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REVIEW ARTICLE

Do techniques based on ^{15}N enrichment and ^{15}N natural abundance give consistent estimates of the symbiotic dependence of N_2-fixing plants?

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Abstract
Aims The primary aim of this review is to determine if methods based on ^{15}N enrichment (*E*) and ^{15}N natural abundance (*NA*) give consistent estimates of the proportional dependence of N_2-fixing species on biological N_2 fixation (P_{atm}), and secondly to attempt to explain any inconsistencies that may be found.
Methods Published estimates of the symbiotic dependence of N_2-fixing plants based on *E* and *NA* techniques applied in the same experiment were compared across scales from glasshouse pots to field plots to landscapes in agricultural and forest ecosystems, which included grain legumes, pasture and forage legumes, and woody perennials. A meta-analysis of the published data was based on correlation coefficients, box-plots and confidence intervals of means.
Results In some studies, estimates were reference plant dependent for both *E* and *NA* techniques, indicating temporal and/or spatial variations in the natural and artificial distribution of ^{15}N, which can sometimes result in erroneous negative estimates of symbiotic dependence. While significant correlations were obtained between *E* and *NA* estimates of P_{atm} for each of the three groups of N_2-fixing species, the probability that the methods provided estimates of P_{atm} within −5 to +5 % of each other was 0.29 or was 0.54 within −10 to +10 % of each other.
Conclusions We have identified a number of interacting factors that may contribute to the inconsistent agreement between estimates of P_{atm} by *E* and *NA* techniques, which underlines the need for a re-examination of the fundamental assumptions on which each method is based, and whether those assumptions are valid in any given situation.

Keywords Symbiotic dependence · ^{15}N enrichment · ^{15}N natural abundance · $\delta^{15}N$ · Legumes · Alder

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Introduction

The ^{15}N isotope dilution estimation of the proportional dependence of legumes on biological N_2 fixation (BNF) was first reported by McAuliffe et al. (1958). The method is based on ^{15}N enrichment of the soil with a labelled fertilizer and the use of paired plots, one containing the legume and the other a non-N_2-fixing reference plant. The ^{15}N isotope dilution technique has been used extensively to estimate BNF in cropping, pastoral, forestry and silvo-pastoral systems, including legumes, actinorhizal plants and tropical C_4 grasses, and several

critical reviews have been written about the technique (e.g., Chalk 1985; Chalk and Ladha 1999).

The use of ^{15}N natural abundance (*NA*) to estimate legume BNF is a more recent development compared with ^{15}N enrichment (*E*), with the first soil-based experiments reported by Amarger et al. (1979) and Kohl et al. (1980). The method also requires the use of a non-N_2-fixing reference plant, and in addition, it requires the determination of the isotopic fractionation which occurs during BNF (the B-value). The *NA* method has also been widely applied in systems that include grain and forage legumes, and woody perennials that include legumes and actinorhizal plants, and several critical reviews have been published (e.g., Shearer and Kohl 1986; Boddey et al. 2000).

Both *E* and *NA* techniques depend on the use of a non-N_2-fixing reference plant to estimate de facto the ratio of labelled (isotope-derived) to unlabelled (soil-derived) N assimilated by the legume. The major assumption in both techniques is that the reference plant accesses the same available soil N pool as the legume. However, as discussed in many previous publications, the application of isotope to confined micro-plots in the field perturbates the system under study and results in a non-uniform temporal and spatial (vertical) distribution of ^{15}N in the soil. Estimates of symbiotic dependence are therefore reference plant-dependent (Chalk 1985; Chalk and Ladha 1999) because of differences in relative rates of N uptake and soil volumes explored by roots of the two species.

On the other hand, there is no disturbance when the *NA* method is used, and it has been claimed that natural variations in ^{15}N abundance are relatively uniform over time and with soil depth (e.g., Ledgard and Steele 1992). Therefore if this assertion, which was originally based on limited published data, is generally valid, estimates of symbiotic dependence, unlike those obtained with the *E* method, should be less sensitive to the choice of the reference plant. To our knowledge, this hypothesis has not been tested by comparison of the many published estimates obtained by *E* and *NA* methods, particularly those where several reference plants were used. The objective of this review is therefore firstly to determine if the two methods give consistent estimates of symbiotic dependence over a range of scales and N_2-fixing systems, and secondly to attempt to explain any observed inconsistencies by considering, where possible, variations in the temporal and spatial distribution of ^{15}N. Attention will also be focused on the *NA* technique with respect to the reference plant δ-value and the B-value and the method used to determine the B-value.

Methodology

^{15}N enrichment method (*E*)

The proportion of legume N derived from the atmosphere (P_{atm}) is estimated by Eq. 1, which gives a yield-independent estimate of P_{atm}.

$$P_{atm} = 1 - \frac{E_{legume}}{E_{reference\ plant}} \quad (1)$$

where *E* is ^{15}N enrichment expressed as excess atom fraction ^{15}N.

In a single case of comparing *E* and *NA* estimates of P_{atm} (Stevenson et al. 1995), the A-value modification of the *E* technique was used, whereby a higher rate of ^{15}N-enriched fertilizer was applied to the reference plant compared with the legume (see review of Chalk 1996).

