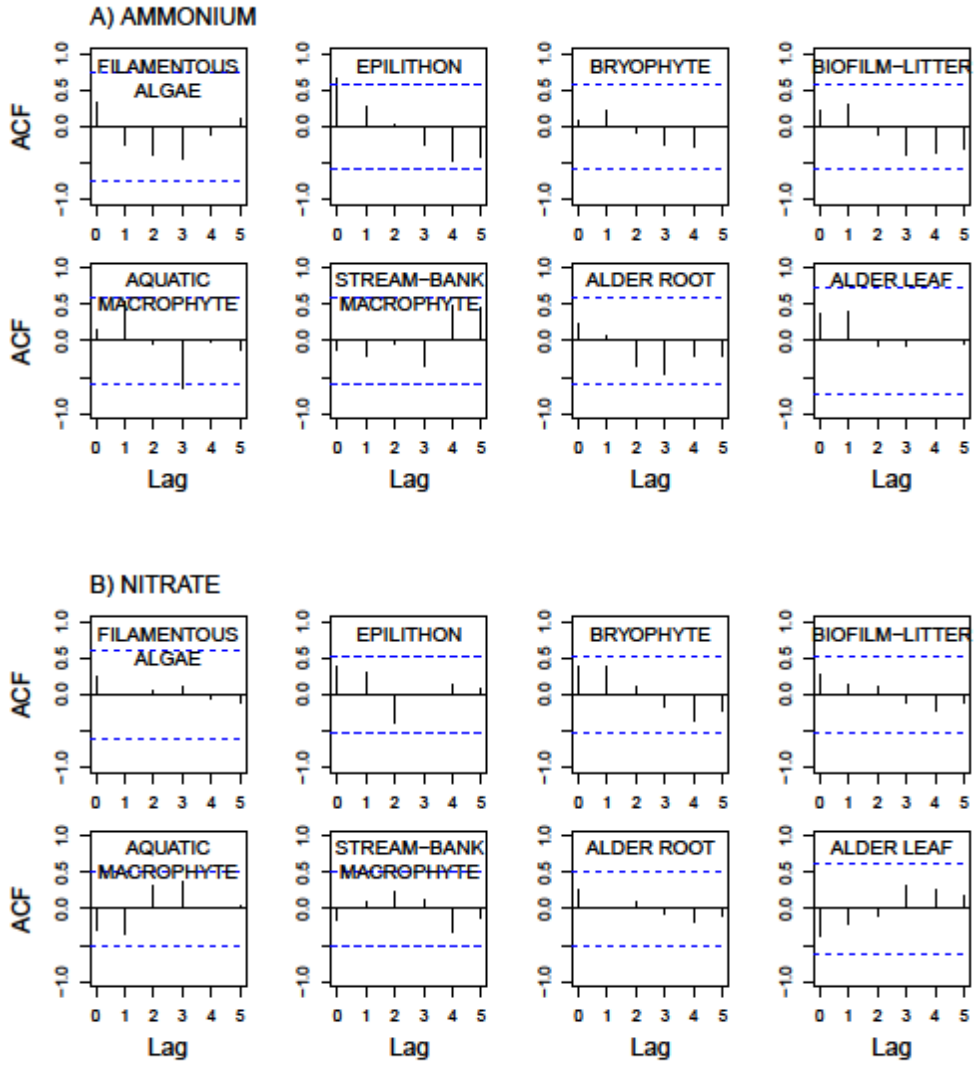

Figure SB.8 Temporal cross-correlations between $\delta^{15}N$ of each PUC type and $\delta^{15}N\text{-}NH_4^+$ (A) and $\delta^{15}N\text{-}NO_3^-$ (B) for AGR stream. Blue dashed lines correspond to 95% confidence interval for an uncorrelated series.

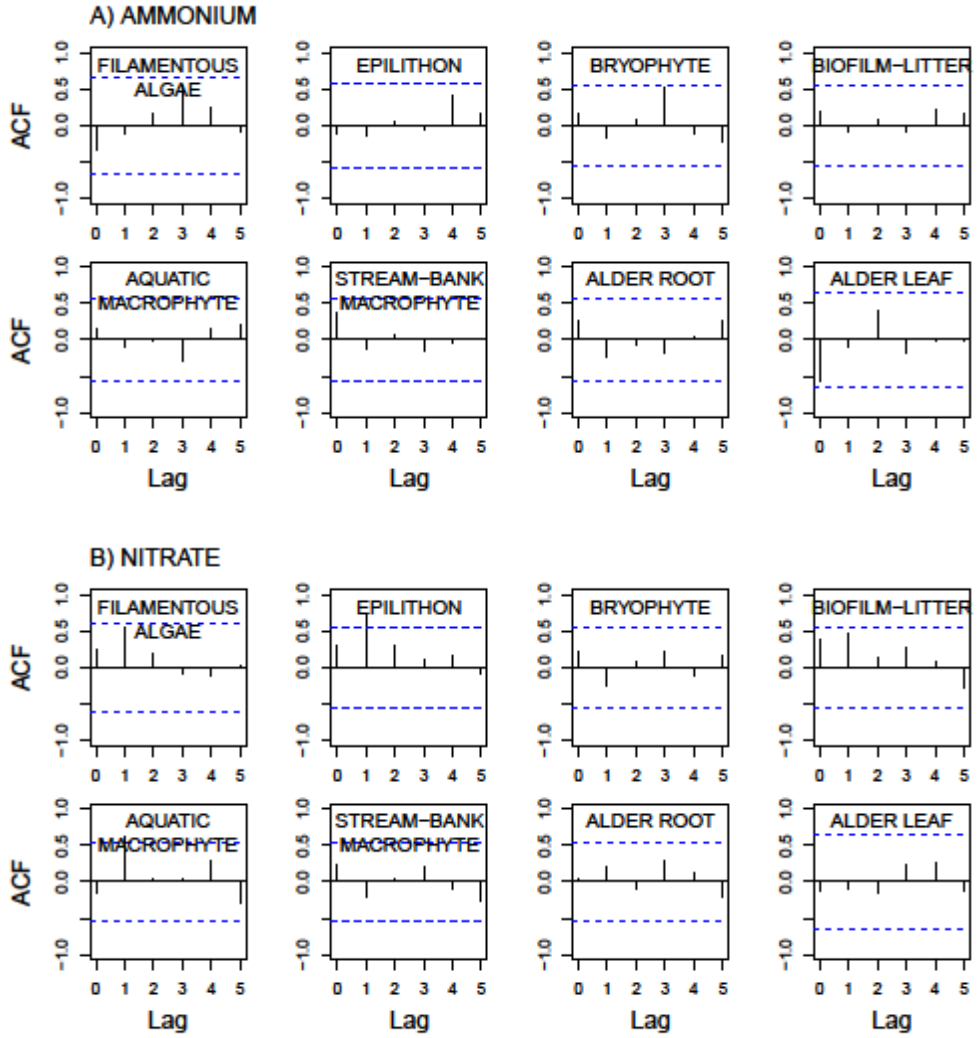

Figure SB.9 Temporal cross-correlations between $\delta^{15}N$ of each PUC type and $\delta^{15}N\text{-}NH_4^+$ (A) and $\delta^{15}N\text{-}NO_3^-$ (B) for URB stream. Blue dashed lines correspond to 95% confidence interval for an uncorrelated series.