



Dynamic species-specific metabolic changes in the trees exposed to chronic N+S additions at the Bear Brook Watershed in Maine, USA

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Abstract

• **Key message** Red spruce (*Picea rubens* Sarg.) and American beech (*Fagus grandifolia* Ehrh.) nutritional imbalances observed during 1998–2000 in response to nitrogen additions beginning in 1989 at Bear Brook Watershed in Maine, USA, were reversed by 2013. However, nitrogen-containing metabolites continued to accumulate to detoxify ammonia. While sugar maple (*Acer saccharum* Marsh.) was N-limited and benefitted from N+S additions, spruce and birch established new homeostatic status via adjusting cellular metabolism.

• **Context** Increased deposition of atmospheric N leads to changes in forest productivity. Effects of added N+S on changes in cellular metabolism will yield information on species-specific sensitivity to N+S.

• **Aims** To evaluate foliar metabolic changes in American beech (*Fagus grandifolia* Ehrh.), sugar maple (*Acer saccharum* Marsh.), and red spruce (*Picea rubens* Sarg.) that were exposed to ammonium sulfate [(NH₄)₂SO₄]; ~28.8 kg S ha⁻¹ yr⁻¹ and 25.2 kg N ha⁻¹ yr⁻¹] additions at West Bear Watershed (WBW) starting in 1989 until the end of this experiment, while East Bear Watershed served as a reference.

• **Methods** Foliage was collected in 1998–2000 and 2013. Sapwood plugs were also collected in 2013. All were analyzed for ions and metabolites using HPLC and ICP.

• **Results** During 1998–2000, only N+S-treated beech and spruce foliage had a reduction in Ca and Mg. All species had significantly higher content of N-rich metabolites. In 2013, ammonia detoxification continued in the absence of nutrient deficiencies. Significant changes in growth promoting metabolites occurred only in maple throughout this study.

• **Conclusion** Metabolic changes indicated that sugar maple at this site was and still is N-limited, whereas red spruce and American beech had to make metabolic adjustments in order to survive under chronic N+S inputs. We conclude that even in the absence of knowledge about individual species tolerance limits for nutrients and critical N load for the site, monitoring with a suite of metabolites that are centrally connected to both C and N pathways could be a very useful tool in assessing stress from nutrient imbalance in various tree species.

Keywords American beech · Bear brook watershed in Maine · Foliar metabolism · Nitrogen plus sulfur addition · Red spruce · Sugar maple

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Abbreviations

FAAs	free amino acids
BAI	basal area increment
BBWM	Bear Brook Watershed in Maine
EBW	East Bear Watershed
PAs	Polyamines
Put	Putrescine
Spd	Spermidine
Spm	Spermine
WBW	West Bear Watershed

1 Introduction

Increased deposition of atmospheric nitrogen (N) from anthropogenic sources has been implicated in changes in forest ecosystem functions and productivity in parts of the northeastern USA and Europe (Fernandez et al. 2010; Minocha et al. 2015; Quinn et al. 2010; van Diepen et al. 2015; Vitousek et al. 1997). Acid deposition enters these ecosystems as clouds, gases, rain, fog, and snow that contain N and sulfur (S) in the form of nitric and sulfuric acids, respectively (Driscoll et al. 2003). This leads to the acidification of soil and surface waters, and increases the amount of plant available total N, both of which can impact critical ecosystems functions. Depending upon the initial soil N status, land use history, species composition, and the rate and duration of N deposition to forests, effects may be positive (Hogberg 2007; Lloyd 1999; Quinn et al. 2010) or negative (Bauer et al. 2004; Fernandez et al. 2010; Minocha et al. 2015 and references therein; Wallace et al. 2007). Although most temperate forests are N-limited, chronic exposure to N deposition could alter the ratio of essential nutrients and ultimately drive these ecosystems towards N saturation, leading to a multitude of harmful consequences for forest health (Aber et al. 1998; Frey et al. 2014). Surplus N can also occur under conditions when other nutrients are limiting and/or when plants cannot utilize the available N because other environmental conditions for growth are not optimum (Näsholm et al. 2000). Availability of surplus N may influence competitive relationships among different species within a forest (Elvir et al. 2006; Quinn et al. 2010). At the cellular level, excess N taken up by the leaves and roots is converted into amines and amides with the simultaneous release of hydrogen ions or organic acids, and in the process soluble carbohydrates are consumed (Nihlgård 1985). Toxic excess N-related wastes that cannot be excreted during dry periods accumulate in the leaves; premature leaf drop may be a mechanism to dispose of this waste (van Diepen et al. 2015).