^{15}N natural abundance method (*NA*)

The proportion of legume N derived from the atmosphere (P_{atm}) is estimated by Eq. 2, which also gives a yield-independent estimate of P_{atm}.

$$P_{atm} = \frac{\delta^{15}N_{reference\ plant} - \delta^{15}N_{legume}}{\delta^{15}N_{reference\ plant} - B} \quad (2)$$

where $\delta^{15}N$ is the $\frac{15_N}{14_N}$ ratio of the sample relative to the $\frac{15_N}{14_N}$ ratio of the international standard, atmospheric N_2 (Eq. 3).

$$\delta^{15}N = \frac{\frac{15_N}{14_N}sample}{\frac{15_N}{14_N}standard} - 1 \quad (3)$$

where by definition, $\delta^{15}N_{standard}$ is zero excess atom fraction ^{15}N.

Several methods have been proposed for determining the 'B-value' in Eq. 2 which represents the isotopic fractionation which may occur during the N_2 fixation process and subsequently during the translocation of biologically-fixed N from the nodulated roots to shoots (Unkovich et al. 2008). The direct and most commonly-used technique (Amarger et al. 1979; Bergersen and

Turner 1983; Method 1), particularly with grain legumes, involves growing the legume in pots in a glasshouse in either sterilized sand culture inoculated with the appropriate rhizobial strain or in solution culture, so that BNF is the sole source of N. A second method (Doughton et al. 1992; Method 2) involves paired treatments where symbiotic dependence is estimated by *E* ($P_{atm(E)}$) and *NA* ($P_{atm(NA)}$) methods in the glasshouse. The B-value is then estimated by Eq. 4 in the form proposed by Okito et al. (2004) by assuming that $P_{atm(E)}=P_{atm(NA)}$. The derived value is then applied in field studies.

$$\mathrm{B-value}\ (‰) = \delta^{15}\mathrm{N}_{\text{reference plant}} - \frac{\delta^{15}\mathrm{N}_{\text{reference plant}} - \delta^{15}\mathrm{N}_{\text{legume}}}{P_{\text{atm }(E)}} \quad (4)$$

A third method involves the determination of an 'apparent B-value' equal to the lowest δ value of the legume measured across the experimental area (Eriksen and Høgh-Jensen 1998; Riffkin et al. 1999; Method 3). Another approach is to use a value or the mean of the range of values from the published literature for a given N_2-fixing species (Unkovich et al. 2008; Method 4) or to assume B is equal to zero or another value (e.g., Høgh-Jensen and Schjoerring 1994; Jacot et al. 2000; Issah et al. 2014; Method 5).

In some publications (e.g., Bergersen and Turner 1983; Ledgard et al. 1985a; b), *NA* values for the reference plant were expressed as atom % ^{15}N, the absolute ^{15}N abundance, $x(^{15}\mathrm{N})$. These data were converted to relative δ (‰) values using the expression (Eq. 5) given by Chalk et al. (2015), after ^{15}N natural abundance (0.3663 atom %) was subtracted from the sample ^{15}N abundance to give ^{15}N enrichment, $x^{\mathrm{E}}(^{15}\mathrm{N})$, as atom % excess ^{15}N.

$$\delta^{15}\mathrm{N}\,/\,‰ = 2740 \times x^{\mathrm{E}}\left(^{15}\mathrm{N}\right)_{\text{sample / air}}\,/\,\% \quad (5)$$

A meta-analysis of the published data was based on Pearson correlation coefficients for $P_{atm(E)}$ vs. $P_{atm(NA)}$, box-whisker plots for B-values and reference plants δ-values, and confidence intervals of means of differences in $P_{atm(E)} - P_{atm(NA)}$. Minitab 17 ® software was used to construct the Figs. and all statistical analysis.

Comparison of estimates of P_{atm} using *E* and *NA* techniques

Published estimates of P_{atm} using the two techniques for grain legumes, pasture and forage legumes and woody perennials are summarized in Tables 1, 2 and 3, respectively. The scale of studies ranged from glasshouse pot experiments, to field microplots (unconfined or confined by barriers, usually in the m^2 scale) to landscape investigations (Tables 1, 2 and 3).

Negative estimates of symbiotic dependence

As discussed in the Introduction, fixing and reference plants superimposed on the non-uniform temporal and/or spatial distribution of ^{15}N at artificial or natural abundance levels can have a profound effect on estimates of P_{atm}, resulting in negative estimates or a marked reference plant dependency. Thus, while Doughton at al. (1995) found close agreement between *NA* and *E* estimates of symbiotic dependence in chickpea at P_{atm}>30 %, the *E* method gave impossible negative values when P_{atm} was in the lower range, whereas the *NA* method provided realistic estimates over the whole range. Chalk (1985) similarly reviewed several studies where negative estimates of P_{atm} were obtained with the *E* method.

The *NA* method was reported to yield negative estimates in white clover – perennial ryegrass swards under grazing (Hansen and Vinther 2001), where the variation of δ^{15}N in the grass varied from −7.0 to +5.7 ‰, most likely due to the random distribution of ^{15}N-depleted urinary N voided on the pasture. Therefore, this result could be considered as atypical of estimates normally obtained by the *NA* technique in the absence of the confounding effect of grazing. However, negative estimates in some treatments were previously reported for the *NA* method (Amarger et al. 1979).

