Review Paper

 $^{15}\text{N}_2$ as a tracer of biological N_2 fixation: A 75-year retrospective Phillip M. Chalk^{a, b, *}, Ji-Zheng He^{a, b}, Mark B. Peoples^c, Deli Chen^a^a Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville 3010, Victoria, Australia^b State Key Laboratory of Urban and Regional Ecology, Research Centre for Eco-environmental Science, Chinese Academy of Sciences, Beijing 100085, PR China^c CSIRO Agriculture & Food, PO Box 1600, Canberra, ACT 2601, Australia

ARTICLE INFO

Article history:

Received 4 September 2016

Received in revised form

14 December 2016

Accepted 17 December 2016

Available online 24 December 2016

Keywords:

Biological N_2 fixation $^{15}\text{N}_2$

Phototrophic

Heterotrophic

Diazotrophs

Chambers

ABSTRACT

$^{15}\text{N}_2$ has played a crucial role in fundamental studies of biological N_2 fixation. However, due to operational constraints, it has more often served as a qualitative rather than a quantitative tracer of biologically-fixed N (BFN). Therefore indirect methods based either on ^{15}N -enrichment or ^{15}N -natural abundance have assumed a dominant role in quantifying N cycle processes involving BFN. However, it is only through the direct $^{15}\text{N}_2$ approach that biological N_2 fixation can be traced through the various components of the soil-plant system. Technological advances in the automated control of the chamber environment have made the $^{15}\text{N}_2$ technique more attractive to long-term studies. Thus the need to enclose plants in a chamber and maintain conditions conducive to plant growth should no longer be seen as a major obstacle to the use of $^{15}\text{N}_2$. The way is now open to evaluate the efficacy of indirect methods used to estimate the contribution of BFN to the N economies of crop and pasture systems, and the dynamics of BFN in agroecosystems. In addition, new applications of $^{15}\text{N}_2$ such as stable isotope probing are emerging, which have the potential to characterize non-cultivated diazotrophs in a range of environments. The role of biological N_2 fixation in the formation of reactive N in the environment and its relationship with the emission of the greenhouse gas N_2O requires further investigation.

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1. Introduction

$^{15}\text{N}_2$, or to be more precise $^{15}\text{N}_2$ reduction, has played a key role in studies of biological N_2 fixation over the past 75 years, since publication of the seminal paper of [Burris and Miller \(1941\)](#). Several general reviews have been written about the measurement of N_2 fixation using ^{15}N techniques, including $^{15}\text{N}_2$ ([Bergersen, 1980](#); [Focht and Poth, 1987](#); [Weaver and Danso, 1994](#); [Zehr and Montoya, 2007](#)). However, a contemporary overview of the subject is lacking, particularly the role of $^{15}\text{N}_2$ as the only direct ^{15}N method. The major drawback of using $^{15}\text{N}_2$ is the high cost of the isotope which necessitates exposure of the plant to the substrate in a leak-free closed chamber, thus precluding the use of more realistic experimental set-ups such as open-top or flow-through chambers.

[Millbank and Olsen \(1981\)](#) and [Warembourg \(1993\)](#) provided descriptions of (i) systems to prepare, purify and store $^{15}\text{N}_2$ (ii) the

types of enclosures used for biological materials (iii) methods of environmental control of enclosures and (iv) operating procedures. Many other enclosure systems that were not reviewed by [Millbank and Olsen \(1981\)](#) or [Warembourg \(1993\)](#) have been described in the literature. Consequently we will provide an overview of the distinctive features of diverse systems used with vascular plants, including legumes and non-legumes.

In addition, we will explore the usage of $^{15}\text{N}_2$ in fundamental studies of N_2 fixation, including confirmation of putative N_2 fixation in various biological systems, the calibration of assays to estimate the rate of biological N_2 fixation, the identification of the biochemical products of N_2 fixation and assimilation pathways within the plant, $^{15}\text{N}_2$ stable isotope probing to identify uncultured diazotrophs in the biosphere and the role of $^{15}\text{N}_2$ in quantifying N_2 fixation and the dynamics of BFN within various components of terrestrial and fresh water ecosystems. We will discuss the practical problems encountered when employing $^{15}\text{N}_2$ as a quantitative tracer, and highlight the problems associated with alternative indirect ^{15}N methods. The focus of the review is terrestrial ecosystems, particularly agroecosystems, which also include flooded rice, but we do not cover the marine environment.

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2. Identifying N₂-fixing associations – A brief history of ¹⁵N₂ usage

2.1. Cultures, excised nodules/nodulated roots, soil, and non-vascular plants

Burris and Miller (1941) were the first to report N₂ fixation by a culture of *Azotobacter vinelandii* by exposure to ¹⁵N₂, while Burris et al. (1943) exposed excised nodules and nodulated roots of pea to ¹⁵N₂ to demonstrate legume-*Rhizobium* symbiotic N₂ fixation. Subsequent studies with ¹⁵N₂ confirmed biological N₂ fixation with lichens and the liverwort *Blasia* (Bond and Scott, 1955), and free-living heterotrophs in soil (Delwiche and Wijler, 1956). Later work with ¹⁵N₂ focused on N₂ fixation in non-vascular plant-Cyanobacterial associations, including the water fern *Azolla* (Peters et al., 1977) and the moss *Sphagnum* (Basilier, 1980).

2.2. Vascular plants

In addition to the legume-*Rhizobium* symbiosis, several higher plant-microbe associations are able to fix atmospheric N₂ symbiotically. For example, Bond (1955) demonstrated that actinorhizal plants belonging to several genera (*Alnus*, *Myrica*, *Hippophaë*) fix ¹⁵N₂ in specialized root structures containing the actinomycete *Frankia*. Subsequently, two vascular plant-Cyanobacterial associations were identified. Bergersen et al. (1965) demonstrated that blue-green algae inhabiting the coralloid roots of the Cycad *Macrozamia communis* fixed ¹⁵N₂ which was then redistributed within the plant. A few years later, Silvester and Smith (1969) showed that the Cyanobacterium, *Nostoc*, present in glands at the base of the leaves of the herbaceous Angiosperm *Gunnera* could fix ¹⁵N₂ which was then quickly incorporated in the host. While these non-legume N₂-fixing symbiotic relationships are undoubtedly important in natural ecosystems, it is the legume-*Rhizobium* symbiosis, the *Azolla*-*Anabaena* symbiosis, and non-legume endophytic/phyllospheric associations that are most important in agroecosystems.

3. Systems used to expose biological materials to ¹⁵N₂

Various types of chambers have been constructed to enclose the roots or the roots + tops of the plant, with considerable variation in chamber volume (Table 1). Warembourg (1993) outlined the factors to be considered when deciding on chamber volume. Often relatively small volumes (<3 l) were used to reduce the cost of the isotope (e.g. Bond, 1955; De-Polli et al., 1977; Ruschel et al., 1979; Frey and Schüepp, 1992; Bremer et al., 1995), but some chambers were much larger varying from 60 l (Hopmans et al., 1983) to almost 100 l (McNeill et al., 1993) to more than 300 l (Bei et al., 2013). The plant root medium varied from N-free liquid culture (with or without perlite) to soil or an artificial sand-manure mixture (Table 1). When only plant roots were enclosed various compounds were used to seal the plant stem from the root compartment (Table 1), although it is doubtful that such compounds can guarantee a leak-proof seal, especially as the stem elongates and movement of the foliage occurs. Indeed, Sims et al. (1986) concluded that the majority of leaks in their system arose at the interface of the plant stem and the chamber.

When photosynthetic plants are exposed to ¹⁵N₂, environmental control within the chamber is essential, including atmospheric concentrations of CO₂ and O₂, temperature and humidity, light intensity and the need to supply plants with water and nutrients. The chamber atmosphere is often circulated by the use of an internal fan, and can be modified internally or externally. For example, condensers can be located within the chamber (e.g. Ross et al., 1964; Hopmans et al., 1983; McNeill et al., 1993), or the chamber

atmosphere can be circulated through a condenser located externally (Eskew et al., 1981; Warembourg et al., 1982). Whenever possible, internal control is preferable in order to reduce the possibility of leakage through connections. Sometimes innovative solutions emerge. For example, Ross et al. (1964) avoided a hard-wire electrical connection to an internal circulating fan, by using a magnetic connection.

3.1. Composition of the chamber atmosphere

In order to reduce the amount of expensive ¹⁵N₂ required, a part of the N₂ present in a normal atmosphere (79%) has been replaced by argon (Ar) in several studies (Table 2). McNeill et al. (1993) studied the effect of reduced partial pressure of N₂ on the dry matter and N yield of white clover and perennial ryegrass (Table 2). A rapid visual effect was observed with clover but not with ryegrass, with some leaves folding downwards along the petiole. These same effects were observed at 22 and 39% N₂ with both Ar and He as partial replacement gases. After 11–16 days exposure, dry matter production and N content of clover shoots were significantly reduced. Possible reasons including impurities in the gases used, stomatal responses and plant water relations were discussed. On the basis of these results McNeill et al. (1993) concluded that the use of inert gases to partially replace N₂ was not a viable proposition.

3.2. Environmental control

3.2.1. CO₂

The CO₂ concentration within the chamber is governed by the balance between two efflux rates (i) root and soil respiration and (ii) dark shoot respiration, and one influx rate via shoot photosynthesis. It is necessary to monitor the CO₂ concentration within the chamber by Infra Red Gas Analysis or by Gas Chromatography (Table 1), and to control it by some means. The net change observed during daylight was CO₂ depletion due to photosynthesis, while at night a net CO₂ increase occurred due to respiration (McNeill et al., 1993).

A degree of control can be achieved by circulating the chamber atmosphere through buffer solutions (Yoshida and Yoneyama, 1980; Hopmans et al., 1983). However, a more reliable technique is to circulate the chamber atmosphere through an external soda lime trap to absorb CO₂, which is then replenished in the chamber with pure CO₂ from a cylinder (e.g. Ross et al., 1964; Ito et al., 1980; McNeill et al., 1993; Bei et al., 2013) according to a set time sequence or automatically through a CO₂ sensor.

3.2.2. O₂ and pressure

O₂ should be monitored using a sensor (McNeill et al., 1993). Chamber pressure will be affected by O₂ produced by photosynthesis, and therefore requires monitoring and control. Pressure has been monitored in many chambers with a simple Hg manometer to indicate pressure relative to atmospheric (Table 1).

