Stable isotopes as tracers in aquatic ecosystems
Salvador Sánchez-Carrillo and Miguel Álvarez-Cobelas

Abstract: The addition of stable isotopes (SI) of $^{13}$C and $^{15}$N has been used to study several aquatic processes, thus avoiding environmental disturbance by the observer. This approach, employed for the last three decades, has contributed to expanding our knowledge of food-web ecology and nutrient dynamics in aquatic systems. Currently, SI addition is considered a powerful complementary tool for studying several ecological and biogeochemical processes at the whole-aquatic-ecosystem scale, which could not be addressed otherwise. However, their contributions have not been considered jointly nor have they been evaluated with a view to assessing the reliability and scope of their results from an ecosystem perspective. We intend to bridge this gap by providing a comprehensive review (78 scientific publications reporting in situ $^{13}$C/$^{15}$N additions at the whole-aquatic-ecosystem scale) addressing the main results arising from their use as tracers. Specifically, we focus on: (i) reasons for SI additions at the whole-ecosystem scale to study ecological processes, (ii) the paradigms resulting from its use and the insights achieved, (iii) uncertainties and drawbacks arising from these SI addition experiments, and (iv) the potential of this approach for tackling new paradigms. SI tracer addition at the ecosystem scale has provided new functional insights into numerous ecological processes in aquatic sciences (importance of subsidies in lakes; heterotrophy dominance in benthic food webs in lakes, wetlands and estuaries; the decrease in N removal efficiency in most aquatic ecosystems due to anthropogenic alteration; the recognition of hyporheic zones and floodplains as hot spots for stream denitrification; and high rates of internal N recycling in tidal freshwater marshes). However, certain constraints such as the high cost of isotopes, the maintenance of the new isotopic steady state, and avoidance of biomass changes in any compartment or pool during tracer addition bear witness to the difficulties of applying this approach to all fields of aquatic ecology and ecosystems. The future development of this approach, rather than expanding to larger and complex aquatic ecosystems, should include other stable isotopes such as phosphorus ($^{31}$P/$^{32}$O).

Key words: $^{13}$C, $^{15}$N, tracer addition experiments, food webs, biogeochemistry.

Mots-clés: $^{13}$C, $^{15}$N, expériences d’ajout de traceurs, réseaux trophiques, biogéochimie.

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Introduction

In aquatic science, the ecosystem scale approach is the ideal framework for the study of all ecological interactions among biotic and abiotic components and their processes within defined boundaries (Likens 1992). The main benefit of the whole-ecosystem scale approach is that it encompasses cross-habitat interactions (e.g., littoral–pelagic), which are critical for trophic interactions (Jeppesen et al. 1998). Another benefit of this approach is that nominal physical conditions are maintained (e.g., light, thermal structure, mixing) that affect growth, nutrient cycling, and succession (Carpenter et al. 2001). Although experimental manipulations of entire ecosystems entail certain constraints (for example, pseudoreplication), carefully designed experiments may be considered one of the most powerful approaches for studying process-level topics relative to ecosystems (Likens 1992). Several nutrient enrichment experiments (N and P fertilizations) at the ecosystem level have contributed to increase our knowledge of aquatic-ecosystem metabolism and trophic cascade effects, hence enabling recommendation of policies for eutrophication remediation (Falkowski et al. 2000; Elser et al. 2007; Schindler 2012; Rosemond et al. 2015; Cough et al. 2016). However, fertilization increases ecosystem disturbances—modifying process rates and fates and altering food-web structure through several feedbacks—and this can be good or bad depending on study goals (e.g., in oligotrophic systems when phytoplankton response is the goal and biomass needs to be increased or when studying some biogeochemical processes and rates are altered if the available substance is oversaturated).

The addition of stable isotopes (SI) of 13C and 15N has been used during the last three decades to study several aquatic processes, thereby avoiding disturbance. The temporal stability of SI and the processes governing isotopic fractionation make them useful as tracers, thus allowing measurement of simultaneous ecological and biogeochemical processes at the whole-ecosystem scale (Peterson and Fry, 1987; Schimel 1993; Fry 2006). Since Hershey et al. (1993) and Kling (1994) published their seminal papers using 13C and 15N additions at the whole-ecosystem scale, many other studies have been reported.

The whole-ecosystem isotope enrichment approach is a robust way to examine nutrient flows through multiple pools simultaneously while maintaining natural hydrologic and biogeochemical gradients (Tobias et al. 2003). In situ 13C and 15N additions at the whole-ecosystem scale have provided valuable results to advance (i) the study of carbon uptake, taking the relationships between terrestrial and lake biomes into account (e.g., Cole et al. 2002; Kritzberg et al. 2004; Pace et al. 2004; Carpenter et al. 2005; Taipale et al. 2008); (ii) the uptake, turnover, and retention processes of nitrogen (e.g., Tank et al. 2000a; Merriam et al. 2002; Hall et al. 2009b; Hadwen and Bunn 2005; Hughes et al. 2000; Gribsholt and Boschker 2005); and (iii) the trophic dynamics and food-web structure (e.g., Raikow and Hamilton 2001; Hamilton et al. 2004; Galván et al. 2012). Much of our current knowledge of freshwater ecology is grounded on the results provided by whole-ecosystem SI experimental additions. Now the time has come to evaluate the validity and scope of the results of these complex experiments to assess their impact on studies of aquatic ecology. However, the emerging success of SI addition experiments has not yet been viewed as a whole or evaluated to assess the reliability and scope of their results. This assessment is fundamental to decide the suitability of SI additions at the ecosystem scale for building in current and future paradigms in aquatic science. Here, we intend to bridge this gap by addressing the main subjects arising from the use of 13C and 15N as tracers at the whole-ecosystem scale. Since the field of aquatic science is very broad we have focused on contributions to the knowledge of freshwater ecosystems, considering all those located in the continental context, including water bodies located in coastal areas with varying degrees of tidal influence (i.e., saltmarsh, tidal flats, and estuaries). Our approach is ecologically based to address the structure and functioning of ecosystems and, therefore, we have not addressed the study of groundwater, where studies also use SI as tracers together with their natural contents (e.g., Aravena and Robertson 1998; Smith et al. 2004). Specifically, we will focus on the following questions. (i) Why have SI additions been used at the whole-ecosystem scale in freshwaters to study ecological processes? (ii) To what, and which paradigms have emerged at the experimental ecosystem scale using 13C and 15N additions and what insights have been achieved? (iii) What uncertainties and deficiencies have arisen from those SI addition experiments that should be considered when interpreting the scope of their results? (iv) Can (or should) this approach continue to be used to tackle new paradigms in the future? To answer these questions, we have reviewed 78 scientific publications reporting in situ 13C/15N additions to aquatic continental systems and their contributions have carefully been discussed.