Spatial variability in ^{15}N abundance

Spatial (horizontal and vertical) variability in ^{15}N abundance should be negligible in pots of well-mixed soil, so any variation in estimates of P_{atm} should be due to temporal non-uniformity in the distribution of ^{15}N. In a pot experiment reported by Ledgard et al. (1985 b) estimates of P_{atm} for sub clover using two reference plants, annual ryegrass and *Phalaris*, which were sampled at 16 and 32 days after sowing (DAS) were

Table 1 Estimates of symbiotic dependence (P_{atm}) of grain legumes obtained by *E* and *NA* techniques

Reference (chronological)	N_2 fixing plant[a]	Reference plant[b]	Scale[c]	Plant		$P_{atm}\times100$ (%)[e]		B value		Reference plant / ‰
				Part[d]	Age (d)	*E*	*NA*	‰	Method[j]	
Evans et al. (1987)	Lupin	Wheat	F	T	128,193	78, 85	88, 81	−0.5	1	+3.8–+4.1
Ofori et al. (1987)	Cowpea	Maize	P	G, G+S	120	41, 36	49, 47	+0.2–+0.3	1	+2.4–+4.1
			F		150	57, 53	68, 69			+5.0–+7.6
Bremer and van Kessel (1990)	Lentil	Several	F, S	G	Maturity	35–81[g]	28–80[g]	0–+0.5	1	+4.5–+8.5
Tobita et al. (1994)	Pigeonpea	Sorghum	F	G	Maturity	54–81	44–61	−1.6	1	+5.0
Androsoff et al. (1995)	Field pea	Canola	F, S	G	91	50[h]	45[h]	+0.9	1	+6.7
Stevenson et al. (1995)	Field pea	Canola	F, S	G+S	Flowering	**92 (1)** *84 (1)*	**72 (3)** *70 (2)*	+0.3	2	+11.0
					Maturity	**84 (2)** *72 (2)*	**83 (2)** *84 (2)*			
Doughton et al. (1995)	Chickpea	Barley	F	T	130	40–60[i]	40–60[i]	−2.1	1	+6.1
Cadisch et al. (2000)	Peanut 1995	Peanut $_{(nn)}$,	F	T+R	Maturity	46 (4)	53 (4)	−1.8–−1.0	1	+12.1, +9.8
	1996	Maize				23 (6)	44 (5)			
		Peanut $_{(nn)}$,				33 (3)	21 (4)			
		Maize				32 (4)	16 (9)			
Oberson et al. (2007)	Soybean	Weeds[f]	F	T+R	104	15–36	15–46	−1.2	1	+3.2–+4.9
					145	24–44	31–54	−0.9–−0.5		+4.0–+6.5

[a] Lupin, *Lupinus angustifolius*; Cowpea, *Vigna unguiculata*; Lentil, *Lens culinaris*; Pigeonpea, *Cajanus cajan*; Field pea, *Pisum sativum*; Chickpea, *Cicer arietinum*; Peanut (groundnut), *Arachis hypogaea*; Soybean, *Glycine max*

[b] Wheat, *Triticum aestivum*; Maize, *Zea mays*; Several, wheat, linseed (flax), *Linum usitatissimum*, uninoculated lentil, barley, *Hordeum vulgare*; Sorghum, *Sorghum bicolor*; Canola, *Brassica napus*; Peanut $_{(nn)}$, non-nodulating peanut

[c] F, field plot; S, spatial variability across the landscape; P, Pot

[d] T, tops (leaves+stems+grain or pods); G, grain; R, roots; S, stover

[e] Data in parentheses are standard errors of the mean; data in bold are landform shoulders; data in italics are landform footslopes

[f] 16 species across 14 genera for the *NA* method; 2 species for the *E* method

[g] Estimates of P_{atm} were not significantly different between *E* and *NA* in 18 out of 21 comparisons

[h] Median values; no correlation between paired *E* and *NA* values across the landscape

[i] Treatments of≤100 kg NO_3^--N at crop establishment

[j] Method: 1, N-free medium; 2, Doughton et al. 1992

Mean values separated by a hyphen indicate the range and when separated by a comma may indicate two different reference plants, two different harvests, or two different plant parts (e.g., grain or grain+stover) as indicated within the line

Table 2 Estimates of symbiotic dependence (P_{atm}) of pasture and forage legumes obtained by *E* and *NA* techniques