Ross et al. (1964) designed an elaborate system whereby excess O₂ was removed by burning pure H₂ at the end of a capillary in a microburner, a technique which was subsequently adopted by Eskew et al. (1981) as an alternative to venting. Recently Bei et al. (2013) recommended H₂ burning as a better way to control long-term O₂ buildup. McNeill et al. (1993) removed excess O₂ from the chamber by diverting the circulating flow through a reduction column of Cu at 350 °C. In contrast, when only the root system is enclosed O₂ can quickly become limiting and a system to monitor and admit O₂ to the enclosure is required (Warembourg, 1993).

Ross et al. (1964) dismissed the idea of using expandable rubber or plastic bags to maintain the initial chamber pressure because of

Table 1Principal characteristics of chambers used to expose vascular plants to $^{15}\text{N}_2$.

Vol. (litre)	Growth medium ^a	Plant ^b	Plant part ^c	Sealant ^d	Contact $^{15}\text{N}_2$ (d) ^e	Place ^f	Temp (°C)	Pressure control ^g	L/D (h) ^h	Light (lux) ⁱ	CO ₂ Control ^j	Special equipment ^k	Reference
8.84	Soil	Ma, Rg	T + R		13–26, S	GC	27–32	H ₂ burn		2000 fc	I, C,	F, Cc, Th	Ross et al., 1964
≤0.25	NFLC	A, Mg, H	R	P	3–5, S	G	21						Bond (1955)
1–4	Soil	Bg, Pg	T + R		1.3–3.3, S	GC	25–36		15/9	3–4 × 10 ⁴		Pe, Pc, Hg, T	De-Polli et al., 1977
2.0		Bean, soy	T + R		1–3, S	GC	22–32		14/10	15 × 10 ³		Pe, Pc, Hg, T	Ruschel et al., 1979
18.2	NFLC	Rice	T + R		7, S	G	25–40			Sunlight	C, GC, S	Cc, Pc, Hg, S	Ito et al., 1980
27.4	Soil		R, T + R	Cs	7–13, S	P	25	Vent, H ₂ Plastic bag Rubber bag	12/12	550 pfd Sunlight	I, B1	Pe, Pc	Yoshida and Yoneyama, 1980
			T + R		3–13, S	GC	21–28				I, S	Pc, R,	Eskew et al., 1981
			R	Mc	9, M	F						Pc	Watanabe and Ventura, 1982
60	Sand + M	Soybean	R	Si-1, T	1, S			P-Al-P bag	12/12	140 pfd	S	F, Pc, O	Warembourg et al., 1982
		Sclover	T + R		5, S	GC	20–24				B2, GC	Cc, Pc	Hopmans et al., 1983
		Sorghum	R	Si-2	3, S						GC, S	Pc, Hg,	Giller et al., 1984
15.7	Soil	Pea	R	Silastic	64, S	F		P-Al-P bag		Sunlight	S	Pc,	Sims et al., 1986
1.39	NFLC	Berseem	R	Gtw	5, M	GC	16–22			400 pfd			Frey and Schüepp, 1992
94.5	NFLC + P	Wc + Pr	T + R		11–14, S	GC	15–20		16/8	320 pfd	I, C, S	F, Pc, S, O, P	McNeill et al., 1993
	Soil	Alfalfa	T + R	Caulk	2, M	G	17–27	Saran bag	14/10	Sun + Na	I,	Pc	Russelle et al., 1994
2.78		Wheat	R	Si-3	21–28, M	G	20–25	Cryovac bag			S	F, O	Bremer et al., 1995
173		Wc + Pr	T + R		14, M	GC	20–15	Tedlar bag	16/8	300 par	I, S	F, Pc, S, Cp	Carter and Ambus, 2006
314		Rice	T + R		70, S	F		Al foil bag		Sunlight	I, C, S	F, Pc, D, R	Bei et al., 2013

^a NFLC, N free liquid culture; M, farmyard manure; P, perlite.^b Ma, *Macroptilium atropurpureus* (Siratro), Rg, Rhodes grass (*Chloris gayana*); Bg, Bahiagrass (*Paspalum notatum*), Pg, Pangola grass (*Digitaria decumbens*); A, *Alnus glutinosa*, Mg, *Myrica gale*, H, *Hippophaë rhamnoides*; Bean, *Phaseolus vulgaris*; Soy, soybean, *Glycine max*; Rice, *Oryza sativa*; Clover, subclover (*Trifolium subterraneum*); Sorghum (*Sorghum bicolor*); Pea, *Pisum sativum*; Berseem, *Trifolium alexandrinum*; Wc, white clover (*Trifolium repens*), Pr, perennial ryegrass (*Lolium perenne*); Alfalfa (*Medicago sativa*); Wheat, *Triticum aestivum*.^c Plant part exposed to $^{15}\text{N}_2$; T, tops; R, roots.^d P, plasticine; Cs, calk strip; Mc, modelling clay; Si-1, RTV silicone rubber; T, Terostat (physiological molding material); Si-2, silicone rubber; Silastic, 738RTV (Dow Corning); Gtw, grafting tree wax; Caulk, MSDF compound (3 M); Si-3, silicone MDX-4-4210 (Dow) + lanolin/wax.^e S, single addition of isotope; M, multiple additions of isotope.^f GC, growth chamber/room; G, greenhouse; P, phytotron; F, field.^g P-Al-P bag, trilaminate of polypropylene-Al foil-polyethylene; Saran bag, polyvinylidene 50 µm thick.^h L, light; D, dark.ⁱ fc, foot-candles; pfd, photon flux density, µE m⁻² s⁻¹; Sun + Na, Sunlight + high pressure Na lamp; par, µmol m⁻² s⁻¹.^j C, CO₂ cylinder; GC, gas chromatograph; I, IRGA monitor; S, external soda-lime trap; B1, Buffer solution 1 (0.05 M K₂CO₃ + 0.1 M KHCO₃); B2, Buffer solution (KHCO₃ + HCl + triethanolamine).^k F, fan; Cc, Cu cooling coils; Th, thermocouple hygrometer (humidity); Hg, Mercury manometer; T, Toepler pump; S, solenoid valve; Pe, evacuating pump; Pc, circulating pump; D, data logger; R, rotameter; O, O₂ sensor and analyser; P, pressure sensor; Cp, Computer.

Table 2

The composition of chamber atmospheres.

Gaseous composition (%)				Exposure time (d)	¹⁵ N enrichment (atom % excess)	Reference
Ar (He)	O ₂	N ₂	CO ₂			
Balance	~21	16, 18, 29		13, 26, 23	7.1, 4.1, 3.4	Ross et al., 1964
—	10	90		4	19.6	Bond, 1955
30	20	50		1.08	95.6	De-Polli et al., 1977
10.8	2.2	85.2	1.8	2.25	95.0	
11.2	3.7	84.5	0.6	3.23	91.7	
51.4	2.6	42.8	3.2	3	85.5	
28	15	55	0.01	3	76.2	Ruschel et al., 1979
20	12	66	0.2	1	43.8	
—	20	80	0.1	7	58–72	Ito et al., 1980
—	21	79	0.08	7–13	14.1–22.7	Yoshida and Yoneyama, 1980
53–43	15–25	33		6.8	79–63	Eskew et al., 1981
51–46	17–23	32–31		3	65–60	
56–52	18–22	25		12.8	49–45	
—	21	79	0.034	14		McNeill et al., 1993
57		22		11		
39		40		14		
(42)		37		13		
44		35		16		
18		60			5.0	Russelle et al., 1994

possible diffusion of gases. Nonetheless, bags have been used in several studies (Table 1). On the basis of the results of a study of gaseous diffusion through plastic bags conducted by van Kessel (1983) using ¹⁵N-depleted N₂, Russelle et al. (1994) used a Saran (polyvinylidene, 50 µm thick) bag to enclose the foliage of alfalfa (lucerne), but the system was not leak-proof. Sims et al. (1986) and Frey and Schüepp (1992) used tri-laminated bags of aluminium foil sandwiched between two layers of polyester. Bremer et al. (1995) used a Cryovac gas bag, but due to system leakage 30–60 ml of ¹⁵N₂ (99 atom %) was added per day. Bei et al. (2013) used a 30 L Al foil bag to prevent over-pressure, but specifications were not provided. Whether system leakage is due to diffusion of gases through bags or due to leakage from other system components or couplings generally remains unknown. However, bags should be tested before use (van Kessel, 1983).

3.2.3. Temperature, humidity and water

Cooling of the chamber was achieved by Ross et al. (1964) by circulating cold water (5–10 °C) through silver-plated Cu condensing coils inside the chamber. Transpired water vapour condenses on the coils and can be collected in a siphon and returned to the soil. The siphon avoids the return of cold water to the soil, and by changing its capacity, the cycle of wetting/drying can be altered (Ross et al., 1964). Humidity can be varied by changing the water temperature. A similar but less sophisticated system was adopted by Ito et al. (1980) and Hopmans et al. (1983). McNeill et al., (1993) used an internally-located heat exchanger controlled by a thermoregulatory unit. Bei et al. (2013) also used within-chamber cooling coils charged with a glycol-water mixture which was chilled by a refrigerator. When the temperature in the chamber rose to 2 °C higher than the outside temperature, a data-logger would trigger a relay to activate the circulation of liquid coolant until the internal temperature was 2 °C lower than outside.

A stainless steel condenser located externally and cooled to 1 °C was used by Eskew et al. (1981), with the condensed water being returned to the chamber. Warembourg et al. (1982) used an externally-located heat exchanger cooled with water from a refrigeration unit. Relative humidity was monitored in several systems (Ross et al., 1964; McNeill et al., 1993; Bei et al., 2013) and was reported to vary between 60 and 80% (Eskew et al., 1981), while Bei et al. (2013) reported a daytime average of 60%. Only rarely has

the soil water potential been monitored, with Sims et al. (1986) installing a tensiometer block.

3.2.4. Light

It is desirable that the enclosure be exposed to sunlight to allow normal plant growth, although artificial light has been employed throughout plant growth (Table 1) or used to extend the photoperiod (Russelle et al., 1994). The lighting system can be ventilated (Ross et al., 1964) or the chamber can be placed in an air-conditioned room or growth chamber to dissipate heat (e.g. Eskew et al., 1981; McNeill et al., 1993).