SI addition experiments in freshwater: trends, compounds used, ecosystems studied, and geographic location

Figure 1 shows the trends in publications, from 1990, reporting whole-ecosystem experiments in aquatic systems using 13C and 15N additions. Most experimental additions were carried out from 2000 to 2009 with an increasing application to lentic systems. Although the use of both 13C and 15N additions displays similar trends, 15N experimental additions are more frequent. This is because 15N was used as a tracer of nitrogen dynamics in streams in two large-scale experiments (see below). The joint use of both SI is uncommon.

In situ 13C enrichments have been performed in aquatic ecosystems through manipulation of the dissolved inorganic carbon pool (DI13C; Carpenter et al. 2005), the dissolved organic carbon pool (DO13C; Hall 1995) and the organic particulate pool (PO13C; Bartels et al. 2012; Table 1). Enriched sodium bicarbonate (NaH13CO3 as 98 atom % 13C) has been used extensively in lakes, tidal marshes, and estuaries to trace C transfer flows through food webs (Fig. 2; Cole et al. 2002, 2006; Pace et al. 2004; Carpenter et al. 2005; Kritzberg et al. 2004, 2006; Pace et al. 2007; Taipale et al. 2008). Labile organic compounds such as sodium acetate (CH3COONa as 99 atom % 13C), glucose (13C6H12O6 as 99 atom % 13C), and compounds with a distinctive δ13C stable isotope value, such as corn (a C4 plant) starch and dextrose, have also been added to study the microbial food web in streams (Hall 1995; Hall and Meyer 1998;
In situ 15N additions to aquatic ecosystems are performed to modify the dissolved inorganic nitrogen pool (DI15N) via either nitrate (15NO3) or ammonium pools (15NH4). Modification of the nitrogen organic pool has seldom been undertaken and is based on 15N labelling of algal compartment and subsequently used as enriched detritus (e.g., Li et al. 2010; Yu et al. 2013; Table 2 and Fig. 2). Potassium/sodium nitrate (K/Na 15NO3 as 10–99 atom% 15N), ammonium chloride (15NH4Cl as 10–99 atom% 15N), and ammonium sulphate (15NH4SO4 as 10.7 atom% 15N) have been used in estuaries, lakes, tidal marshes, and streams (Fig. 2) to assess aquatic food-web structure (Hamilton et al. 2004; Galvan et al. 2008) and nitrogen-cycling (Peterson et al. 1997; Hall et al. 1998; Holmes et al. 2000; Sanzone et al. 2001; Gribsholt and Boschker 2005; Hall et al. 2009a; Fig. 2).

Geographically, experiments using 13C and 15N additions to aquatic ecosystems are performed to modify the dissolved inorganic nitrogen pool (DI15N) via either nitrate (15NO3) or ammonium pools (15NH4). Modification of the nitrogen organic pool has seldom been undertaken and is based on 15N labelling of algal compartment and subsequently used as enriched detritus (e.g., Li et al. 2010; Yu et al. 2013; Table 2 and Fig. 2). Potassium/sodium nitrate (K/Na 15NO3 as 10–99 atom% 15N), ammonium chloride (15NH4Cl as 10–99 atom% 15N), and ammonium sulphate (15NH4SO4 as 10.7 atom% 15N) have been used in estuaries, lakes, tidal marshes, and streams (Fig. 2) to assess aquatic food-web structure (Hamilton et al. 2004; Galvan et al. 2008) and nitrogen-cycling (Peterson et al. 1997; Hall et al. 1998; Holmes et al. 2000; Sanzone et al. 2001; Gribsholt and Boschker 2005; Hall et al. 2009a; Fig. 2).

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Table 2. Summary of $^{15}$N tracer addition experiments conducted in aquatic continental ecosystems.