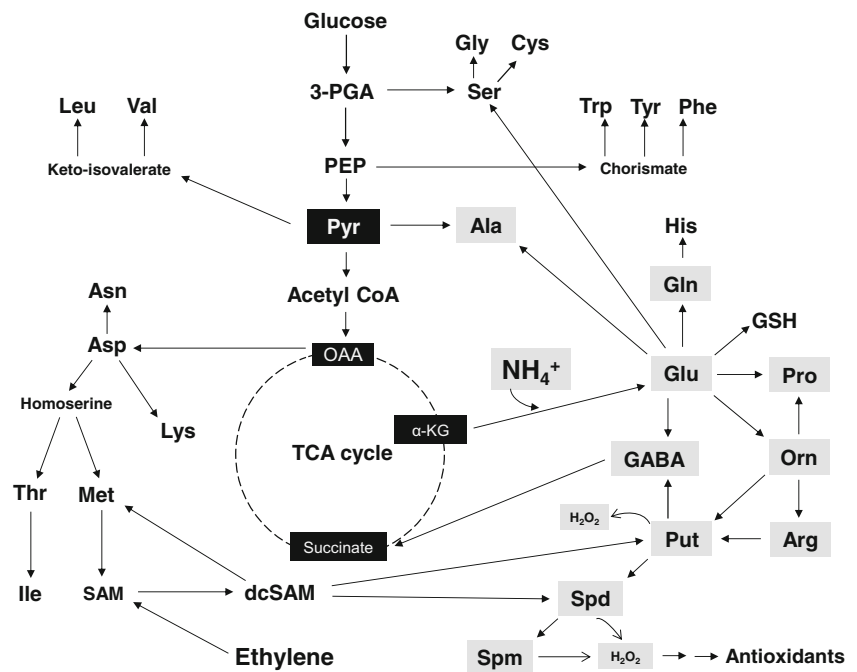
Acidity associated with acid deposition mobilizes aluminum (Al) which in turn competes with Ca and Mg at soil cation exchange sites (Shortle and Smith 1988). This accelerates cation leaching which leads to reduced base cation availability (Fernandez et al. 2003). If Ca and Mg are displaced from soil

at a faster rate than their replenishment through weathering or atmospheric deposition, this can also add to base cation depletion (Driscoll et al. 2003; Johnson and Fernandez 1992). Insufficient Ca is known to predispose trees to disease and pathogen infection, and can increase susceptibility to freezing injury as seen in American beech (*Fagus grandifolia* Ehrh.), red spruce (*Picea rubens* Sarg.), and sugar maple (*Acer saccharum* Marsh (DeHayes et al. 1999; Hallett et al. 2006; Long et al. 1997; Schaberg et al. 2011; Schaberg et al. 2001). A combination of one or more of the abovementioned factors leads to measureable changes in plant metabolism that could be used as indicators of stress from change(s) in nutrient status over time (Minocha et al. 2000, 2010, 2015).

In physiologically stressed trees, changes in metabolite concentrations often occur before any visual symptoms become evident. Hence, measurement of these metabolic changes may serve as indicators of overall health of a species of concern at the time of sampling of forest stands. Common metabolites such as cellular polyamines (PAs) [putrescine (Put) and spermidine (Spd)] and amino acids (AAs) [arginine (Arg), γ -aminobutyric acid (GABA), and glutamine (Gln)] play significant roles in the growth and development of all living organisms but can also act as metabolic sinks for excess N (Alcázar et al. 2006; Ericsson et al. 1993). Organic acids, free PAs and AAs have long been used as indicators of various kinds of biotic and abiotic stress; e.g., Ca depletion, high N, high Al, heavy metal toxicity (Minocha and Long 2004a; Minocha et al. 2010, 2015; Näsholm et al. 1997; Pinchot et al. 2017; Thangavel et al. 2007). Changes in N metabolism are intrinsically connected with changes in C metabolism (Fig. 1). Changes in concentrations of chlorophyll and cellular soluble proteins have also been used to indicate environmental stress (Guy 1990; Lichtenthaler and Rinderle 1988a; Lichtenthaler and Rinderle 1988b; Parida and Das 2005; Parvaiz and Satyawati 2008).