3.2.5. Plant growth

Although plants have been reported to be healthy after enclosure for 13–26 d (Ross et al., 1964), or for longer periods (Bremer et al., 1995) others have observed abnormal growth of plants in controlled-environment chambers. For example, Eskew et al. (1981) reported that all the flag leaves of rice died during exposure for 12.8 d to an artificial atmosphere composed of 56% Ar, 18% O₂ and 25% N₂, while Mohr et al., (1998) observed that alfalfa plants appeared to be under some stress after prolonged continuous exposure to ¹⁵N₂ for 7 weeks.

Several authors have made direct comparisons of plant growth inside and outside of chambers under a normal atmosphere. For example, Ito et al. (1980) observed spindly growth of rice inside the chamber with a reduced dry matter of shoots and roots, with root growth relatively more suppressed compared to outside. In contrast, Sims et al. (1986) reported that provided that seedlings of field pea established well initially in the chamber, there was no difference in the field average at the final harvest. Similarly, McNeill et al., (1993) found that there were no differences in dry matter or N yield of white clover or perennial ryegrass grown in a chamber for 14 days compared with those grown in a standard growth cabinet.

There is always a possibility that legume growth may be reduced by insect or disease attack. For example, Carter and Ambus (2006) reported that the yield of white clover was reduced by a severe aphid attack which peaked at 26 weeks but was still present 36 weeks after emergence. Control of pests and disease may prove difficult under the humid conditions within the chamber.

3.3. Recovery of residual $^{15}\text{N}_2$ in the chamber

Because $^{15}\text{N}_2$ is expensive, attempts have been made to return unused $^{15}\text{N}_2$ at the end of an experiment to the supply reservoir (e.g. Ito et al., 1980; Giller et al., 1984). However, residual $^{15}\text{N}_2$ must be contaminant-free and of sufficient ^{15}N enrichment to make the exercise worthwhile. Ito et al. (1980) filled the chamber with water and pushed the gas back into the supply reservoir through a liquid N_2 trap and a heated reduced Cu column to remove contaminants.

3.4. Chamber leakage

Initially, an attempt was made to make the growth chamber gas-tight, i.e. leak-proof or leak-free (Ross et al., 1964). In a series of three experiments spanning 13–26 days, it was shown that the decrease in the amount of excess ^{15}N in the atmosphere was equal to the increase in the amount of excess ^{15}N in the soil-plant system, thus confirming the absence of leakage. Other systems have also been described as ‘gas-tight’ (Yoshida and Yoneyama, 1980; Ito et al., 1980; Eskew et al., 1981), but there is ample evidence that this was not the case because the ^{15}N abundance of the atmosphere decreased during the experiments, i.e. the ^{15}N -enriched atmosphere was diluted with externally-derived unlabelled N_2 . Processes such as atmospheric N_2 fixation or plant mineral N uptake that remove N from an homogeneous ^{15}N labelled pool do not in themselves change the ^{15}N abundance of the pool, although the amount of ^{15}N will decrease over time (Barraclough, 1991). In the study of Yoshida and Yoneyama (1980) with paddy rice the average daily decrease in ^{15}N abundance was much higher when the root system was exposed to $^{15}\text{N}_2$ compared with exposure of the whole plant, presumably because of either free gas exchange between the roots and the atmosphere through the aerenchyma tissue and/or leakage around the plant stem sealant.

Because of leakage several strategies have been adopted to maintain the concentration of $^{15}\text{N}_2$ within the chamber, including pulse labelling 10 times over 5 days (Frey and Schüepp, 1992), complete replacement at 2–3 day intervals (Watanabe and Ventura, 1982) or a daily top-up (Bremer et al., 1995).

Symbiotic dependence (P_{atm}), the proportion of plant N derived from atmospheric N_2 , is estimated according to Eq. 1

$$P_{\text{atm}} = \frac{E_{\text{plant}}}{E_{\text{atmosphere}}} \quad (1)$$

Where $E = ^{15}\text{N}$ enrichment expressed as excess atom fraction ^{15}N .

When leakage occurs, a problem arises because $E_{\text{atmosphere}}$ is not constant and decreases with time. The rate of decrease has sometimes been linear (McNeill et al., 1993, 1994) or almost linear (Ito et al., 1980; Eskew et al., 1981; Sims et al., 1986) but other studies have shown a variable rate (e.g. Yoshida and Yoneyama, 1980; Hopmans et al., 1983). The decrease in ^{15}N abundance was relatively slow from 5 (Eskew et al., 1981) up to 57 days (Sims et al., 1986), but in other cases it was very fast over a period of one week (Ito et al., 1980). $E_{\text{atmosphere}}$ has been estimated by the arithmetic mean of measurements taken during the experimental period (e.g. Ito et al., 1980; Yoshida and Yoneyama, 1980; Hopmans et al., 1983; McNeill et al., 1994) or by a time-weighted mean (Russelle et al., 1994).

Chamber leakage will cause error in the estimation of P_{atm} because of the temporal decrease of $E_{\text{atmosphere}}$ (Eq. (1)), which can only be approximated by mean values. A mathematically integrated value of $E_{\text{atmosphere}}$ over the exposure period would be a better

approach. However, in a system where the whole plant is exposed to $^{15}\text{N}_2$, as opposed to roots only, an additional error may arise through disequilibrium in isotopic composition between the above- and below-ground atmospheric components of the system.

3.5. Sources of $^{15}\text{N}_2$, contaminants and clean up procedures

$^{15}\text{N}_2$ is available commercially in lecture bottles or break-seal flasks from several suppliers. A delivery system for transferring $^{15}\text{N}_2$ from a 400 ml lecture bottle to a field chamber with little or no gas wastage was described by Harker and Wullstein (1981). $^{15}\text{N}_2$ can also be produced in the laboratory by reaction of an ammonium salt with alkaline hypobromite according to Eq. (2).

Wood and Kennedy (2001) and Diocares et al. (2006) have each described an apparatus for generating and then dispensing small quantities of $^{15}\text{N}_2$ gas produced according to the above reaction. Production of $^{15}\text{N}_2$ in the laboratory may offer several advantages over purchase including reduced cost and the flexibility of generating the amount and ^{15}N enrichment of the gas when required.

However, it is essential that the $^{15}\text{N}_2$ either produced in the laboratory or purchased commercially is free of contaminants such as $^{15}\text{NH}_4^+$ or $^{15}\text{NO}_3^-$ which can be absorbed by plants and thus be wrongly attributed to biological N_2 fixation. Wood and Kennedy (2001) tested for contamination by exposing sterile wheat plants to the ^{15}N -enriched gas, which they deduced was contaminant-free as the ^{15}N abundance of the wheat did not differ from background. Burris (1972) recommended that commercial supplies be passed through an alkaline KMnO_4 solution to remove oxides of N and then through an acidified Na_2SO_4 solution to remove NH_3 . Several authors have followed this procedure (Yoshida and Yoneyama, 1980; Eskew et al., 1981; Watanabe and Ventura, 1982; Warembourg, 1993), while Diocares et al. (2006) sparged the generated $^{15}\text{N}_2$ first with acidified KMnO_4 and then with acidified H_2O to remove contaminants.

An alternative approach is to pass the $^{15}\text{N}_2$ gas through a liquid N_2 trap to remove any impurities before admittance to the chamber (Ross et al., 1964; De-Polli et al., 1977; Ito et al., 1980; Warembourg, 1993; McNeill et al., 1994; Bremer et al., 1995; Bei et al., 2013). Dabundo et al. (2014) confirmed that $^{15}\text{N}_2$ supplies obtained from three commercial companies were contaminated to a greater or lesser extent with NH_4^+ , $\text{NO}_3^-/\text{NO}_2^-$ and N_2O , which were assimilable by the green algae *Dunaliella tertiolecta*, thus echoing the warning given by Burris (1972).

4. Identification of the biochemical products of biological N_2 fixation and assimilation pathways

Various physiological studies since the mid 1940's have supplied $^{15}\text{N}_2$ to free-living N_2 -fixing micro-organisms (Burris and Wilson, 1946; Zelitch et al., 1951), detached alder nodules (*Alnus glutinosa*; Leaf et al., 1958), detached bog-myrtle nodules (*Myrica gale*; Leaf et al., 1959), detached legume nodules (Aprison et al., 1954; Bergersen, 1965; Kennedy, 1966a,b), isolated bacteroids (the N_2 -fixing forms of rhizobia present in nodule infected cells) from legume nodules (Bergersen and Turner, 1990; Waters et al., 1998; Allaway et al., 2000), and intact nodulated legume root systems (Matsumoto et al., 1977; Herridge et al., 1978; Ohya and Kumazawa, 1978, 1979; Atkins and Thumfort, 2002), to either:

- (i) identify the initial products of N_2 fixation,
- (ii) investigate the subsequent assimilation of the fixed N into more complex organic forms of N, or

- (iii) confirm the main forms of fixed N exported from N₂-fixing nodules to the plant.

4.1. Identifying the initial products of N₂ fixation

Many investigations have identified ammonia (NH₃) as the earliest labelled products of N₂ fixation by the nitrogenase enzyme (EC 1.18.6.1; EC 1.19.6.1) present in free-living N₂-fixing organisms (Burris and Wilson, 1946; Zelitch et al., 1951) and bacteroids of symbiotic root nodules (Bergersen, 1965; Kennedy, 1966a,b), with NH₃ often representing >85% of the total ¹⁵N fixed from ¹⁵N₂ (Bergersen, 1965; Bergersen and Turner, 1990).

Glutamate and/or glutamine frequently represented the first and most highly labelled organic compounds detected in legume (Burris and Wilson, 1946; Aprison et al., 1954; Kennedy, 1966a) and alder nodules (Leaf et al., 1958) following exposure to ¹⁵N₂ suggesting both these compounds were important primary products (Kennedy, 1966b). Initially it was thought that the accumulation of ¹⁵N-label in glutamate may have been catalysed by glutamate dehydrogenase (GDH; EC 1.4.1.3). However, enzymological and tracer studies later provided evidence that the NH₃ (via ammonium; NH₄⁺) was more likely to be assimilated via the coupled activities of glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT; EC 2.6.2.53) (Schubert, 1986; Day et al., 2001). This was confirmed by studies using ¹⁵N₂ (Ohyama et al., 1978) and ¹³N₂, a short-lived radioactive isotope of N (Meeks et al., 1978), which demonstrated that NH₄⁺ was first incorporated into the amide position of glutamine in the reaction catalysed by GS, and that the amide group was subsequently transferred to the 2-carbon of oxoglutarate in the reductive amination reaction carried out by GOGAT.