Reference (chronological)	N_2 fixing plant[a]	Reference plant[b]	Scale[c]	Plant		$P_{atm}\times100$ (%)		B value		Reference plant / ‰
				Part[d]	Age (d)	*E*	*NA*	‰	Method[h]	
Bergersen and Turner (1983)	Sub clover	Perennial ryegrass	F	T	71, 191	92, 97	73, 94	+1.3	1	+16.4, +14.8
Ledgard et al. (1985 a)	Sub clover	AnnRye., Phalaris	F	T	25	70, 50	85, 86	+0.6	1	+4.5, +4.3
	Lucerne	AnnRye., Phalaris				88, 70	81, 64	+1.1		+4.5, +4.5
Ledgard et al. (1985 b)	Sub clover	AnnRye., Phalaris	P	T	16	65, 20	72, 50	+0.6	1	+23.8, +22.5
					32	75, 60	84, 64			+23.8, +22.5
Høgh-Jensen and Schjoerring (1994)	White clover + red clover	Perennial ryegrass	F	T	Cut 3 y^{-1}	50–94	51–78	0.0	5	+4.9
					Cut 6 y^{-1}	64–96	59–73			+5.9
Hossain et al. (1995)	Medic, Luc	Mixture	F	T	1 y	93[e], 82[e]	69[e], 69[e]	−2.0, −3.0	1	+7.4, +9.7
Brendel et al. (1997)	Red clover	Perennial ryegrass	P	T+R	63	58–73	62	−1.0	4	–
Carranca et al. (1999)	SC 1992/93	Perennial ryegrass	F	T	Cut 3 y^{-1}	37	51	−1.1	1	–
	1993/94	+ cocksfoot			Cut 4 y^{-1}	46	46			
Jacot et al. (2000)	9 species	3–9 (*E*), 4–7 (*NA*)	F	T	Cut 1–3 y^{-1}	73–87[f]	56–87[f]	0 or −1.0	5	−1.0–+5.3
Hansen and Vinther (2001)	White clover	Perennial ryegrass	F, S	T	Cut 2–3 y^{-1}	90[f]	70–87[f]	−2.1–−2.6	3	−7.0–+5.7
Huss-Danell and Chaia (2005)	Red clover	Timothy+fescue	F	T+R	34–52	99[g]	95[g]	−0.8	3, 4	+4.7–+6.5
Huss-Danell et al. (2007)	Red clover	Grasses	F	T+R	52	95[g]	75[g]	−1.0	1	+5.5–+7.4
Burchill et al. (2014)	White clover	Perennial ryegrass	F	T	Cut 6 2011	82–42[i]	82–39[i]	−1.1–−1.6,	1, 3, 5	+1.6–+8.7
					Cut 7 2012	82–64[i]	90–71[i]			

[a] Sub clover, *Trifolium subterraneum*; Lucerne (alfalfa), *Medicago sativa*; White clover, *T. repens*; Red clover, *T. pratense*; Medic (snail medic, *M. scutellata*+barrel medic, *M. truncatula*); Luc, Lucerne; SC, sub clover

[b] Perennial ryegrass, *Lolium perenne*; AnnRye., annual ryegrass, *L. rigidum*; Phalaris, *Phalaris aquatica*; Mixture (purple pigeon grass, *Setaria incrassata*+Rhodes grass, *Chloris gayana*); Cocksfoot, *Dactylis glomerata;* Timothy, *Phleum pratense*; Fescue, *Festuca pratensis*

[c] P, pot; F, field plot; S, spatial variability

[d] T, tops (leaves+stems); R, roots

[e] 1988 results

[f] 1998 results

[g] Weighted mean of shoots+roots at August harvest

[h] Method: 1, N-free medium; 3, Apparent value; 4, from the published literature; 5, assumed value

[i] Range of values from 0 to 280 kg N ha^{-1}

–, not reported; mean values separated by a hyphen indicate the range and when separated by a comma may indicate two different legumes, two different reference plants, or two different harvests as indicated within the line

Table 3 Estimates of symbiotic dependence (P_{atm}) of woody perennials obtained by *E* and *NA* techniques

Reference (chronological)	N_2 fixing plant[a]	Reference plant[b]	Scale[c]	Plant		$P_{atm}\times100$ (%)		B value		Reference plant / ‰
				Part[d]	Age / mo	*E*	*NA*	‰	Method[e]	
Domenach et al. (1989)	Alder	White poplar	P	L	12	97±14	110±20	−1.9	4	+3.6
Kurdali et al. (1990)	Alder	Black poplar	P	T+R	5–6	74±30	43±17	−1.9	4	+0.8
Peoples et al. (1996)	Calliandra Gliricidia	Senna	F	T-regular pruning	16	40±3 49±3	24±2 56±2	−1.3 −1.5	1 1	+3.8
	Calliandra Gliricidia				30	58±4 67±5	56±4 61±2			+4.4
Hairiah et al. (2000)	Flemingia Gliricidia	Yellow batai	H		20	32±4 55±4	24±11 37±12	−1.1 −1.3	1	–
Bouillet et al. (2008)	Acacia	Eucalyptus	F	T	30	58	14	−0.3	4	+0.8 to +2.2
Issah et al. (2014)	Caragana Sea buckthorn	Chokecherry	P	T+R	5	65 73	59 70	0.0	5	–

[a] Alder, *Alnus glutinosa*; Calliandra, *Calliandra calothyrsus*; Gliricidia, *Gliricidia sepium*; Flemingia, *Flemingia congesta*; Acacia, *Acacia mangium*; Caragana, *Caragana arborescens;* Sea buckthorn, *Hippophae rhamanoides*

[b] White poplar, *Populus alba*; Black poplar, *Populus nigra*; Senna, *Senna spectabilis*; *Yellow batai, Peltophorum dasyrachis*; Weeds, *Echinochloa colona*; *Erigeron philadelphicus*, *Cyperus esculantus* (6 months); *Rumex japonicus*, *E. colona*, *Commelina diffusa* (7 months); Eucalyptus, *Eucalyptus grandis*; Chokecherry, *Prunus virgiana*

[c] P, pot; F, field plot; H, hedgerows in field

[d] L, leaves; T, tops (leaves+stems); R, roots

[e] Method: 1, N-free medium; 4, from the published literature; 5, assumed value

–, not reported

strongly reference plant dependent for both *E* and *NA* methods at each sampling time (Table 2). Therefore in the presumed absence of spatial variability, these pot experiment results suggest that both *E* and *NA* methods were affected by temporal ^{15}N variability. Indeed, Feigin et al. (1974) demonstrated that the $\delta^{15}N$ values of NO_3^- released from four Illinois soils increased during the first 35 days of incubation before becoming constant with time.