<table>
<thead>
<tr>
<th>Aquatic ecosystem type</th>
<th>Isotope compound</th>
<th>Number of experiments</th>
<th>Isotopic target level ($\Delta^{15}N$)</th>
<th>Addition method</th>
<th>Manipulated pool</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stream</td>
<td>$^{15}$NO$_3$ (10–99 Atom % $^{15}$N)</td>
<td>15</td>
<td>2000–10 000‰</td>
<td>Daily short pulses (2–24 hours) from one day to several weeks</td>
<td>DIN (NO$_3$)</td>
<td>Peterson et al. (2001); Böhlke et al. (2004); Mulholland et al. (2004, 2006, 2008, 2009); O’Brien et al. (2007, 2012); Bernt et al. (2006); Hamilton and Ostrom (2007); Hall et al. (2009a, 2009b); von Schiller et al. (2009); Hubbard et al. (2010); Sobota et al. (2012)</td>
</tr>
<tr>
<td>Tidal marsh</td>
<td>$^{15}$NH$_4$Cl (10–99 Atom % $^{15}$N)</td>
<td>20</td>
<td>500–1100‰</td>
<td>Continuous pulses for several weeks</td>
<td>DIN (NH$_4$)</td>
<td>Hershey et al. (1993); Peterson et al. (1997); Hall and Meyer (1998); Wollheim et al. (1999); Tank et al. (2000a, 2000b); Mulholland et al. (2000a, 2000b); Raikow and Hamilton (2001); Sanzone et al. (2001, 2003); Hamilton et al. (2001, 2004); Merrian et al. (2002); Webster et al. (2003); Ashkenas et al. (2004); Parkyn et al. (2005); Morrall et al. (2006); Riis et al. (2012); Sánchez-Carrillo et al. (2017)</td>
</tr>
<tr>
<td>Estuary</td>
<td>$^{15}$NO$_3$ (10–99 Atom % $^{15}$N)</td>
<td>5</td>
<td>650–1000‰</td>
<td>Daily short pulses (hours) for a couple of weeks</td>
<td>DIN (NO$_3$)</td>
<td>Tobias et al. (2001); Galván et al. (2008, 2012); Drake et al. (2009)</td>
</tr>
<tr>
<td>Lake</td>
<td>$^{15}$NO$_3$ (10–99 Atom % $^{15}$N)</td>
<td>1</td>
<td>*</td>
<td>Continuous addition for 10 days</td>
<td>DIN (NO$_3$)</td>
<td>Epstein et al. (2012)</td>
</tr>
<tr>
<td>Hyporheic zone</td>
<td>$^{15}$NH$_4$Cl (10–99 Atom % $^{15}$N)</td>
<td>2</td>
<td>*</td>
<td>Continuous pulses for several weeks</td>
<td>DIN (NH$_4$)</td>
<td>Kling (1994); Armengol et al. (2012);</td>
</tr>
<tr>
<td></td>
<td>$^{15}$N-organic (Microcystis)</td>
<td>2</td>
<td>1800‰</td>
<td>Single injection</td>
<td>PON</td>
<td>Li et al. (2010); Yu et al. (2013)</td>
</tr>
</tbody>
</table>

Note: Values in brackets in the aquatic ecosystem type column represent the number of stable isotope addition experiments conducted as reported in the literature. DIN, dissolved inorganic nitrogen; PON, particulate organic nitrogen. *Data not found in the literature.
What do SI tracer additions at the whole-ecosystem scale have that other approaches lack?

Isotopes as tracers have been used complementary to other studies to solve some of the main issues in freshwater ecology that could not be addressed otherwise. When isotopes are used at the whole-ecosystem scale, results are supported by all possible biotic and abiotic interactions occurring in the studied system. The addition of SI is most useful when employed to: (i) make parsimonious model choices (thus simplifying predictor variables), (ii) calibrate other more elegant and scalable approaches (like natural abundance SI interpretation; Galvan et al. 2012), and (iii) obtain in situ actual rates that cannot be obtained by either net mass balances and benchtop work or natural abundance measurements.

In the natural environment, fractionation can be a huge concern in SI studies. The overall goal of doing added tracer experiments is to discard ambiguity resulting from fractionation and source overlap using SI natural abundance. Due to kinetic isotope effects [KE = (kD/kH₂O - 1) × 1000], which measures the ratio of reaction rate constants (k) of heavy (kD) and light (kH₂O) isotopologues, expression in units of per thousand (‰), the isotope fraction becomes enriched in the lighter isotope relative to the reactant. Furthermore, if the reactant pool is limited because it is in a closed system (e.g., anaerobic substrate compartments, along the groundwater flowpath in aquifers and riparian zones, and occasionally in river sediments; Kellman and Hillaire-Marcel 1998), the reactant becomes enriched in the heavier isotope. However, this could vary depending on the environmental conditions and whether the reaction is an equilibrium process (e.g., Mariotti et al. 1981; Mariotti et al. 1988; Fustec et al. 1991). In open-system experiments, the simultaneous addition of reactant may confound the natural isotope fraction of a given process. The reactant pool is changing because of the downstream transport of upstream substances into the system reach and the diffusion from the water column; neither process can be discriminated isotopically. For example, out of all N removal processes from rivers, only denitrification is accompanied by significant isotope fractionation enriching the remaining nitrate in 15N (Böttcher et al. 1990). However, the concomitant admixture of nitrate from other sources tends to increase riverine nitrate concentrations and this simultaneous addition of nitrate would readily mask any isotope denitrification signal from SI natural abundance analyses (Mayer et al. 2002). Therefore, when adding SI as tracers, the target enrichment is great enough to clearly differentiate the pool of interest, making any potential fractionation trivial. There are two main situations where the contribution of SI tracer addition at the ecosystem scale has been crucial for incorporating new functional insights in aquatic ecology: food-web ecology and nutrient dynamics.

New approaches to the analysis of nutrient fluxes and transformations

The addition of SI as tracers in whole-ecosystem experiments has provided new insights into nutrient dynamics, particularly for the aquatic nitrogen cycle. Although traditional methods for the study of N-cycling (input–output budgets and experimental additions of ammonium or nitrate; Grimm 1987; Marti and Sabater 1996) have provided valuable information on N dynamics, they were found to have major shortcomings: (i) since processes of uptake, release, and downstream transport occur simultaneously in all ecosystem compartments, ambient rates of uptake, transformation, and retention could not be measured independently through mass balances, or were substantially altered by nutrient enrichment; (ii) the key role of specific biomass pools in N uptake and retention could only be indirectly inferred; and (iii) N turnover rates and the fate of N taken up could not be explicitly determined (Mulholland et al. 2000c; Drake et al. 2009). These shortcomings were overcome by using nutrient SI tracers as we will reveal below. Addition of ammonium or nitrate with high 15N-enrichment levels results in a negligible increase in ammonium or nitrate concentrations, avoiding the stimulatory effects of added nutrients. 15N additions have been used successfully to trace most N-cycle processes, allowing assessment of N uptake, turnover, and retention processes at whole-ecosystem scale in wetlands, lakes, and streams (Gribsholt and Boschker 2005; Mulholland et al. 2009; Sánchez-Carrillo et al. unpublished results) as well as in hydrodynamic complex systems such as estuaries, hyporheic zones, and floodplains (Holmes et al. 2000; Hall et al. 2009b; Hubbard et al. 2010; Zarnetske et al. 2011a, 2011b).