Bacteroids inside the infected root cells of legumes are surrounded by a symbiosome or peribacteroid membrane of plant origin that effectively controls the transfer of compounds between the bacteroids from the plant cytoplasm (Day et al., 2001). Given that GS and GOGAT activities are repressed in bacteroids and elevated in the plant fraction of infected cells, it has long been believed that the NH₃ produced in the bacteroid diffuses into the peribacteroid space where it is protonated in the acidic environment, before the NH₄⁺ is transported across the peribacteroid membrane into the plant cytoplasm where it is first assimilated into glutamine, and then converted to glutamate in the plastids of the infected cell (Schubert, 1986; Day et al., 2001). The resulting pools of glutamine and glutamate subsequently provided suitable substrates for further synthesis of other amino acids and nitrogenous compounds (Schubert, 1986).

However, the notion that NH₄⁺ was the main source of fixed N released from bacteroids was challenged by a study by Waters et al. (1998) who reported that sucrose-gradient-purified soybean bacteroids excreted ¹⁵N-labelled alanine rather than NH₃ when provided ¹⁵N₂ under conditions of oxygen deprivation in a closed system. Subsequent experimentation undertaken using similarly purified pea bacteroids demonstrated that NH₄⁺ was the sole secretion product at low to medium bacteroid densities in a closed system, but that alanine synthesis began at higher densities (Allaway et al., 2000). Both NH₃ and alanine pools became highly labelled when supplied with ¹⁵N₂, which was consistent with NH₃ being the direct precursor of alanine. It was speculated that the switching on of alanine synthesis at high bacteroid densities was caused by the accumulation of NH₄⁺ in the fixed volume of the assay system. The findings of Waters et al. (1998) were further explored in an elegant investigation using steady state ¹⁵N₂ labelling of the nodulated roots of intact cowpea plants (*Vigna unguiculata*) under conditions of high rates of N₂ fixation (Atkins and Thumfort, 2002).

In this particular study root systems were frozen in liquid N₂ after 5, 10, 20, 30, 45 and 60 min and the nodules removed; nodule solutes were then extracted for isotopic analysis by GC-MS. Atkins and Thumfort (2002) found rapid and high labelling of the amide group of glutamine within 5 min of the ¹⁵N₂ being supplied. The rate and extent of label accumulation in other amino acids over the duration of the time-course of ¹⁵N₂ labelling indicated: glutamate > aspartate > glycine > alanine. This was consistent with the acquisition of N from the amido group of glutamine through GOGAT and the activity of aminotransferases, and provided no evidence that supported the idea that alanine could be a precursor of the amide group of glutamine.

4.2. Determining the forms of fixed N exported from legume nodules

The process of export of N from root nodules appears to involve the secretion of nitrogenous compounds into the xylem by specialised pericycle cells of the nodular vascular tissue (Pate, 1980). The xylem-borne N is then transported to the shoot in the transpiration stream to undergo further metabolism prior to synthesis into protein, and/or exchange with phloem (Pate et al., 1981; Atkins et al., 1982, 1983). Presumed products of N₂ fixation can be inferred by analysing xylem bleeding sap from detached nodules for the dominant N solutes, or comparisons of the N composition of xylem exuding from decapitated roots above and below the nodulated zone of plants growing in N-free rooting medium, (Herridge et al., 1978; Pate, 1980; Pate et al., 1981). However, it is only with use of ¹⁵N₂ can the origin of specific N solutes be confirmed as the principal products of fixed N exported from nodules (Schubert, 1986). In some cases ¹⁴CO₂ has also been used to distinguish current products of N₂ assimilation from other forms of N transported in the xylem (Gardner and Leaf, 1960; Herridge et al., 1978).

Collectively these experiments indicate that the forms of fixed N transported from nodules are species specific (Pate, 1980; Schubert, 1986; Unkovich et al., 2008). Asparagine and glutamine appear to be the main compounds exported from nodules of many temperate legumes (e.g. field pea, white lupin; *Lupinus albus*; Kennedy, 1966b; Pate et al., 1981), while the ureides, allantoin and allantoic acid, are the main compounds in xylems carrying fixed N in certain tropical legumes (e.g. soybean, cowpea; Matsumoto et al., 1977; Herridge et al., 1978; Ohyama and Kumazawa, 1978, 1979), and citrulline has been found to be rapidly labelled in nodules following ¹⁵N₂ feeding and subsequently transported to the shoots in *Alnus-Aciti-nomycete* associations (Leaf et al., 1958; Gardner and Leaf, 1960).

In some cases ¹⁵N₂ experiments have also provided supporting evidence for some of the metabolic pathways involved in the synthesis of the products of N₂ fixation to ultimately be transported from the nodule. For example, in the nodules of ureide-exporting legume species the amide group of glutamine is believed to be used by two amidotransferases of the *de novo* purine pathway in plastids in infected cells to form IMP that is subsequently oxidized to xanthine and ureides (Schubert, 1986). The steady state ¹⁵N₂ feeding time-course study of Atkins and Thumfort (2002) found that the rate of accumulation of double-labelled xanthine molecules was far greater than either single-labelled molecules or those with 3 or 4 ¹⁵N atoms was consistent with this presumed pathway. Furthermore, the slightly slower labelling kinetics of xanthine than observed for glutamine or glutamate would also be expected for an intermediate of a pathway that was some 16 enzyme steps removed from the second of the glutamine-dependent steps.

5. Calibration of assay procedures using ¹⁵N₂

Numerous studies that were designed to calibrate the acetylene reduction assay were conducted with ¹⁵N₂ using microbial cultures,

Table 3
Calibration of the C₂H₂ reduction assay with ¹⁵N₂.

N ₂ -fixing system ^a	Plant part	Exposure time		C ₂ H ₂ :N ₂	Reference
		C ₂ H ₂	¹⁵ N ₂		
Cycad	Coralloid root	4 h	4 h	5.8	Halliday and Pate, 1976
Azolla	3–4 fronds	5 h	5 h	1.7	Peters et al., 1977
	<i>Anabaena azollae</i>			2.5	
Common bean	Nodulated root	2 h	2 h	7.3–8.3	Saito et al., 1980
Rhizobium strains	Agar cultures	1 d	4 d	7–18	Kaneshiro et al., 1980
Sphagnum-Nostoc	30 branches	2 h	2 h	4.0–5.1	Basilier, 1980
Lichens, 3 spp	Thalli 10 g	25 min	14 d	8–10	Millbank, 1981
Wood litter	22 samples	1 d	5–9 d	1.3–26.5	Roskoski, 1981
Lichens, 2 spp.	12 thallus discs	0.5–0.73 h	1.5–3.8 h	4.3–4.8	Horstmann et al., 1982
Field pea	Excised nodules	40 min	45–50 min	3.3–5.1	Skot, 1983
Red clover			40 min	2.5–7.9	van Kessel and Burris, 1983
Alsike clover				4.9–6.7	
Soybean				4.6–5.2	
Trees, 6 spp.		1 h	1 h	2.2–4.6	van Kessel et al., 1983
Sub clover	Whole plant		5 d	5.1 ± 0.4	Hopmans et al., 1983
Grey alder	Intact roots	2 h	2 h	2.7–4.4	Sellstedt, 1986
Sesbania	Stem segments			2.0	Becker et al., 1990
Curly indigo				1.75	

^a Cycad, *Macrozamia riedlei*; Azolla, *Azolla caroliniana*; Common bean, *Phaseolus vulgaris*; Sphagnum, *Sphagnum riparium*; Lichens 3 spp., *Peltigera membranacea*, *P. polydactyla*, *Lobaria pulmonaria*; Wood litter, Sugar maple, *Acer saccharum*, Yellow birch, *Betula alleghaniensis*, American beech, *Fagus grandifolia*; Lichens 2 spp., *Lobaria pulmonaria*, *L. oregana*; Field pea, *Pisum sativum*; Red clover, *Trifolium pratense*, Alsike clover, *T. hybridum*, Soybean, *Glycine max*; Trees 6 spp., *Acacia pennatula*, *Albizia lebeck*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, *Leucaena leucocephala*, *Inga jinicuil*; Sub clover, *T. subterraneum*; Grey alder, *Alnus incana*; Sesbania, *Sesbania rostrata*; Curly indigo, *Aeschynomene afraspera*.

excised nodules, excised nodulated roots, plant litter, stem segments, intact root systems and whole plants (Table 3). Calibration involved the simultaneous determination of the ratio of C₂H₂ reduced to N₂ fixed. The ratios obtained were extremely variable being either higher or lower than the theoretical 3:1 (Table 3). Many factors were responsible for this variation, particularly the conditions under which the assay was performed. These efforts were largely abandoned following the publication of several articles which discredited the way in which the assay was being used (e.g. Minchin et al., 1983, 1986; Giller, 1987). Therefore it is doubtful that any degree of confidence can be attached to quantitative estimates of biological N₂ fixation obtained by extrapolating the C₂H₂ assay using the experimentally determined ratio of C₂H₂ reduced to N₂ fixed. Nonetheless, the assay is still used together with ¹⁵N₂ in studies of symbiotic N₂ fixation, due to its high sensitivity (Keuter et al., 2014), although Montoya et al. (1996) claimed that the sensitivity and precision of the ¹⁵N₂ tracer method for marine plankton were comparable to, or better than, the C₂H₂ reduction assay.