Established in 1989, the present study is part of an ongoing long-term experiment at the Bear Brook Watershed in Maine (BBWM) to assess the effects of acid deposition and N+S enrichment from ammonium sulfate additions on the response and recovery of whole ecosystem on a decadal time scale. Soil chemistry and stream chemistry have been previously described for this study (Fernandez et al. 2010; SanClements et al. 2010). Jefts et al. (2004) found that after 12 years of treatment, continued accumulation of N at West Bear Watershed (WBW) led to higher N cycling rates (higher net N mineralization and nitrification) than in the reference East Bear Watershed (EBW), which in turn resulted in higher annual release of N into stream water in both mixed hardwood and softwood stands. Other reports from this site have shown that increased export of Ca and Mg into stream water was the initial response in the N+S-treated WBW; this was the result of anion adsorption (primarily SO_4^{2-}), base cation desorption (mainly Ca and Mg), and Al desorption and mobilization (Fernandez et al. 2010). Elvir et al. (2005) showed reduced

Fig. 1 Role of glutamic acid as a precursor of biosynthesis of polyamines and amino acids (modified from (Mohapatra et al. 2010)). Metabolites in polyamine and amino acids pathways connected directly or indirectly with their synthesis and/or catabolism are shown in gray boxes while metabolites that connect C and N pathways are shown in black boxes. Although solid arrows are drawn between metabolites, most reactions are multistep



amounts of total Ca and Mg in the foliage of red spruce and American beech from WBW compared to EBW.

The specific objectives of the present study were to 1) determine the effects of decades of (NH₄)₂SO₄ additions on changes in foliar and sapwood soluble inorganic nutrients and N-related metabolites in American beech, sugar maple, and red spruce; and 2) establish, for each species, if there is a relationship between the observed metabolic changes and tree vigor and productivity reported by other researchers during this time. We hypothesized that the effects of chronic N+S additions on soluble nutrition and metabolites will not only be species-specific but will also be modulated/readjusted over time in order to attain a different homeostatic or steady state. Cellular homeostasis as stated here means the tendency of individual cells to regulate its internal chemical processes in order to maintain its health and functions under a given environment. On the other hand, an organism's homeostasis may require sacrificing functioning of non-vital parts for the survival of vital parts, e.g., under nutrient deficiency, leaves may turn yellow and die because their energy is needed by stem and roots. We also hypothesized that these changes will reflect a unique metabolic state for each species at the time of sampling. The present study focuses mainly on the effects of N+S additions on the N metabolism.

2 Materials and methods

2.1 Site description

The BBWM is located in eastern Maine (44° 52' 15" N, 68° 06' 25" W) approximately 60 km from the Atlantic Ocean. It is

located on the southeast slope of Lead Mountain (maximum elevation 475 m). The BBWM is the site of a long-term paired watershed manipulation study designed to investigate the effects of N and S deposition. It is comprised of two contiguous forested watersheds, WBW (10.3 ha) and EBW (11.0 ha). Both watersheds have similar hydrological, topographical, soil, and vegetative characteristics making them ideal for long-term comparative studies. East Bear Watershed is the untreated reference site while WBW has been treated bi-monthly with granular ammonium sulfate [(NH₄)₂SO₄; ~28.8 kg S ha⁻¹ yr⁻¹ and 25.2 kg N ha⁻¹ yr⁻¹] applied aeri-ally beginning in November, 1989, and throughout the duration of the study reported here. Both watersheds received ~8.4 kg N ha⁻¹ yr⁻¹ of combined ambient wet plus dry deposition at the beginning of the study (Norton et al. 1999). Since the beginning of this study in 1989, reductions in ambient N and S deposition levels (<https://cfpub.epa.gov/roe/indicator.cfm?i=1#6>; <https://cfpub.epa.gov/roe/indicator.cfm?i=1#5>) have been observed in the northeastern USA. However, both watersheds would have been equally affected, which means that these reductions did not differentially affect WBW. Lower elevations are dominated by hardwood stands (~40–60 years old) including American beech, yellow birch (*Betula alleghaniensis* Britt.), red maple (*Acer rubrum* L.), and sugar maple. Softwood stands (~80–100 years old) including red spruce, and to a lesser degree, balsam fir (*Abies balsamea* (L.) Mill), are the dominant species at higher elevations (DeWalle et al. 1999). Soils are mainly coarse, loamy, mixed, frigid typic Haplothorids formed from compact basal till (Rustad et al. 1993).