In experiments conducted in field plots, estimates of P_{atm} for peanuts (Cadisch et al. 2000), sub clover and lucerne (Ledgard et al. 1985 a) were similarly reference plant dependent for both *E* and *NA* methods (Tables 1 and 2, respectively). Cadisch et al. (2000) found significant variation in the distribution of $\delta^{15}N$ of total N in *E* and *NA* plots between 0–10, 10–20 and 20–30 cm depth, with decreasing values for *E* and increasing values for *NA* (+5.7, +7.0 and +9.2 ‰, respectively). However, there was no significant depth difference in the $\delta^{15}N$ signature of mineral N released during incubation of *NA* soil samples for 21 days. Huss-Danell and Chaia (2005) similarly reported increasing $\delta^{15}N$ values for total N between 0–20 and 20–30 cm from +4 to +7 ‰.

In several studies, samples were taken during crop development and at maturity, or several cuts were taken from pastures during the growing season (Tables 1 and 2). The $\delta^{15}N$ value of both grain and pasture legumes and reference species exhibit a marked seasonal variation (Pate et al. 1994), but in this study the legumes always had lower $\delta^{15}N$ values than the reference plants. However, seasonal trends were not consistent among reference plants. Agreement between *E* and *NA* estimates tended to improve with plant age, in line with a concomitant increase in symbiotic dependence (e.g., Bergersen and Turner 1983; Evans et al. 1987; Peoples et al. 1996). When experiments were conducted in successive years (e.g., Cadisch et al. 2000; Carranca et al. 1999) agreement between *E* and *NA* techniques were inconsistent.

Lateral variability in ^{15}N abundance

Lateral variability in $\delta^{15}N$ signatures has been studied at different scales ranging from the experimental site (Oberson et al. 2007) to landscapes (Bremer and van Kessel 1990; Androsoff et al. 1995; Stevenson et al. 1995) to regions (Pate et al. 1994). Oberson et al. (2007) found large variations in the $\delta^{15}N$ values of 16 reference weed species across the experimental treatments in their legume-based sward, which

ranged from +2.6±0.2 ‰ to +8.1±3.3 ‰. On the other hand, Pate et al. (1994) found less variation at the regional scale encompassing a range of agricultural ecosystems across south-west Australia, with the δ^{15}Nvalue of a more restricted number of weed species varying from +2 to +5 ‰.

At the landscape scale, Bremer and van Kessel (1990) found that variability in δ^{15}N of reference plants was site and season dependent. Seasonal patterns among six reference plants were inconsistent, in agreement with the finding of Pate et al. (1994). The variability of δ^{15}N among reference plants differed between three sites with the greatest range at one site being from +2.8 to +9.3 ‰ over a distance of 67 m. At one site, the δ values of pea and flax were well separated across horizontal distance with the value for pea always less then flax, but at two other sites the δ values of pea and flax or lentil and wheat were not well separated and some crossover occurred, which would give erroneous negative estimates of P_{atm} at some individual sampling points. However, Bremer and van Kessel (1990) found that mean *E* and *NA* estimates of P_{atm} for field pea and lentil were not significantly different in 18 out of 21 comparisons despite the site and seasonal dependency of reference plant δ^{15}N.

Additional studies with field pea were conducted in the same rolling (undulating) landscape by Androsoff et al. (1995) and Stevenson et al. (1995). On a 90×100 m sampling grid with 10 m spacing, Stevenson et al. (1995) found poor agreement between *E* and *NA* estimates of P_{atm} at flowering at both landform footslopes and shoulder positions, while at maturity agreement between the two methods was only close in the shoulder position (Table 1). Both Stevenson et al. (1995) and Androsoff et al. (1995) found no correlation between individual *E* and *NA* estimates of P_{atm} across the landscape, and concluded that while symbiotic dependence of field pea was partly controlled by topography due to the divergent availability of water and mineral N, other unspecified factors operating at the plot scale (i.e., within 3 m) exerted a stronger influence. In an earlier microcosm experiment in the glasshouse, Brendel et al. (1997) also found that there was no correlation between individual estimates of P_{atm} of red clover when *E* treatments were imposed at two levels of ^{15}N abundance (atom fraction ^{15}N of 0.005 and 0.05) and one ^{15}N depleted level (−16.5 ‰).

Determination of the B-value

In all but one of the studies with grain legumes the B-value was determined by growing the legume in an N-free medium (Table 1). In contrast, in several of the studies with pasture and forage legumes (Table 2) and with woody perennials (Table 3), the B-value was not determined in the same way, but was either an apparent value, a value taken from the published literature or an assumed value. There is therefore a degree of uncertainty with regard to the efficacy of the B-values in such studies, in contrast to the B-values determined directly for the grain legumes.

Several studies have shown that B-values are dependent on the rhizobial strain used as the inoculum in the N-free medium. e.g., groundnut (Cadisch et al. 2000) and soybean (Pauferro et al. 2010). Since this finding is generally applicable to other legumes, it could pose problems, particularly for pasture legumes, where multiple strains could infect the host plant. This is perhaps a further reason for inconsistent agreement between *E* and *NA* techniques for pasture and forage legumes. In an attempt to circumvent this problem, Unkovich et al. (2008) recommend that a mixed or soil inocula should be used to determine the B-value if the infecting strains are unknown.