6. Stable isotope probing (SIP) with ¹⁵N₂

Despite the vital importance of biological N₂ fixation in maintaining terrestrial ecosystem sustainability, the taxonomic identity of the microorganisms involved has usually been confined to a small fraction of the microbiota that can be isolated and cultivated. The recent development in the coupling of molecular biological methods with SIP in biomarkers has provided a cultivation-independent means of linking the identity of bacteria with their function in the environment (Radajewski et al., 2000). This method relies on the incorporation of a substrate that is highly enriched in a stable isotope, such ¹³C or ¹⁵N, and the identification of active microorganisms by the selective recovery and analysis of isotope-enriched cellular components (Radajewski et al., 2000, 2003; Dumont and Murrell, 2005). The N₂ fixation *nifH* gene, which encodes a subunit of the nitrogenase enzyme, provides a useful marker that can be used to study the distribution and diversity of diazotrophs without the need for cultivation. Surveys of *nifH* diversity in soil commonly reveal sequence types that correspond to

diverse unidentified diazotrophs (Hsu and Buckley, 2009). The SIP technique has now been used in conjunction with metagenomics to establish links between the microbial identity and particular metabolic functions.

Initially, SIP was carried out using ¹³C (Radajewski et al., 2000, 2003; Orphan et al., 2001, 2002), but there is potential to use other stable isotopes such as ¹⁵N. ¹⁵N₂ when used with SIP provides a powerful tool for characterizing free-living diazotrophs in natural ecosystems. In the first application of ¹⁵N₂-DNA-SIP to soil, three groups of free-living diazotrophs that are actively involved in soil N₂ fixation were identified (Buckley et al., 2007). Subsequently, Buckley et al. (2008) explored the linkage between different C sources and N₂ fixation by specific diazotroph populations using ¹⁵N₂-DNA-SIP. Addison et al. (2010a,b) applied ¹⁵N₂-DNA-SIP to investigate the diversity and abundance of active but non-cultivated diazotrophs in New Zealand pulp and paper wastewater. Therefore, ¹⁵N₂-DNA-SIP can be used to link particular 16 S rRNA genes and the *nifH* gene to characterize non-cultivated diazotrophs in a range of environments.

¹⁵N₂-DNA-SIP is distinguished from other SIP applications by several attributes. First, because atmospheric N₂ can be totally replaced with ¹⁵N₂, incubations can be conducted at realistic concentrations. Second, isotope dilution is likely to be less of a problem with ¹⁵N₂ than with ¹³C-labelled substrates, as N₂ fixation can be inhibited by high mineral N contents. Third, cross-feeding is not a significant concern with ¹⁵N₂-DNA-SIP since unlabelled ¹⁴N₂ can be removed completely by evacuation and since the majority of N₂ fixation occurs only when fixed (and unlabelled) forms of N are unavailable to the cell. Therefore, only those organisms that are actively engaged in N₂ fixation should become heavily labelled with ¹⁵N in response to incubation with ¹⁵N₂.

The application of ¹⁵N₂-SIP needs to become widespread and may make it possible to identify previously uncharacterized groups of nitrogenase genes and to reveal the underlying microbial mechanisms of N cycling processes. However, this method is not without its limitations and will require further developments to assess its applicability to natural N₂-fixing environments (Addison et al., 2010b). Large, reliable databases will also be required to be assembled so that organisms assimilating ¹⁵N₂ can be catalogued.

Such databases may ultimately lead to hypotheses about the physiological and genetic bases for ecological fitness.

7. The quantitative estimation of symbiotic dependence (P_{atm}) using $^{15}\text{N}_2$

7.1. Endophytic diazotrophs

$^{15}\text{N}_2$ has been used worldwide to identify endophytic biological N_2 fixation in a range of cereals and tropical grasses. Chalk (2016) reviewed 22 published papers in which the $^{15}\text{N}_2$ technique confirmed putative N_2 fixation. Because a control or reference treatment is not required, N_2 fixation can be measured either under natural (undisturbed) or imposed (e.g. inoculated) treatments. Exposure times were generally of short duration, usually a few days but seldom exceeding 1–2 weeks (Chalk, 2016). Therefore the method has generally not provided a quantitative estimate of the contribution of biological N_2 fixation to the N nutrition of the plant over its life cycle. However, the study of Bremer et al. (1995) with wheat was an exception. Wheat roots of a single cultivar (Lancer) were exposed to $^{15}\text{N}_2$ at vegetative and reproductive growth stages for 28 and 22 days when plants were 13 and 54 days old, respectively. Fixation of $^{15}\text{N}_2$ did not occur at either growth stage. However, indirect ^{15}N enrichment (E) and ^{15}N natural abundance (NA) techniques have been widely used to estimate associative biological N_2 fixation of cereals, forage grasses, industrial crops, fruits, vegetables and forest species (Unkovich et al., 2008; Chalk, 2016).

7.2. Free-living heterotrophs in soil

In a review of the literature covering symbiotic and non-symbiotic N_2 fixation in 23 natural ecosystems worldwide (Cleveland et al., 1999), the majority of estimates of non-symbiotic N_2 fixation were obtained using the C_2H_2 reduction assay, while none of the reported 106 studies used $^{15}\text{N}_2$. While $^{15}\text{N}_2$ has been used in studies of asymbiotic N_2 fixation in agroecosystems, its application has been mainly confined to laboratory incubation studies (e.g. Chang and Knowles, 1965; Brouzes et al., 1969; Unkovich and Baldock, 2008; Hsu and Buckley, 2009; Gupta et al., 2014), or for calibration of the C_2H_2 reduction assay (e.g. Keuter et al., 2014).

Nevertheless, $^{15}\text{N}_2$ was used to quantify the post-harvest decomposition of wheat straw and roots in soil which was continuously labelled for 4 weeks from 76 to 104 days after sowing (DAS) (Bremer et al., 1995). Appreciable ^{15}N enrichments were observed in residual straw and roots and in surrounding soil indicating non-symbiotic N_2 fixation, but the estimated amount (26 g N ha^{-1}) was agronomically insignificant. Therefore, due to the lack of similar studies there remains a knowledge gap in respect of the contribution of asymbiotic N_2 fixation to agroecosystem N inputs. Unkovich and Baldock (2008) discussed the challenges faced in obtaining realistic estimates, but concluded that it is worth persevering with $^{15}\text{N}_2$ studies because the method is highly sensitive and should enable rates of N_2 fixation equivalent to ca. $0.3 \text{ mg N m}^{-2} \text{ day}^{-1}$ ($1 \text{ kg N ha}^{-1} \text{ y}^{-1}$) to be quantified.

7.3. Phototrophic diazotrophs

7.3.1. Terrestrial

Soils of arid regions often have surface crusts of lichens, Cyanobacteria and other cryptogams covering as much as 30% of the total area (Rogers et al., 1966). These authors were able to demonstrate fixation of $^{15}\text{N}_2$ during laboratory incubation of a surface crust from the southern central arid zone of Australia, which was composed of the lichen *Collema coccophorus* with the

blue-green algae symbiont, *Nostoc* sp. Similarly, Holst et al. (2009) demonstrated $^{15}\text{N}_2$ fixation in biological soil crusts from an Inner Mongolian steppe during laboratory incubation.

In contrast to the above studies Rousk et al. (2016) attempted $^{15}\text{N}_2$ labelling for 24 h in mesocosm soil cores containing either a naturally-occurring legume, a lichen, a moss or free-living from a N-limited subarctic tundra site. Mesocosms were sampled 3 days and 5 weeks after labelling and the label was traced into the soil, microbial biomass and associated non- N_2 -fixing plants. Three days after labelling one half of the fixed $^{15}\text{N}_2$ was found in the soil in legume mesocosms, while after 5 weeks free-living N_2 fixers released two-thirds of the fixed ^{15}N into the soil. In contrast, the moss and the lichen retained the fixed ^{15}N . These results demonstrate that different N_2 fixing associations release fixed N at different rates, and also show the importance of field measurements.

7.3.2. Fresh water

Bei et al. (2013) studied the relative contribution of heterotrophic and phototrophic N_2 fixation to the N nutrition of paddy rice in China by exposure to $^{15}\text{N}_2$ for 70 days. Four treatments were included: fallow (not planted) and planted, each uncovered or covered with black cloth to shade out phototrophic organisms. The following calculations were made:

$$\text{Heterotrophic } ^{15}\text{N}_2 \text{ fixation} = ^{15}\text{N}_2 \text{ fixed}_{\text{covered}} \quad (3)$$

The effect of rice on heterotrophic biological N_2 fixation was calculated by Eq. (4).

$$\Delta \text{Heterotrophic}_{\text{rice}} = ^{15}\text{N}_2 \text{ fixed}_{\text{rice-covered}} - ^{15}\text{N}_2 \text{ fixed}_{\text{fallow-covered}} \quad (4)$$

$$\text{Phototrophic } ^{15}\text{N}_2 \text{ fixation} = ^{15}\text{N}_2 \text{ fixed}_{\text{uncovered}} - ^{15}\text{N}_2 \text{ fixed}_{\text{covered}} \quad (5)$$

The effect of rice on phototrophic biological N_2 fixation was calculated by Eq. (6).

$$\Delta \text{Phototrophic}_{\text{rice}} = (^{15}\text{N}_2 \text{ fixed}_{\text{rice-uncovered}} - ^{15}\text{N}_2 \text{ fixed}_{\text{rice-covered}}) - (^{15}\text{N}_2 \text{ fixed}_{\text{fallow-uncovered}} - ^{15}\text{N}_2 \text{ fixed}_{\text{fallow-covered}}) \quad (6)$$

The estimated amounts and proportions of $^{15}\text{N}_2$ fixed by heterotrophic and phototrophic organisms in fallow and rice-based systems are shown in Table 4.

In the fallow treatment the phototrophs contributed a significantly greater proportion (0.67) compared with the heterotrophs to the total N_2 fixed, whereas in the presence of rice plants the proportional contributions from each N_2 fixing system were equal (Table 4). Both heterotrophic and phototrophic biological N_2 fixation increased significantly in the presence of the rice plant compared to the fallow treatment. However, the effect of rice on the difference between fallow and planted treatments for either heterotrophic (Eq. (4)) or phototrophic (Eq. (6)) biological N_2 fixation was not significant.

The contribution of endophytic biological N_2 fixation to the N nutrition of paddy rice has been well documented by the use of the indirect E methodology (Chalk, 1991, 2016) with marked differences between rice cultivars in their ability to support biological N_2 fixation. However, it is recognised that the E methodology as

Table 4The proportional distribution of $^{15}\text{N}_2$ fixed between heterotrophic and phototrophic diazotrophs in fallow and rice-based systems. (After [Bei et al., 2013](#)).