Main findings of 13C and 15N tracer additions at the whole-ecosystem scale advances aquatic science

Carbon subsidies supporting food webs

For a long time, ecologists viewed ecosystems as autotrophic or supported by energy derived from photosynthetic carbon fixation within the system. However, several facts revealed by metabolic studies (based upon bottle incubations, gas saturation, dissolved oxygen, and carbon dioxide diel changes) suggest that many lakes are net heterotrophic, meaning that system respiration (R) exceeds gross primary production (GPP; Jansson et al. 1999; J.L. Riera et al. 1999; Carignan et al. 2000; Cole et al. 2000). This net hetero-
trophy was only maintained if R was supported by allochthonous organic matter. 13C tracer additions at the whole-lake scale demonstrated that terrestrial (allochthonous) DOC is respired by bacteria and that this external carbon subsidizes food webs, being that this allochthony is inversely proportional to lake nutrient enrichment (Table 3; Cole et al. 2002, 2006; Kritzberg et al. 2004, 2006; Pace et al. 2004; Carpenter et al. 2005; Taipale et al. 2008). Moreover, experimental additions have shown that terrestrial carbon inputs subsidize benthic food webs and from here upward the pelagic habitats in oligotrophic lakes (Bartels et al. 2012), because this allochthony is inversely related to NO3 concentration (2006) in tidal flats demonstrated that bacterial carbon is a primary sink of organic carbon in the food web, with recycling of nitrogen within stream ecosystems (e.g., Davis and Minshall 1990). In estuarine environments, studies tracing 13C revealed the temporary strong coupling between bacterial and algal production during phytoplankton blooms, which influences the feeding strategies of mesozooplankton (van den Meersche et al. 2011). Bacterivory in coastal sediments was considered a marginal source for benthic heterotrophs (Hamels et al. 2001); however, the advent of assays using additions of enriched 13C organic dissolved compounds has enabled this assertion to be tested. Experiments performed by Van Oevelen et al. (2006a, 2006b) and Veuger et al. (2006) in tidal flats demonstrated that bacterial carbon is a primary sink of organic carbon in the food web, with recycling of carbon within the dissolved organic carbon–bacteria loop and bacterial respiration as the main loss process from this loop (Table 3).

**Table 3.** Summary of the main contributions of 13C and 15N tracer additions at the whole-ecosystem scale for the advancement of aquatic sciences.

<table>
<thead>
<tr>
<th>Research area and topic</th>
<th>Ecosystem</th>
<th>Stable isotope</th>
<th>Finding</th>
<th>Earlier reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food webs:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allochthony</td>
<td>L</td>
<td>13C</td>
<td>Ecosystem net heterotrophy is supported by allochthonous C</td>
<td>Cole et al. (2002)</td>
</tr>
<tr>
<td>Bacterial supports</td>
<td>S</td>
<td>13C</td>
<td>Invertebrates derive a large C fraction from bacteria in headwater streams</td>
<td>Hall (1995)</td>
</tr>
<tr>
<td>CW 13C</td>
<td>Microphytobents support meiofauna but bacterial C is mainly a sink</td>
<td>Middleburg et al. (2000) and van Oevelen et al. (2006a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nutrient dynamics:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-cycling</td>
<td>S</td>
<td>15N</td>
<td>NH4 depends upon the physical environment</td>
<td>Mulholland et al. (2000a)</td>
</tr>
<tr>
<td>S 15N</td>
<td>NO3 depends upon the chemical and biological environment</td>
<td>Böhlke et al. (2004)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 15N</td>
<td>Denitrification depends upon physical, chemical and biological factors</td>
<td>Mulholland et al. (2009)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 15N</td>
<td>Hotspots for denitrification mostly occur in transient storage zones (hyporheic zone and floodplain)</td>
<td>Zarnetske et al. (2011a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S, E, CW 15N</td>
<td>N dynamics (cycling, uptake and sequestration) is inversely related with NO3 concentration</td>
<td>Mulholland et al. (2008) and Drake et al. (2009)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E, L 15N</td>
<td>Long-term N retention occurs in the benthic habitat (epiphytes or macrophytes)</td>
<td>Gribsholt et al. (2005), Epstein et al. (2012) and Sánchez-Carrillo et al. (2017)</td>
<td></td>
<td></td>
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</table>

**Note:** L, lake; S, stream; CW, coastal wetland; E, estuary.

**The importance of bacteria supporting food webs in forest streams**

In headwater forest streams, microbes can be more productive than primary producers, representing the trophic base of the ecosystem (net heterotrophic; Cummins 1974). In these systems, consumers feed on an assemblage of bacteria and detritus, and for years researchers have believed they rely mostly on detritus (plant debris) but were unaware of the trophic link between bacteria and larger consumers ( meiofauna, macroinvertebrates, and protozoa); Hall (1995). For years, the fraction of invertebrate carbon derived from bacteria was a subject of controversy limited by the available methodology to determine bacterial consumption by grazers (visible bacterial tracers such as fluorescence or isotopic labelling of bacteria in laboratory assays during very short time periods; Hall et al. 1996). Additions of 13C enriched in stream-scale (whole-system) experiments succeeded in labelling sediment bacteria, including biofilms and exopolymers, and were able to determine the fraction of invertebrate carbon derived from bacteria (Table 3). Studies using 13C tracer additions have highlighted the importance of heterotrophic biofilms as the basic organic source supporting food webs in forest streams, suggesting that invertebrates derive between 10% and 100% of their carbon from bacteria (Hall and Meyer 1998; Simon et al. 2003; Parkyn et al. 2005; Wilcox et al. 2005), with exopolymers (exopolysaccharides) reported as the basic organic source for both biofilm scrapers and predatory invertebrates (Hall 1995; Parkyn et al. 2005; Hall and Meyer 1998; Hall et al. 2000).