The B-value depends on the particular part of the plant that is sampled (Cadisch et al. 2000; Huss-Danell et al. 2007). B-values are usually determined on the shoot material which may or may not be grown to maturity. Therefore the estimated isotopic fractionation may not necessarily correspond to the actual fractionation that occurs if the legume is grown for a different period of time or if a different plant sample is collected. As can be seen in Tables 1, 2 and 3, there is considerable variation in these parameters among the published data.

The reference plant δ-value

According to Unkovich et al. (2008) the reference plant exerts a strong influence on estimates of P_{atm} when δ^{15}N is <4 ‰, but does not have a large effect if δ^{15}N is >6 ‰. Since the relationship between $\delta^{15}N_{legume}$ and P_{atm} is linear for a given $\delta^{15}N_{reference\ plant}$, the sensitivity of the final estimate will also be proportional to the $\delta^{15}N_{reference\ plant}$ (Unkovich and Pate 2000). The higher the δ^{15}N value of the reference plant, the more precise the estimate of P_{atm} will be. Unkovich et al. (1994)

suggested that, given the analytical precision of±0.2 ‰, a reference $\delta^{15}N$ of at least 2 ‰ (about 10 times the precision of measurement) would be required to detect a theoretical change in P_{atm} of around 10 %. Therefore we can assume that estimates of P_{atm} for grain legumes were more precise overall than those of the other categories, as no values of $\delta^{15}N_{reference\ plant}$ were<2 ‰, whereas several $\delta^{15}N_{reference\ plant}$ values for the other groups were below this value, and in several cases the reference plant δ values were not given (Tables 2 and 3).

One problem when comparing *E* and *NA* techniques in the same experiment is the possibility of cross contamination if labelled treatments are randomized with unlabelled treatments. Brendel et al. (1997) recognized this possibility and separated the individual ^{15}N treatments within different compartments of the same glasshouse. However, it appears that cross contamination may have been a factor in some experiments, as Ledgard et al. (1985 b) reported reference plant δ values of +22.5 to +23.8 in the *NA* treatment in a pot experiment involving subclover-annual ryegrass and subclover-Phalaris associations, values well outside the normal range expected in plants grown in unlabelled soil. Somado and Kuehne (2006) also found that $\delta^{15}N$ values of the tops and roots of the reference plant (rice) in the *NA* treatment pots randomized with the *E* treatment pots fell within the atypical range of +12.7 to +26.4 ‰ when estimating P_{atm} for a green manure legume (*Aeschynomene afraspera*). Similarly, the reference plant δ values reported by Bergersen and Turner (1983) in the *NA* treatment also fell outside the expected range in a field study of a subclover-ryegrass sward on a lower footslope position that included confined *E* treatment microplots (Table 2). These results suggest that estimates of P_{atm} using the *NA* technique should be treated with caution when reference plant δ values are atypical.

Statistical analysis

Correlations between estimates of P_{atm} using *E* and *NA* techniques

Significant correlations were found when estimates of P_{atm} were compared between *E* and *NA* methods for

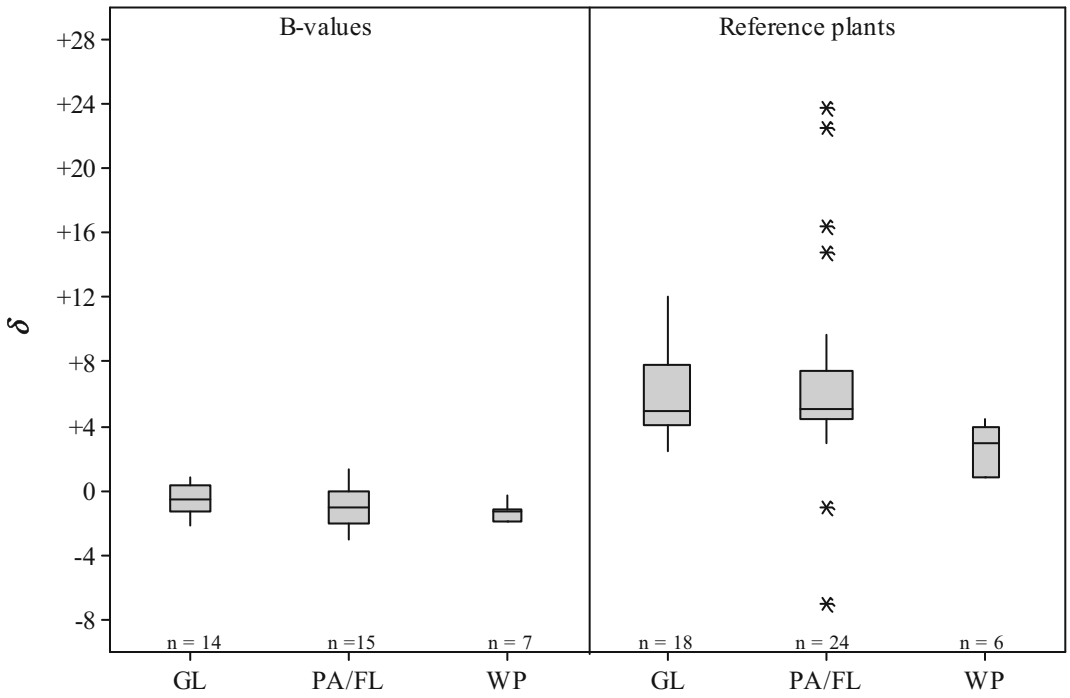

Fig. 1 Box-whisker plots of B-values and reference plant δ-values ($\delta^{15}N$ / ‰) reported in the literature for the ^{15}N natural abundance (*NA*) method (*GL* Grain legumes; *PA/FL* Pasture and forage legumes; *WP* Woody perennials). Outliers are denoted by *. *n*= number of observations