System	N_2 fixation	$^{15}\text{N}_2$ fixed (mg N pot $^{-1}$) ^a	Proportion of total N_2 fixed
Fallow	Heterotrophic	0.45 $\pm$ 0.14 b B	0.33
	Phototrophic	0.91 $\pm$ 0.19 aB	0.67
Rice-based	Heterotrophic	2.15 $\pm$ 0.07 aA	0.50
	Phototrophic	2.18 $\pm$ 0.19 aA	0.50
Effect of rice	Δ Heterotrophic	1.70 $\pm$ 0.20 a	
	Δ Phototrophic	1.27 $\pm$ 0.38 a	

^a Data are means $\pm$ SD (n = 3). Different lower case letters denote significant differences ($P < 0.05$) between heterotrophic and phototrophic in the same system, while different upper case letters denote significant differences ($P < 0.05$) between fallow and rice based systems in the same BNF process.

previously applied cannot differentiate between endophytic and phyllospheric biological N_2 fixation ([Chalk, 2016](#)) and that the floodwater must be covered to separate phototrophic from heterotrophic biological N_2 fixation ([Chalk, 1991](#)). Thus the direct long-term study of [Bei et al. \(2013\)](#) using $^{15}\text{N}_2$ represents a significant advance in our understanding of the respective roles of heterotrophic vs. photodependent N_2 fixation in the N nutrition of paddy rice, and highlights the need for further work to quantify the relative contributions of endophytic and phyllospheric biological N_2 fixation.

7.4. Legumes

As far as we know, the direct estimation of symbiotic dependence of a legume by exposure to $^{15}\text{N}_2$ has been reported in only one long-term study. [Sims et al. \(1986\)](#) exposed two lysimeters containing field pea to $^{15}\text{N}_2$ for 64 days, and recorded the symbiotic dependence as $> 90\%$. Although [Mohr et al. \(1998\)](#) also exposed alfalfa to $^{15}\text{N}_2$ for a long period (74 days) they did not report symbiotic dependence *per se*, but focused instead on the distribution of fixed N within the soil-plant system.

8. The quantitative estimation of biological N_2 fixation dynamics in ecosystems using $^{15}\text{N}_2$

8.1. The distribution of biologically-fixed N_2 in the soil-plant system

8.1.1. Studies with rice

The distribution of $^{15}\text{N}_2$ fixed between above- and below-ground components of paddy rice was determined by short- and long-term exposure to $^{15}\text{N}_2$ at different growth stages ([Table 5](#)). The highest proportion of N_2 fixed for short-term exposure was in the soil ($>75\%$), with a greater proportion in tops than in roots, irrespective of growth stage, exposure time or whether roots or tops + roots were exposed to $^{15}\text{N}_2$ ([Table 5](#)).

For a long-term exposure to $^{15}\text{N}_2$ (10 weeks) a different distribution emerged compared to short-term exposure ([Bei et al., 2013](#); [Table 5](#)). A much higher proportion of fixed N_2 occurred in tops, with a correspondingly much lower proportion in the soil, although the proportion in roots was similar (≤ 0.10). Covering the surface of the pots with black cloth markedly changed the distribution of fixed N_2 with a greater proportion in rice tops and less in the soil, which reflected the absence of photosynthetic Cyanobacterial diazotrophs. These results reinforce the general observation of [Mohr et al. \(1998\)](#) that long-term exposure to $^{15}\text{N}_2$ would be more representative of the ultimate partition of fixed N_2 among the various components of the soil-plant system.

8.1.2. Studies with legumes

A quantitative measurement of the entry of fixed N_2 into any pool in the soil-plant system can be obtained by exposure of nodulated roots or whole plants to $^{15}\text{N}_2$. In a series of 3 experiments involving a legume and a grass grown together, [Ross et al. \(1964\)](#) found that from 9 to 32% of fixed $^{15}\text{N}_2$ was recovered in the soil and 68–91% in the legume + grass after exposure times of 13–26 days ([Table 1](#)). However, it was not clear as to whether the soil compartment included roots or not.

Using much shorter exposure times of 24–72 h, [Ruschel et al. \(1979\)](#) found a relatively constant proportion of fixed N was located in the tops of common bean and soybean (0.67–0.71) and that variable proportions were distributed between roots (0.12–0.30) and soil (0.03–0.17). The difficulty of separating roots from soil may have contributed to the latter variability. The significance of the data is that from one-quarter to one-third of the N_2 fixed by the legume in the short term was located below-ground, and that the majority of the fixed N was quickly transferred to the legume tops ([Table 6](#)).

[Russelle et al. \(1994\)](#) similarly used a short-exposure time of 48 h with the perennial forage legume alfalfa. A few days after exposure to $^{15}\text{N}_2$ the majority of the fixed N was present in the tops

Table 5Distribution of $^{15}\text{N}_2$ fixed among the tops and roots of rice (*Oryza sativa*) and the soil.

Cultivar	Treatment ^a	Plant age (d)	Growth stage ^b	Exposure time (d)	Plant part ^c	Proportion of N_2 fixed			Reference
						Tops	Roots	Soil	
Koshi-hikari	Uncovered	91	EM	13	R	0.11	0.08	0.81	Yoshida and Yoneyama, 1980
		108	GF	7	T + R	0.11	0.08	0.81	
		113	FL	13		0.15	0.10	0.75	
IR 2037–117–2	Covered	41	TL	12.8		0.14	0.02	0.84	Eskew et al., 1981
		73 ^d	HD	4.1		0.05	0.03	0.92	
		37 ^e	TL	70		0.43	0.04	0.53	
Zhendao 11	Uncovered					0.64	0.07	0.29	Bei et al., 2013
	Covered								

^a Floodwater uncovered or covered with black cloth.

^b EM, early milk; GF, grain filling; FL, flowering; TL, tillering; HD, heading.

^c Plant part exposed to $^{15}\text{N}_2$, R, roots; T + R, tops + roots.

^d Harvested at maturity.

^e Harvested at milk filling stage.

Table 6Distribution of $^{15}\text{N}_2$ fixed among the tops and roots of grain and forage legumes and the soil.

Plant species ^a	Plant age (d)	Exposure time (d)	Plant part ^b	Harvest age (d)	Proportion of N_2 fixed			Reference
					Tops	Roots	Soil	
Common bean	35	3	T + R	38	0.67	0.30	0.03 ^c	Ruschel et al., 1979
	25	1		27	0.71	0.12	0.17 ^c	
Soybean	20			22	0.69	0.18	0.13 ^c	Russelle et al., 1994
Alfalfa	70	2		74	0.59 ^d	0.39	0.02 ^f	
				105 ^e	0.53 ^d	0.46	0.01 ^f	
Wclover	25	49	R	74	0.35	0.53	0.12 ^g	Mohr et al., 1998
	60	3	R	63	0.72	0.25 ^h	0.03 ^h	Lesuffleur et al., 2013

^a Common bean, *Phaseolus vulgaris*; Soybean, *Glycine max*; Alfalfa, *Medicago sativa*; Wclover, white clover, *Trifolium repens*.^b Plant part exposed to $^{15}\text{N}_2$, R, roots, T + R, tops + roots.^c Soil data were corrected for any N_2 fixed in soil-only or maize (*Zea mays*) controls.^d Saranac cultivar, herbage + crown.^e Regrowth after the first harvest of topgrowth.^f Described as "rhizosphere soil", "bulk soil" value were not reported.^g "Rhizosphere soil" proportion of fixed N_2 was reported as 0.02.^h Stolons were designated as roots; Soil was an artificial rooting medium composed of a 1: 1 mixture of attapulgite and clay balls (2–6 mm diameter).

(foliage + crown) + roots with only 2% in the rhizosphere soil (Table 6). Rhizosphere soil was defined as soil adhering to roots and nodules during sequential sieving of soil + roots through 10-, 5- and 2-mm screens. Rhizosphere soil was separated from roots by 60 s sonication in sodium phosphate buffer, pH = 7.0. When sampled 5 weeks later after regrowth, the partition was similar with 1% of the label present in rhizosphere soil, 53% in the tops and the remainder in roots. Russelle et al. (1994) estimated that between 36 and 46% of the fixed N initially present in crowns and roots was re-translocated to support herbage regrowth. It was not clear in this particular study how much isotope was recovered in non-rhizosphere soil. Using a short $^{15}\text{N}_2$ exposure time of 3 days with the perennial forage legume white clover, Lesuffleur et al. (2013) found very little fixed N in the artificial rooting medium (Table 6), results consistent with those of Russelle et al. (1994) for alfalfa.

As pointed out by Mohr et al. (1998) only the N assimilated during a brief exposure period can be traced directly, and that continuous and prolonged exposure would label a greater proportion of symbiotically-fixed N and apply a label more representative of the N fixed throughout alfalfa growth. Thus in contrast to the above studies, Mohr et al. (1998) used an exposure time of 7 weeks for alfalfa. The subsequent analyses indicated that the greatest proportion of the fixed $^{15}\text{N}_2$ occurred in roots (53%), and 12% was recovered in the soil (Table 6), 2% being associated with rhizosphere soil – a value remarkably consistent with that previously reported by Russelle et al. (1994).

8.2. The distribution of biologically-fixed N_2 among plant organs

In addition to data on the distribution of fixed $^{15}\text{N}_2$ in plant tops, roots and soil (Tables 5 and 6) several authors have provided

detailed information on the ^{15}N enrichment and partitioning of fixed N_2 between various plant organs of soybean (Warembourg et al., 1982), tropical grasses (De-Polli et al., 1977) and rice (Ito et al., 1980; Yoshida and Yoneyama, 1980; Eskew et al., 1981; Watanabe and Ventura, 1982). Although these studies are of limited value because of short-term exposure to $^{15}\text{N}_2$, they nevertheless served to identify N_2 fixing hot spots in rice foliage, including leaf blades and sheaths (Ito et al., 1980; Watanabe and Ventura, 1982) which could harbour endophytic and/or phyllospheric diazotrophs.

Warembourg et al. (1982) provided time series data on the distribution of fixed $^{15}\text{N}_2$ in soybean labelled for 24 h at 63 and 85 days after sowing (DAS) which showed the redistribution of fixed N within the plant tissues. At 63 DAS the distribution of fixed N (%) was leaves > nodules > stems = roots > flowers + pods, while at 85 DAS the distribution was stems > seeds > leaves = nodules > roots = flowers + pods.