**The old paradigm of the “detritus-based ecosystem” in coastal environments**

Coastal food-web studies using the natural abundance of stable isotopes have brought into question the historical assumption regarding the dietary predominance of cordgrass (Spartina) in salt marshes, revealing the importance of benthic microalgae and phytoplankton in food webs (e.g., Haines and Montague 1979). Galvan et al. (2012) and P. Riera et al. (1999) measured natural abundance isotopes and performed experiments using 13C additions in coastal water bodies, demonstrating that macrophyte detritus was less important as a food source for most infaunal species than algal sources. Microphytobenthos moderate carbon flow through benthic heterotrophs with a rapid, direct linkage between algae and bacteria via exudates (Table 3; Middleburg et al. 2000). In estuarine environments, studies tracing 13C revealed the temporary strong coupling between bacterial and algal production during phytoplankton blooms, which influences the feeding strategies of mesozooplankton (van den Meersche et al. 2011). Bacterivory in coastal sediments was considered a marginal source for benthic heterotrophs (Hamels et al. 2001); however, the advent of assays using additions of enriched 13C organic dissolved compounds has enabled this assertion to be tested. Experiments performed by Van Oevelen et al. (2006a, 2006b) and Veuger et al. (2006) in tidal flats demonstrated that bacterial carbon is a primary sink of organic carbon in the food web, with recycling of carbon within the dissolved organic carbon–bacteria loop and bacterial respiration as the main loss process from this loop (Table 3).

**Key factors controlling N-cycling in streams**

Before the use of 15N as tracer, our knowledge of the N-cycle in lotic systems was often incomplete. Streams were viewed as conduits for nitrogen from terrestrial to marine ecosystems (e.g., Howarth et al. 1996), but numerous studies clearly demonstrated the complexity and efficiency of the transformation and assimilation of nitrogen within stream ecosystems (e.g., Davis and Minshall 1999; Dent and Grimm 1999; Mulholland et al. 2000a; Peterson et al. 2001). Most information has been provided from LINX
projects (Lotic Intersite Nitrogen eXperiments; www.faculty.biol.
vt.edu/webster/linx), two large-scale 15N addition experiments carried out in 15 headwater streams (LINX I using 15NH4+), and 72 streams (LINX II using 15NO3−) throughout US. The addition of 15N at the stream scale elucidated how uptake and removal of NH4+ and NO3− depend on different variables (Table 3): the former on physical environment (stream size and hydrology, decreasing ammonium uptake and retention in large streams; Mulholland et al. 2000a; Tank et al. 2000b; Peterson et al. 2001; Wollheim et al. 2001; Dodds et al. 2002; Ashkenas et al. 2004; Hamilton et al. 2004; Morrall et al. 2006) and the latter on chemical and biological variables (nitrate concentration and autotrophic assimilation; Böhleke et al. 2004; Mulholland et al. 2008; Hall et al. 2009b; Mulholland et al. 2009). 15N tracer additions revealed produced of autochthonous dissolved organic nitrogen represents a substantial transformation of stream N (a similar magnitude to those of nitrification and denitrification), depending on ecosystem heterotrophy (Johnson et al. 2013). 15N tracer experiments have demonstrated that a combination of hydrological (flow velocity x depth), chemical (NO3− and NH4+ concentrations), and biological (ecosystem respiration rate) factors are the most important controls of stream denitrification, with in situ denitrification from stream water accounting for a small fraction of nitrate removal (=16%; Böhleke et al. 2004; Mulholland et al. 2009; Table 3) and with a neglected effect of DOC on nitrate stimulation (Johnson et al. 2012). Transient storage zones (including hyporheic zones) were recognized as hot spots for stream denitrification (Zarnetske et al. 2011a, 2011b). 15N addition assays demonstrated that hyporheic zones and floodplains acted as nitrogen sinks, decreasing nitrogen removal capacity along with anthropogenic alteration of streams (Hall et al. 2009a; Hubbard et al. 2010). Other experimental additions of 15NH4+ have helped to confirm previous observations in relatively unexplored topics such as subsidies from aquatic to terrestrial habitats. In this respect, Sanz and others (2003) demonstrated that invertebrate invertebrates (such as spiders and odonates) facilitate energy transfer from aquatic to terrestrial habitats by consuming emerging aquatic insects along the stream edge.

The role of the benthic zone in nutrient dynamics of lentic systems

Historically, research has neglected the role of the benthic zone (including the littoral) in nutrient dynamics of lakes. Vertical fluxes (sedimentation, eddy diffusion, fall turnover) have been considered largely responsible for controlling nutrient concentrations and therefore plankton production (Horne and Goldman 1994). However, the majority of lakes worldwide are relatively small and shallow, with extensive littoral zones, which may be important for nutrient uptake and recycling (Downing et al. 2006). 15N additions in lakes are very scarce but experimental tracing has also shed light on nitrogen partitioning and transport; in oligotrophic lakes nitrogen enters the food web fast via the seston (pelagic primary producers) but is retained for longer periods within the benthic zone (Epstein et al. 2012; Sánchez-Carrillo unpublished results; Table 3). Experiments undertaken in Lake Taihu (China) using detritus from cyanobacteria (Microcystis) cultures enriched with 32P have demonstrated the key role of cyanobacterial detritus from blooms as a food source for both planktonic and benthic food webs, including quick assimilation by submerged and emergent macrophytes (Li et al. 2010; Zhang et al. 2010; Yu et al. 2013).