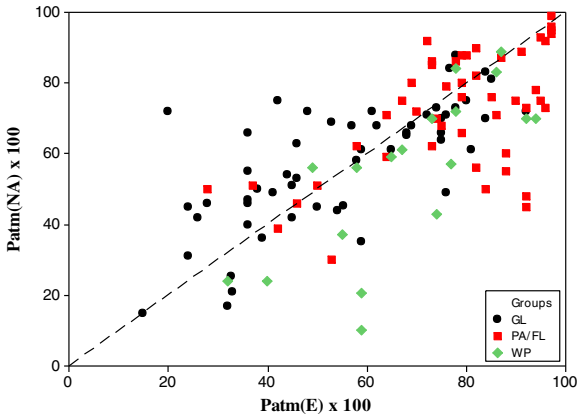

Fig. 2 Scatter plot of $P_{atm\ (E)}$ vs. $P_{atm\ (NA)}$. The *dashed line* represents the hypothetical boundary along which estimates are equal (i.e., where $P_{atm\ (E)}=P_{atm\ (NA)}$). The dispersion of points around the line shows the magnitude of the discrepancy between the estimates. *GL* grain legumes; *PA/FL* pasture and forage legumes; *WP* woody perennials

each category of N_2-fixing species (grain and pasture/forage legumes) and woody perennials, and for all categories combined. The data used for these comparisons were taken from individual treatments within each publication rather than means or selected treatments as shown in Tables 1, 2 and 3. The correlations were higher for grain legumes (r=0.72, p<0.001, n=54) and woody perennials (r=0.76, p<0.001, n=18) than for pasture and forage legumes (r=0.45, p<0.004, n=40). These significant correlations obtained from published data contrast with data obtained in some studies where the individual estimates of P_{atm} obtained by *E* and *NA* methods were not correlated (e.g., Stevenson et al. 1995; Androsoff et al. 1995; Brendel et al. 1997).

Apart from the dependency of P_{atm} on the reference plant per se for both methods due to the non-uniform temporal and spatial distribution of ^{15}N as discussed previously, there are other possible reasons for inconsistencies between the two methodologies, which relate to the determination of the B-value and the reference plant δ-value for the *NA* technique.

Variability of B-values and reference plant δ-values

Box-plots show that the variability of reference plant δ-values was much larger compared to the variability of B-values in the published data (Fig. 1). For example, Ofori et al. (1987) found that the B-values of cowpea varied from +0.2 to +0.3 ‰, while the maize reference plant δ-values ranged from +5.0 to +7.5 ‰ in a field study and from +2.4 to +4.1 ‰ in a glasshouse experiment. Similarly, Oberson et al. (2007) showed the δ-values of weeds used as reference plants ranged from +3.2± 0.4 to +6.5±1.5 ‰ in different treatments (cropping system and growth stage), while B-values of soybean varied from only −1.2 to −0.9 ‰ at flowering and maturity, respectively.

Data for pasture and forage legumes show higher variability of B-values and reference plant δ-values than grain legumes and woody perennials, including the outliers (Fig. 1). This high variability for δ-values may partly explain the weaker correlation of *E* and *NA* estimations for pasture/forage legumes than for grain legumes and woody perennials. Therefore, we believe that the impact of the reference plant δ-value on estimates of P_{atm} by the *NA*

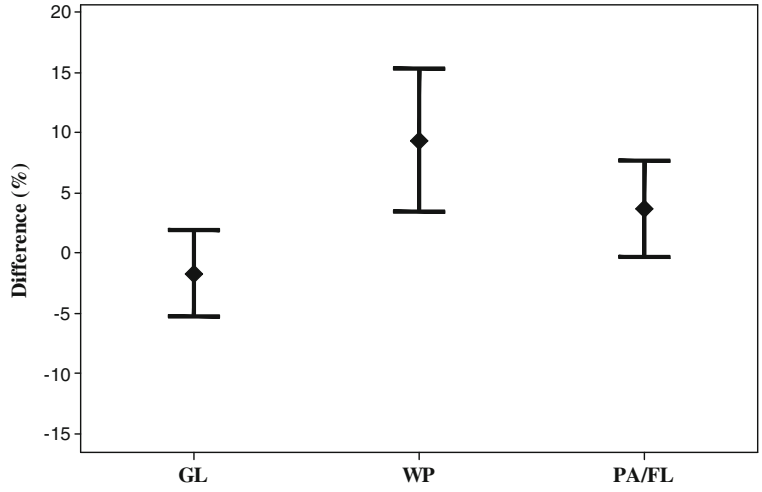

Fig. 3 Means and 95 % confidence intervals of the differences $P_{atm(E)}-P_{atm(NA)}$ for each group of species. *GL* grain legumes; *WP* woody perennials; *PA/FL* pasture and forage legumes