Two experiments involving long-term exposure to $^{15}\text{N}_2$ provide data on the incorporation of fixed N_2 into plant tissues, one involving alfalfa (Mohr et al., 1998) and the other rice (Bei et al., 2013). The aerial tissues (leaf and stem) showed fairly uniform ^{15}N enrichments in both studies (Table 7), while crowns and roots tended to be less enriched. This difference was particularly marked in the case of paddy rice. Fixed N_2 was most abundant in the taproot and fine roots of forage alfalfa which represented regrowth after defoliation 10 days previously, while it was most abundant in the aerial tissues of rice, particularly in the leaf blade (Table 7).

8.3. The transfer of biologically-fixed N_2 in legume–non-legume associations

The transfer of biologically-fixed N from legumes to companion

Table 7 ^{15}N enrichment and distribution of $^{15}\text{N}_2$ fixed among plant organs in long-term $^{15}\text{N}_2$ exposure experiments.

Plant ^a	$^{15}\text{N}_2$ (d) ^b	^{15}N enrichment (excess atom % ^{15}N) ^c					Reference
		Leaf	Stem	Crown	Fine root	Taproot	
Alfalfa	49	4.26 (18)	4.04 (8)	3.38 (12)	3.32 (32)	3.85 (30)	Mohr et al., 1998
Rice ^d	70	Panicle	Leaf blade	Leaf sheath	Stem	Root	Bei et al., 2013
		1.16 (15)	1.36 (40)	1.09 (22)	1.08 (14)	0.446 (9)	

^a Alfalfa, *Medicago sativa*; Rice, *Oryza sativa*.^b Exposure time; Plant age, growth stage, harvest age and plant part exposed to $^{15}\text{N}_2$ for rice and alfalfa are given in Tables 5 and 6, respectively.^c Data in parentheses are % distribution in plant organs.^d Floodwater not covered with black cloth.

non-legume species has been estimated directly by exposure of the legume to $^{15}\text{N}_2$. Chalk et al. (2014) reviewed seven published papers involving transfer from forage/pasture legumes to grasses or cereals. A single dose or several pulses of $^{15}\text{N}_2$ was generally given over a relatively short time period (<1 up to several days) although in a few cases exposure times lasted for 2–4 weeks (Chalk et al., 2014). The percentages of non-legume N derived from the transfer of biologically-fixed N were low, ranging from 0 up to 7.6%. Significant transfer generally occurred following the death of the legume while only modest amounts were transferred between actively growing species.

8.4. Biologically-fixed N as a source of N_2O ?

As far as we are aware, only one study has utilised $^{15}\text{N}_2$ to specifically explore whether biologically-fixed N could be a source of the greenhouse gas N_2O (Carter and Ambus, 2006). A mixture of white clover and perennial ryegrass was exposed to $^{15}\text{N}_2$ for 14 days at each of 3 growth stages at 16, 26 and 36 weeks after emergence. Pots were removed from the labelling chamber and placed in a static chamber for 180 min and the ^{15}N abundance of the emitted N_2O was measured. Only a small percentage ($2.1 \pm 0.5\%$) of the total N_2O emitted was derived from biologically-fixed N_2 . Although other non-isotopic studies have not necessarily substantiated the significance of the potential emissions of N_2O during biological N_2 fixation (Jensen et al., 2012), the work of Carter and Ambus (2006) is an important initial study, and further long-term research is warranted given that biologically-fixed N, like synthetic fertilizer N, is a major source of reactive N in the environment.

8.5. The transfer of biologically-fixed N_2 in crop sequences

The methodology for estimating the transfer of N in legume-based crop rotations has usually involved the addition of ^{15}N -enriched legume residues to soil, and measurement of the ^{15}N recovery by a subsequent non-leguminous crop. Legume tissues can be labelled through uptake from ^{15}N -labelled fertilizer added to soil or an artificial growth medium, or through ^{15}N -foliar labelling. This technique estimates the transfer of labelled legume N, and does not estimate the transfer of biologically-fixed N_2 . The only direct method for estimating the transfer of biologically fixed N_2 in a crop sequence involves labelling the N_2 -fixing plant with $^{15}\text{N}_2$, and measuring the uptake of ^{15}N by a succeeding crop following legume harvest or senescence. However, it appears that only once has this *in-situ* approach been adopted.

For example, in a growth room experiment Mohr et al. (1998) measured the uptake of $^{15}\text{N}_2$ fixed by alfalfa by a following crop of barley after the forage legume had been terminated either by application of glyphosate or by tillage. Seedlings of alfalfa (25-day-old) were continuously exposed to $^{15}\text{N}_2$ (4–6 atom % ^{15}N) for 7 weeks using the enclosure system described by Bremer et al. (1995). Two cuts of topgrowth were taken at days 42 and 64 after seeding during the exposure period. Five days after termination, barley was sown and harvested after 4 weeks.

Two treatments were imposed. Labelled legume foliage was transferred to defoliated cylinders containing unlabelled legume roots, and unlabelled legume foliage was transferred to defoliated cylinders containing labelled legume roots. Herbicide termination was simulated by leaving foliage on the soil surface, while tillage was simulated by mixing foliage with the soil.

The percentage of barley N derived from biologically-fixed N_2 in alfalfa was very low for surface applied foliage (herbicide), but was almost 13% for incorporated foliage (tillage), reflecting the intimacy of soil contact with decomposing residues (Table 8). The recovery of labelled foliage N by barley closely reflected these values. Much

Table 8

The percentage of biologically-fixed $^{15}\text{N}_2$ and ^{15}N recovery from the foliage and roots of alfalfa in a subsequent crop of barley (*Hordeum vulgare*) as affected by method of alfalfa termination. (After Mohr et al., 1998).

Treatment	Termination	Ndfa (%) ^a	Recovery (%) ^b
Labelled alfalfa foliage	Herbicide	1.3a	1.2a
	Tillage	12.8b	10.4b
Labelled alfalfa roots	Herbicide	42.4a	6.1a
	Tillage	25.3b	3.6a

^{a,b}Data within a column within a treatment followed by different letters are significantly different ($P < 0.05$).

^a Ndfa, % of N in barley derived from fixed $^{15}\text{N}_2 = (^{15}\text{N} \text{ enrichment of barley} \div ^{15}\text{N} \text{ enrichment of alfalfa}) \times 100$.

^b Recovery = (mg labelled N in barley foliage $\div$ mg labelled N in added alfalfa foliage or roots) $\times 100$.

higher percentages of barley N were derived from biologically-fixed N_2 in alfalfa roots, but the effect of termination method was the opposite, with herbicide having a significantly higher value than tillage, suggesting a direct effect of herbicide on *in-situ* root decomposition (Table 8). Recoveries of labelled root N by barley were, however, relatively low (<7%) and did not significantly differ between termination methods.

9. Do indirect ^{15}N methods give accurate estimates of P_{atm} or N transfer?

9.1. P_{atm} estimated by indirect methods that use a reference plant

Indirect ^{15}N -based methodologies using either *E* or *NA* approaches to estimate P_{atm} by N_2 -fixing plant associations require a non- N_2 -fixing reference plant treatment to act as a surrogate to estimate the proportion of labelled to unlabelled soil N obtained by the N_2 fixer (Unkovich et al., 2008; Chalk et al., 2016). The *E* method is based on the addition of a ^{15}N -enriched material to the soil, but no addition is required with the *NA* method. The *E* method results in a marked temporal and spatial non-uniformity in the distribution of ^{15}N in the soil matrix (Chalk and Ladha, 1999). Although it was originally thought that the isotope would be more uniformly distributed with the *NA* method, this has not proven to be the case (Chalk et al., 2016).

There are numerous reports where estimates of plant reliance upon N_2 fixation using either *E* or *NA* techniques can be greatly influenced by choice of reference plant, indicating that the root systems of different reference plant species are sampling different pools of N, which are themselves likely to be different from that sampled by the N_2 -fixing plant. For the *E* method, P_{atm} is estimated by Eq. (7).

$$P_{\text{atm}} = 1 - \frac{E_{\text{N}_2\text{-fixing plant}}}{E_{\text{reference plant}}} \quad (7)$$

where *E* is the excess atom fraction ^{15}N . E_{plant} is the difference in the ^{15}N abundance (atom fraction ^{15}N) of the plant in the ^{15}N -enriched treatment minus the ^{15}N abundance of the plant in a control (un-enriched) treatment.

For the *NA* method P_{atm} is estimated by Eq. (8).

$$P_{\text{atm}} = \frac{\delta^{15}\text{N}_{\text{reference plant}} - \delta^{15}\text{N}_{\text{N}_2\text{-fixing plant}}}{\delta^{15}\text{N}_{\text{reference plant}} - B} \quad (8)$$

where $\delta^{15}\text{N}$ is the $\frac{^{15}\text{N}}{^{14}\text{N}}$ ratio of the sample relative to the $\frac{^{15}\text{N}}{^{14}\text{N}}$ ratio of the international standard, atmospheric N_2 (Eq. (9)).

$$\delta^{15}\text{N} = \frac{\frac{^{15}\text{N}}{^{14}\text{N}}_{\text{sample}}}{\frac{^{15}\text{N}}{^{14}\text{N}}_{\text{standard}}} - 1 \quad (9)$$

where by definition, $\delta^{15}\text{N}_{\text{standard}}$ is zero excess atom fraction ^{15}N , and B is the isotopic fractionation associated with the process of N_2 fixation, N assimilation and partitioning within the plant. The value of B is usually determined as the $\delta^{15}\text{N}$ abundance of shoots of effectively inoculated plants grown in an N -free medium, where it is assumed that the plant is wholly dependent on N_2 fixation for its N nutrition (Unkovich et al., 2008). However, there is no direct evidence that this is necessarily always the case, as small amounts of available forms of N have been reported to be released from some reputedly “inert” rooting media such as perlite, and plants can potentially also absorb N through the foliage from external sources such as NH_3 (Denmead et al., 1976). Such external inputs of N in addition to possible micro-symbiont strain, plant species, and/or growth effects might explain some of the variation (including some odd positive values) observed in published B values (Unkovich et al., 2008).