The 15NO3− tracer addition technique has also improved our understanding of nitrogen-cycling in complex systems such as estuaries. Studies have revealed the key importance of benthos in the N-cycling of estuaries, which also supports phytoplankton demands (Holmes et al. 2000; Tobias et al. 2003). Further studies in estuaries demonstrate that nitrate loading plays a crucial role in N-dynamics: the efficiency in N-cycling, sediment sequestration, and efficiency of plant uptake in estuaries all decrease in conjunction with high NO3− concentrations (Drake et al. 2009). Also, 15N tracer addition has revealed that the benthic microbial community is the most important mechanism for long-term nitrogen retention in tidal freshwater marshes (Gribsholt et al. 2005, 2006, 2007, 2009; Veufer et al. 2007; Table 3). A decline in denitrification efficiency as NO3− concentration increases has also been observed in coastal marsh ecosystems (Drake et al. 2009).

Fig. 3. Hierarchical distribution of the main topics under consideration in stable isotope (SI) tracer addition at the whole-ecosystem scale to trace ecological processes. The main issues that might currently limit a SI tracer addition experiment are marked with †; the main sources of uncertainty are noted by *.

Constraints of 13C and 15N tracer additions at the whole-ecosystem scale

Target enrichment, dilution of added SI and exchange boundaries

The amount of 13C or 15N to be introduced into the system (∆δi = enriched δ − baseline δ) must have a large enough target enrichment to be unambiguous and measurable (immediate, large, and transient increase in the isotopic signal), but simultaneously avoiding alterations in the ambient concentration by the added substance (Mulholland et al. 2000a; Tank et al. 2000b; Cole et al. 2002). Detectable amounts of SI added are dependent on pool sizes, pool concentrations, and the number of intermediate biogeochemical transformations before the target item is traced (Boschker and Middleburg 2002; Fig. 3). It is logical to think that since 13C has lower fractionation than 15N, lower enrichments are needed (Tables 1 and 2). However, it depends on the target, the compound used, and the system under study (Fig. 3); for example, if 13C is added as inorganic carbon, there is a rapid loss to the atmosphere, and if the zooplankton community or planktivorous fish are the targets, large SI amounts should be added (Cole et al. 2002). In situ 15NO3− tracer studies of denitrification are constrained by two main challenges: contamination of samples by air dinitrogen (N2) and discrete increases in 15NO3−. The measurement of isotope-ratios of N2 after 15NO3− addition is subject to potential for contamination by N2 in air. Dissolved gases need to be extracted into a headspace, and the headspace gas sample needs to be stored until analysis by isotope ratio mass spectrometry in the laboratory. Even seemingly slight contamination by air, either as bubbles in the water sample or from leakage during sample processing or storage, can impair the accuracy of analysis because the partial pressure of N2 in the headspace sample is far less than that in the air. Moreover, dissolved N2 in surface waters is continually exchanging with atmospheric N2, and therefore background level of
and dynamic turnover are high. Consequently, elevated $^{15}$N enrichments are difficult to obtain, and in situ addition experiments commonly involve only discreet increases in $^{15}$N$_2$ (Fig. 3). The amount of added tracer is constrained by the high cost of the isotope and the need to minimize the increase in NO$_3^-$ availability produced by tracer addition (not enhancing denitrification rates; Fig. 3). Thus, isotope addition experiments commonly produce only modest increases in the $^{15}$N$_2$ of dissolved N$_2$, in which case measurement methods have to be optimized to quantify the tracer $^{15}$N at enrichments that may be only a few $\%$ above background (natural abundance; Hamilton and Ostrom 2007).

As previously stated, SI addition experiments make fractionation trivial. However, dilution of the added SI through exchange outside the control volume could be an important issue for in situ experiments (Fig. 3). In salt marshes $^{13}$C additions can only be done at small scales or in some constrained environments because whole-ecosystem scale experiments face the dilution from seawater bicarbonates (Hardison 2009; Hardison et al. 2010). In larger streams and rivers, dilution continues to be an important constraint in $^{15}$N addition experiments. Indeed, it is a simple fact that the amount of $^{15}$N added is not enough to trace N dynamics. Clearly, the addition of a sufficient quantity of $^{15}$N to be able to measure $^{15}$N$_2$ production and food-web relationships would become prohibitively expensive, but not impossible. Currently, it is incredibly difficult to label benthos sufficiently to determine the long-term fate of assimilated $^{15}$N in streams (O’Brien et al. 2012).

Characterizing the exchange boundaries and fluxes outside the control volume is of vital importance in any ecosystem study and also in SI additions at the whole-ecosystem scale (Fig. 3). We have previously pointed out that in open systems the downstream transport of substances, such as nitrate carried from upstream into the study reach or diffusion from the water column, increases riverine nitrate concentration and changes the isotopic signal of denitrification. Sometimes concentrations and signatures can change considerably during the experiment due to the simultaneous addition of nitrate from sewage or manure (elevated $^{15}$N) and this can worsen the accuracy of results and estimates of process rates (Fig. 3). Downstream transport of organic matter (algae and allochthonous organic matter) carried from upstream into the study reach can confound tracer labelling of consumers. Unlabelled material can be ingested by consumers that filter the water or feed on deposited organic matter, which does not reflect the isotopic enrichment of consumers, leading to overestimation of the importance of allochthonous sources (Hamilton et al. 2004).