Table 4 Means ($\bar{x}$), standard deviation (SD) and the estimated probabilities by groups of species that the differences $P_{atm(E)}-P_{atm(NA)}$ will be within the ranges of −5 to +5 % or −10 to +10 %

Data set	n[a]	$\bar{x}$(%) $P_{atm(E)}-P_{atm(NA)}$	SD	Probability $P_{atm(E)}-P_{atm(NA)}$	
				−5 to +5 %	−10 to +10 %
Grain legumes	53	−1.68	12.99	0.30	0.55
Pasture/forage legumes	46	3.65	13.39	0.28	0.53
Woody perennials	16	9.38	11.19	0.25	0.48
All	115	1.99	13.39	0.29	0.54

[a] Outliers were removed

technique may be greater than expected due to the large variability found for this parameter in the published data, although in theory the impact of the B-value should be higher than that of the reference plant δ-value when P_{atm} is >60 % (Unkovich et al. 2008).

Consistency in the estimates of P_{atm}

A scatter plot of $P_{atm(NA)}$ vs. $P_{atm(E)}$ is shown in Fig. 2. It appears that there is a tendency for $P_{atm(NA)}$ to give lower values than $P_{atm(E)}$ at high values of symbiotic dependence (i.e., when $P_{atm(E)}$>60 %), and higher values than $P_{atm(E)}$ when $P_{atm(E)}$<60 %), except for woody perennials that show $P_{atm(E)}>P_{atm(NA)}$ in 15 out of 18 comparisons (Fig. 2).

When the outliers were removed, the differences in $P_{atm(E)} - P_{atm(NA)}$ were found to be within the range from −30.0 to +34.0 %, with 50 % of data from −7.0 to +10.3 %, and the median equal to 2.0 % (normal distribution, Anderson-Darling test, p=0.134). Means and 95 % confidence intervals of the differences in $P_{atm(E)}-P_{atm(NA)}$ for each group of species are shown in Fig. 3. The mean difference for the grain legumes was significantly less (t-test, p<0.05) than for the other groups, but there was no significant difference between woody perennials and pasture/forage legumes. Therefore estimates of $P_{atm(E)}$ and $P_{atm(NA)}$ for grain legumes were more consistent than estimates obtained for the other groups (Fig. 3). For the standardized normal distribution of data for all species combined the probability that the methods gave similar estimates within an arbitrarily selected range of $P_{atm(E)}-P_{atm(NA)}$ of −5 to +5 % was 0.29, while the corresponding probability within the range of −10 to +10 % was 0.54 (Table 4).

Conclusions

On the basis of our examination of the published literature we believe that the methods do not provide consistent estimates of the proportional dependence of N_2-fixing species on biological N_2 fixation over a range of scales and settings. The reasons for the generally poor agreement overall are complex and may include one or more of the following factors: (i) non-uniform temporal and spatial distribution of the ^{14}N and ^{15}N isotopes (ii) asynchrony of mineral N uptake by legume and reference plants (iii) error in the estimation of the B-value (iv) insufficient difference in δ values between the atmosphere and soil available N (v) cross contamination between *E* and *NA* treatments. The limited observations neither contradict nor support the often-stated hypothesis that *NA* should be influenced less by the non-uniform distribution of ^{15}N compared with *E*. While it is possible to identify potential reasons for discrepancies in individual studies, in many cases it is not possible because essential data on B-values, reference plant δ values and the spatial/temporal distribution of the N isotopes were not provided.

The choice of which method to use will ultimately depend on practical considerations such as the cost of ^{15}N-enriched fertilizer, the scale of the experiment, the analytical and instrumental facilities available, and the work required to determine the B-value. Pauferro et al. (2010) and Oberson et al. (2007) considered that *NA* is the most easily applied 'on farm' technique. Oberson et al. (2007) also commented that *NA* was better than *E* for the determination of the amount of N_2 fixed due to restriction of root growth by the laterally-confined microplots often used for the *E* technique. A practical guide for the application of both techniques can be found in Unkovich et al. (2008).

The overall problem of the dependency of estimates of P_{atm} on the reference plant for both *E* and *NA* methods can only be overcome by seeking a way to discard the reference plant altogether. Two approaches have been proposed to make the reference plant redundant. For the *E* method the temporal decline in the ^{15}N enrichment of available N in the topsoil can be accommodated by fitting an exponential equation to the experimental data, which provides an integrated estimate of the ^{15}N enrichment of the available N pool over the measurement period (Chalk et al. 1996). For the *NA* method Wanek and Arndt (2002) demonstrated experimentally that the difference ($\Delta^{15}N$) between the $\delta^{15}N$ values of the shoot and nodulated roots (i.e., $\Delta^{15}N = \delta^{15}N_{shoot} - \delta^{15}N_{nodulated\ root}$) was linearly and highly correlated with reference plant estimates of P_{atm} of soybean in solution and soil (pot) culture. Furthermore, they showed similar significant relationships for published data for soybean at different growth stages under glasshouse or field conditions, for different cowpea cultivars in the field and for tagasaste in hydroponic culture. The authors claimed that this approach overcomes the problem with the *NA* method when the relative ^{15}N abundance of soil mineral N is close to zero. Both of these reference-plant-free approaches represent conceptual advances in response to the reference plant dilemma, aptly described by Chalk and Ladha (1999) as the 'Achilles heel' of the ^{15}N methodology. However, more field testing is required before the potential of these alternative methodologies can be confidently assessed.

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