Several alternative techniques have been proposed to determine the B -value (Chalk et al., 2016). An indirect method (Doughton et al., 1992) involved paired treatments where symbiotic dependence was estimated by E ($P_{\text{atm}(E)}$) and NA ($P_{\text{atm}(NA)}$) methods in the glasshouse, where cross-contamination is a potential problem (Chalk et al., 2016). The B -value is then estimated by Eq. (10) by

$$E_{\text{legume corrected}} = \frac{(\text{Legume N yield}_{t_1} \times E_{\text{legume}, t_1}) - (\text{Legume N yield}_{t_0} \times E_{\text{legume}, t_0})}{\text{Legume N yield}_{t_1} - \text{Legume N yield}_{t_0}} \quad (13)$$

$$E_{\text{reference plant corrected}} = \frac{(\text{Reference N yield}_{t_1} \times E_{\text{reference}, t_1}) - (\text{Reference N yield}_{t_0} \times E_{\text{reference}, t_0})}{\text{Reference N yield}_{t_1} - \text{Reference N yield}_{t_0}} \quad (14)$$

assuming that $P_{\text{atm}(E)} = P_{\text{atm}(NA)}$.

$$B - \text{value} (\text{‰}) = \delta^{15}\text{N}_{\text{reference plant}} - \frac{\delta^{15}\text{N}_{\text{reference plant}} - \delta^{15}\text{N}_{\text{N}_2\text{-fixing plant}}}{P_{\text{atm}(E)}} \quad (10)$$

This approach assumes that the E method gives an accurate indirect estimate of P_{atm} , but there is insufficient evidence to support this assumption. It would be far better to use $^{15}\text{N}_2$ to gain a direct and accurate estimate of P_{atm} . Such an estimate could then be compared with the B -value determined in an N -free medium.

9.2. Comparison of estimates of P_{atm} using $^{15}\text{N}_2$ and the E methodology

In studies where E and NA methods and $^{15}\text{N}_2$ were employed (e.g. Akao, 1991) a comparison of estimates of P_{atm} was not possible because of the markedly different time periods between the short-term $^{15}\text{N}_2$ exposure and the longer-term indirect methods which provide average estimates of P_{atm} over the duration of the experimental period. Ross et al. (1964) suggested that the chamber

technique could be used to test the validity of estimates of P_{atm} obtained by the E method, but so far this has been attempted only once. For example, McNeill et al. (1994) proposed a method based on the incremental differences in N yields and ^{15}N enrichments between a time zero (t_0) control and equivalent later time (t_1) measurements. For the isotope dilution method calculations were required for both fixing and reference species, but for the $^{15}\text{N}_2$ method no reference plant is required.

P_{atm} for the direct $^{15}\text{N}_2$ method is given by Eq. 1, and is independent of the size of the plant N pool at the start of the measurement period. The proportion of the N increment (P_{inc}) derived from N_2 fixation over the period t_1 to t_0 is given by Eq. (11).

$$P_{\text{inc}} = \frac{\text{Legume N yield}_{t_1} \times P_{\text{atm}}}{\text{Legume N yield}_{t_1} - \text{Legume N yield}_{t_0}} \quad (11)$$

For the indirect isotope dilution method P_{atm} is given by Eq. (7). The proportion of the N increment (P_{inc}) derived from N_2 fixation over the period t_1 to t_0 is given by Eq. (12).

$$P_{\text{inc}} = 1 - \frac{E_{\text{legume corrected}}}{E_{\text{reference plant corrected}}} \quad (12)$$

where E is ^{15}N enrichment (excess atom fraction ^{15}N), and $E_{\text{legume corrected}}$ and $E_{\text{reference plant corrected}}$ are given by Eqs. (13) and (14), respectively.

The reference plant was perennial ryegrass either in monoculture or in mixed culture with the legume (McNeill et al., 1994). The authors claimed good agreement between estimates of P_{inc} between the indirect E methodology and the direct method for white clover for both reference criteria, equal to $P_{\text{inc}} = 0.90$ over an experimental period of 19 days. High values of symbiotic dependence are generally independent of the reference plant (Hardarson et al., 1988; Unkovich et al., 2008), and therefore these results do not represent a robust comparative evaluation of the E method with the $^{15}\text{N}_2$ method.

McNeill et al. (1994) also made equivalent comparisons with the woody actinorhizal species alder (*Alnus glutinosa*) using red fescue (*Festuca rubra*) in mixed culture and non-nodulated alder in monoculture as reference plants over an experimental period of 21 days. In this case there was disagreement in P_{inc} between E and the direct method, with a value of P_{inc} of 0.16 for $^{15}\text{N}_2$ and 0.33 or 0.58 for the E method, depending on the reference plant. The authors claim that high variability among replicates coupled with the small increment in N yield over 21 days led to large errors in the estimates of P_{inc} for alder.

Therefore, the results of comparisons of methods for white clover and alder were largely inconclusive. The disadvantage of

using the P_{inc} approach is that the estimates becomes yield-dependent, and therefore the significant advantage of the yield independency of isotope dilution and $^{15}N_2$ methods is lost. In order to avoid this problem, treatments would need to be imposed on seedlings so that antecedent N could be ignored. Therefore there is ample scope for further comparisons of both E and NA methodologies with $^{15}N_2$ for a range of legumes and reference species.

9.3. P_{atm} estimated by indirect methods that do not use a reference plant

In an endeavour to avoid the dilemma of the reference plant, a yield-independent method based on E (Chalk et al., 1996) and another based on NA (Wanek and Arndt, 2002) have been proposed. Chalk et al. (1996) developed a model based on the exponential decline of the ^{15}N -enrichment of the available soil N pool following the addition of labelled fertilizer to the soil. The integral of the equation derived from the experimental results served as the reference criterion.

Wanek and Arndt (2002) demonstrated experimentally that P_{atm} of several legumes was significantly and negatively correlated with the difference ($\Delta^{15}N$) between the $\delta^{15}N$ values of the shoot and nodulated roots (Eq. (15)).

$$\Delta^{15}N = \Delta^{15}N_{shoot} - \Delta^{15}N_{nodulated\ root} \quad (15)$$

Although these methods have been tested by comparison with reference plant derived estimates of P_{atm} using either the E (Chalk et al., 1996) or NA (Wanek and Arndt, 2002) methodologies, their efficacy has not been tested by comparison with direct estimates using $^{15}N_2$.

9.4. N transfer in legume–non-legume associations

The indirect E method have been used to estimate the transfer of biologically-fixed N to a non- N_2 -fixing companion species (Chalk et al., 2014), but the indirect NA method cannot be applied due to the unknown isotopic fractionation that occurs during N transfer (Peoples et al., 2015). However, it is not known how accurate indirect E estimates are, since to our knowledge direct comparisons with $^{15}N_2$ have not been made. It may be possible to estimate isotopic fractionation during N transfer by comparing the NA estimate of P_{atm} with the value obtained using $^{15}N_2$.

9.5. N transfer in crop sequences

Indirect E methods have been used to estimate the transfer of biologically-fixed N and legume N in crop sequences (Chalk, 1998). However it is not known how accurate any of these techniques are, since direct comparisons with $^{15}N_2$ have not been made. As previously discussed, Mohr et al. (1998) made a direct determination of the transfer of biologically-fixed N in an alfalfa–barley crop sequence using $^{15}N_2$, but as far as we are aware this is an isolated example, and additional studies are needed.

10. Conclusions

$^{15}N_2$ has been used on many occasions since 1941 to examine the assimilatory pathways associated with biological N_2 fixation, and to confirm putative N_2 fixation by a range of biological materials. However, because of technical problems associated with the use of enclosures and the high cost of $^{15}N_2$, the direct method has seldom been used to obtain quantitative estimates of N_2 fixed over a plant's life cycle. The relatively small chamber volume has generally restricted the scale of experiments to plants growing in

pots or cylinders. While pot size undoubtedly affects plant growth (e.g. Poorter et al., 2012) and thus the amount of N_2 fixed, it should not compromise the yield-independent estimation of symbiotic dependence (P_{atm}). Therefore, indirect methods based either on E or NA rather than $^{15}N_2$ have been the techniques of choice to obtain field-based quantitative estimates of plant reliance upon N_2 fixation for growth, or the transfer of BFN in mixed stands or crop sequences of legumes and non-legumes (Unkovich et al., 2008; Chalk et al., 2014; Peoples et al., 2015).

Nevertheless, several publications have demonstrated that the practical problems of long-term experiments with $^{15}N_2$ and higher plants are not insurmountable (Sims et al., 1986; Bremer et al., 1995; Mohr et al., 1998; Bei et al., 2013). Technological advances in controlled-environment chambers have made the method more amenable for quantitative estimation of N cycle processes (Bei et al., 2013). There is a clear need to evaluate indirect E and NA methods for quantitative estimation of symbiotic dependence and the dynamics of biologically-fixed N_2 in agroecosystems using $^{15}N_2$. This applies equally to methods that use or do not use a reference plant. In addition, new technologies have emerged for the application of $^{15}N_2$ such as stable isotope probing to identify uncultured diazotrophs.

Therefore, there are sound reasons for a return to fundamental studies involving $^{15}N_2$. This will be most relevant where a pressing issue has still not been resolved. For example, in a 50-year assessment of the global nitrogen budgets for major cereal crops, Ladha et al. (2016) estimated that 29, 38, and 25 kg N ha^{−1} yr^{−1} for maize, rice and wheat, respectively, are contributed by sources other than fertilizer- or soil-N. These authors suggested that about 50% of this N (i.e. 13, 22, and 13 kg N ha^{−1} yr^{−1}, respectively) could be derived from non-symbiotic N_2 fixation, including free-living and endophytic biological N_2 fixation. There is currently no direct evidence available to support or disprove this hypothesis.

A survey of the literature has shown that publication of $^{15}N_2$ chamber studies peaked between 1980 and 1995, with few studies reported before or since. Nevertheless, the demonstrated benefits that can accrue should outweigh the disadvantages. However, it has long been known that commercial supplies of $^{15}N_2$ may contain impurities, and recent research suggests that this may still be the case, so diligence and the use of clean up procedures will be required when using $^{15}N_2$ to provide unequivocal proof of biological N_2 fixation.

Acknowledgements

The senior author thanks the Chinese Academy of Sciences (CAS) for a Visiting Fellowship under the CAS President's International Fellowship Initiative for 2016, Grant No. 2016VMB029.

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