### Turnover rate and experiment duration

All SI addition experiments are designed for matching experiment duration to turnover times in pools of interest (Fig. 3); however, this is not always easy to achieve. The isotopic values of consumers may lag behind those of their food, with the lag period being a function of consumer turnover rates (Weidel et al. 2011). Although in-depth discussion on this topic falls outside the scope of this study (see Martínez del Río et al. 2009 for further information), some considerations are of interest to correctly assess SI additions at the whole-ecosystem scale. The question often arises as to the proper SI addition method based on both the pools under study and the turnover times (Table 3). The isotopic turnover rates and the factors that influence them must be considered: (i) determine the time window through which pools of interest reflect the isotope change, and (ii) assume that variability in resource use by pools or tissues may be related with different turnover rates (Martínez del Río et al. 2009). Pulses with large SI enrichments are suitable for fast reactions like biogeochemical ones, provided that transport and dilution do not reduce them (e.g., Cole et al. 2006; Pace et al. 2007). For example, to study denitrification a ground-water pulse will work, but this will not be useful for surface water because of differences in gas exchange. In theory a pulse or prolonged SI tracer delivery will work for the food web as long as the pulse is sufficiently enriched. Typically, for food-web studies the SI tracer is added for a sufficiently long timescale so that enrichment can be detected in the longest turnover pool of interest (Mulholland et al. 2000b). In practice, that is difficult but the general idea that emerges is that researchers can trade enrichment for duration.

One may expect that pools of interest are closer to the new isotopic steady-state enrichment during SI addition (Table 3). Therefore, an even distribution of SI tracer throughout the enrichment period is a prerequisite to trace the label properly and construct a reliable model for the whole ecosystem. However, numerous SI addition experiments have not reached this new steady-state enrichment for much of the assay duration. To what extent is it important to reach this new steady state? Most biological processes are generally unidirectional and kinetic isotope reaction rates are dependent on the ratios of isotopic masses and concentrations of products and reactants. Usually, in the course of experiments, researchers do not wait to perform sampling until the new steady state takes place, but they wait until they have data showing that the trend towards that new steady state is sufficient to fit a model. Although faster reaction steps (e.g., nitrification and denitrification) reach the new isotopic steady state enrichment early, to maintain it for a long time has proved to be more difficult than in slower reaction steps (e.g., food-web; Mulholland et al. 2009).

### Changes in standing stocks

The assumption of turnover rate steady state would also be rejected if there was substantial biomass growth in any compartment during tracer addition (Hesslein et al. 1993). Standing stock measurements (i.e., changes in algal and microbial biomass) are recommended regularly, and they also have to be taken into account in the model of isotopic turnover (Dodds and Welch 2000).

### Modelling results of SI additions at the whole-ecosystem scale

In the whole-ecosystem approach using $^{13}$C and $^{15}$N additions, box models (named compartmental models in some studies) are commonly used for data treatment. They have been implemented considering several approaches, according to the manipulated and target pools, as well as the process and the physical and biological features of each ecosystem (Carpenter et al. 2005). Due to the dynamic nature of SI addition experiments, steady-state models, like those used in studies of natural isotopic abundance, are considered inappropriate (Carpenter et al. 2005); however, when studying N-cycling, some authors have recently used this approximation to obtain variable daily inputs (e.g., Hall et al. 1998; Böhleke et al. 2004). Box dynamic models have been used extensively in SI addition at the whole-ecosystem scale to study C and N dynamics (Hall et al. 1998; Tank et al. 2000b; Cole et al. 2002; Carpenter et al. 2005; Cole et al. 2006; Pace et al. 2007; Solomon et al. 2008; Weidel et al. 2008). These models are essentially multipool finite difference dynamic models that assign functions (e.g., first order, zero order, or otherwise) to reactions (connecting the boxes), then a mass balance of each isotope is independently computed, and the enrichment at each time step is subsequently recalculated. Parameters are optimized to best fit the data. Models have been designed to simulate a high number of pools simultaneously and have been developed for each particular process to meet both the features of each ecosystem type (e.g., lotic versus lentic) and the aims of each assay (Cole et al. 2002). The first modelling in SI addition was developed by Hall et al. (1998) when studying N-cycling in streams, but they did not consider any transformation rates. The most comprehensive dynamic box model is the one created by Cole at al. (2006) studying C dynamics in four small northern US lakes. The model built was named dual-isotope flow modelling (DIF, as it simulates the flow of total-C ($^{12}$C + $^{13}$C) and $^{14}$C, and was able to compute 12 carbon pools (DIC, pCO$_2$, DOC, ...
The study of N stream dynamics also requires complex box models based on exhaustive reaction rates, which include solute travel times, variable air–water gas exchanges affected by temperature and other gas fluxes, and reach-averaged rates of groundwater discharge, nitrification, and NO₃⁻ assimilation (Böhlke et al. 2004; Mulholland et al. 2004; Mulholland et al. 2008). Also more simple models based on a few assumptions have been developed to predict the response of one pool from one isotope manipulation at a time (e.g., the UNI model; Pace et al. 2004; Carpenter et al. 2005). Like parsimonious models, these overlook information on the dynamics of closely related time series and do not attempt to represent the specific ecological processes (Carpenter et al. 2005). Most problems appear when jointly modelling dynamics of slowly (benthos or fish) and rapidly (DIC) changing pools (Carpenter et al. 2005). An intermediate solution has also been created by incorporating some additional information such as the dynamics of closely related variables using a multivariate autoregression model (Ives et al. 2003). The main weaknesses of these dynamic box models (as in most models) are the assumptions of values and relationships which, whenever possible, are derived from their statistical fit to the data. Nonetheless, results from these models are usually very realistic, since they depend on a large number and diversity of measurements, many of which are based on in situ data; however, potentially complex errors can emerge as Carpenter et al. (2005) acknowledged. Evidently other model approaches could make an improvement but at this moment there is no evidence that they are more robust for interpreting SI data. Furthermore, some software has been developed based on dynamic box models such as Simulation Analysis and Modelling (WinSAAM, http://www.winsaam.org; Hamilton et al. 2004) to simulate and fit data and to model the transfer rate of tracers in a system, without requiring the system to have reached a steady state. Its use in SI additions at the ecosystem-scale is very recent (C flow in food webs; Lee et al. 2011), but results are promising given the implicit simplicity of the model.

Tracer addition assays for which experimental design includes circular statistics (Zar 1996) are becoming widely used to quantify the overall effects of SI addition on stable isotopes (Bartels et al. 2012) because they provide a quantitative understanding of complex isotopic changes at the community and food-web levels in time and (or) space (Schmidt et al. 2007). Stable isotope data have also been incorporated into ecological network analysis and simulation software to assess and identify system interactions and whole-ecosystem properties (Fath et al. 2007). The SI addition study of Lee et al. (2011) combined the software WinSAAM and EcoNet (http://eco.engr.uga.edu/index.html; Kazanci 2007) to access food-web dynamics by providing system indices that quantify flows and food-web interactions. EcoNet uses network environmental analysis and deterministic and stochastic algorithms as well to quantify the actual relationship between compartments and environmental inputs and outputs (Tollner and Kazanci 2007; Ings et al. 2009). Results are interesting since this approach allows concurrent quantitative assessment of food-web structure and ecosystem functioning regardless of whether the system achieves the new isotopic steady state enrichment after SI tracer addition.

**Future challenges of SI addition experiments to produce new paradigms in aquatic science**

Since the first seminal in situ SI tracer addition experiment carried out by Kling (1994), aquatic science topics and paradigms have changed and studies have been modified to solve current environmental and ecological challenges. Today, one of the biggest challenges facing aquatic ecology and biogeochemistry concerns the role of microbial communities sustaining ecosystem functioning (Hall and Meyer 1998). Metabolic rates and dynamics of nutrient transformations in Nature are not well known yet (Dumont and Murrell 2005; Jetten et al. 2009). Although ¹³C and ¹⁵N enrichment experiments are potentially able to solve most uncertainties in aquatic microbial ecology, a greater effort is needed to develop new methods and techniques to improve current results. In fact, SI tracer addition cannot be used everywhere. For example, we currently lack a good method for SI use in larger streams and rivers, which are able to ensure that SI addition is enough to label benthos sufficiently at an affordable cost and to determine the long-term fate of the assimilated ¹⁵N (O’Brien et al. 2012). The use of stable isotope-labelled substrates in combination with molecular tools has allowed quantifying degradation rates and identification of organisms involved (Bull et al. 2000) and thus the co-experimentation of microbial molecular tools with in situ tracer experiments would be a valuable avenue to pursue. Only the effects of simple natural organic substrates such as PO₄ have been simulated using ¹³C additions in benthic food-web experiments, whereas the reproduction of complex substrates like detritus using SI will have to wait. This is complicated because it is not easy to establish a mixture of natural organic substances that show a clear signature contrast (e.g., corn starch −10.4‰ vs particulate organic matter −28.8‰; Bartels et al. 2012) with high insolubility, being a homogeneous material enough to quickly settling to be available to the benthic community alone. Finally, the importance of relatively stagnant N pools in some tissues should be investigated further in controlled laboratory studies, and methods for sampling fast-turnover tissues in small invertebrates should be developed for isotopic analysis studies.

Specifically, some processes, such as denitrification, still require further investigation as do other complex, recently described or identified processes like anammox, dissimilatory nitrate reduction to ammonia, and denitrification by sulfur-oxidizing bacteria (Mulholland et al. 2008, 2009; Burgin et al. 2012). Compound-specific isotope analysis as well as combining the SI addition approach with quantitative PCR of potential genes (e.g., Findlay et al. 2011; Vilar-Sanz et al. 2013) would provide a wide range of possibilities to study these complex microbial transformations in the aquatic environment (Boschker and Middleburg 2002; Johnson et al. 2012). Future assessment of other ecological assumptions that are still open to debate may be supported by results of experiments using ¹³C and ¹⁵N as tracers, for example: the role of allochthonous C inputs in lakes according to their environmental properties (Pace et al. 2007), the effects of increased N loads in lakes on nitrogen-cycling and food-web structure (Pace et al. 2007), the importance of wetlands and hyporheic zones in the nitrogen transformation at landscape scales (Duff and Triska 2000; Böhlke et al. 2009), the metabolic support of food webs (Pace et al. 2007), the N uptake rates and control variables in benthic regions (Wetzel 2001), the mechanisms of N transformation, storage and export in estuaries (Gribsholt and Boschker 2005), or the seasonal variability of food-web structure due to changes in biotic (basal resources, reproductive or stages of development) and abiotic (temperature, runoff, rainfall or evaporation) constraints (Layman et al. 2012).

In natural abundance studies, the dual-isotope approach (¹³C and ¹⁵N simultaneous study) is a common practice to assess trophic pathways in aquatic systems (Yoshii et al. 1999; Post 2002; García et al. 2007; Bratkic et al. 2012). In SI addition experiments, simultaneous (¹³C+¹⁵N) enrichments are still seldom used. Some experiments at the ecosystem-scale have taken advantage of these simultaneous enrichments and their results are highly promising in terms of elucidating organic matter sources, ecosystem processes, food sources, and trophic patterns (Mulholland et al. 2000b; Carman and Fry 2002; Parkyn et al. 2005; Taipele et al. 2008; Hardison et al. 2010; van den Meersche et al. 2011; Galván et al. 2012). However, including a second tracer can drastically increase both the logis-
Most current uncertainties in aquatic microbial ecology can be solved using SI enrichment experiments at the whole-ecosystem scale; however, a greater effort is required to develop new methods and techniques to improve current results (e.g., combined with microbial molecular tools). Furthermore, methods must be improved to properly apply SI tracer addition to ecosystems where it has not been possible to date (large aquatic ecosystems). Concerning phosphorus, which is the main limiting factor of productivity in freshwaters, there is a clear need to undertake SI addition experiments at the whole-ecosystem scale, and although the methodology already exists it has only been used for quantifying natural isotopic contents so far. Finally, despite its high potential to elucidate ecosystem processes and trophic pattern, simultaneous ($^{15}$C and $^{15}$N) SI enrichment experiments are rarely used as we still lack an easy and cost-effective solution to their logistical and economic constraints.

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References