2.2 Foliar and sapwood sample collections

In August of 1998, healthy-looking foliage from mid to upper canopy branches of randomly selected dominant or co-dominant trees (20 trees/species) was collected using a shotgun. Samples were collected for sugar maple and American beech at mid-elevation and red spruce [current year (CY) and one-year-old (1Y)] at high elevation from each watershed. The same trees were sampled again in August of 1999 and 2000. In August of 2013, because the trees at this site had been relabeled by another research group, a different set of randomly selected trees (15 trees/species) were sampled from the same locations. Ten of these 15 trees were also sampled for sapwood in 2013. Minimal American beech mortality was reported from 2000 to 2002 in either watershed (Elvir et al. 2010). However, by 2013, beech bark disease was more evident at this site and thus a potential stressor for this species.

For each foliar sample, a pool of approximately 500 mg fresh weight (FW) of foliage was collected from a single branch from each tree. Needle samples were cut with scissors into 1–2 mm pieces, and 4 mm disks were punched from hardwood leaves with a paper punch while carefully excluding major veins. The clippings were mixed and a sub-sample (~200 mg FW) was placed in a pre-weighed 2 ml microfuge tube and 1 ml of 5% perchloric acid (PCA) was added to each sample. The rest was saved for chlorophyll and protein analyses. For sapwood, three or four ~1.5-cm plugs of wood, each extracted from a different face of a tree using a Hagl of increment hammer, were clipped into ~0.5 cm segments that were placed in a pre-weighed 2-ml microfuge tube and 1 ml of 5% PCA was added (Minocha et al. 2015). All samples were immediately placed on ice for transport to the laboratory and stored at –20 °C until further analysis. The tubes that had PCA added to tissue sample were weighed, thawed, and re-frozen 3 times (each thawing for at least an hour and each freezing event for 4 h or more) as described in details in Minocha et al. (1994) and then centrifuged at 13,000×g for 10 min. The resulting supernatants was used for analyses of PCA-extractable (free) PAs and AAs, and soluble inorganic elements. For each analysis, all extracts were analyzed individually without pooling.

2.3 Soluble inorganic ions

The quantitation of soluble inorganic ions of Ca, Mg, Mn, K, Al, and P (defined as the fraction of total ions within cells that is extractable in 5% PCA) was conducted using a simultaneous axial inductively coupled plasma emission spectrophotometer (Vista CCD, Varian, Palo Alto, CA, USA) and Vista Pro software (version 4.0). Supernatants of 3× frozen and thawed samples in PCA were diluted (foliage 100×, sapwood plugs 50×) with distilled deionized water for estimation of soluble ions.

2.4 Polyamines and amino acids

Polyamines (1998–2000) Perchloric acid extracts were dansylated and quantified according to the procedure described in Minocha et al. (1990). A PerkinElmer (Norwalk, CT, USA) HPLC system consisting of Series 200 pump, autosampler, and LC 240 fluorescence detector with a 20-µl loop (10 µl injection volume) was used for quantification. A pecosphere – 3 × 3 CR C₁₈, 3 µm, 33 × 4.6 mm I.D. analytical column was used for the separation of PAs. Excitation and emission wavelengths were set at 340 and 510 nm, respectively. A Gilson (Middleton, WI, USA) 712 HPLC system controller (version 1.2) was used to integrate the data.

Amino acids (1999–2000) Amino acids data were collected starting in 1999 using PCA extracts that were dansylated and quantified according to the procedure described in Minocha and Long (2004b) with a PerkinElmer pecosphere reduced activity RP C₁₈, 3 µm, 100 × 4.6 mm analytical column.

Simultaneous polyamine and amino acid processing and quantification (2013) Both PAs and AAs were simultaneously dansylated and quantified within a single run using the method described in Minocha and Long (2004b). The dansylation reaction was terminated in these samples using L-asparagine (50 µL of 20 mg ml⁻¹ in water) rather than Alanine (Ala), described in the original method. A new series 200 PerkinElmer HPLC system fitted with a Phenomenex (Torrance, CA, USA) Synergi™ Hydro-RP, 4 µm, 100 × 4.6 mm I.D. analytical column; a C18 Securityguard™, 5 µm, 4 × 3 mm I.D. cartridge guard column; and a PerkinElmer C18 Scavenger, 10 µm, 33 × 4.6 mm I.D. cartridge column was used. Excitation and emission wavelengths were set at 340 and 515 nm respectively. Data were integrated using TotalChrom HPLC software package (Perkin-Elmer, version 6.2.1).

2.5 Total chlorophyll and soluble proteins

For chlorophyll (Chl) analysis, 1 ml of 95% ethanol was added to ~10 mg of thawed foliage. Samples were incubated in the dark in a 65 °C water bath for 16 h, centrifuged (13,000×g for 5 min), and the supernatants scanned for absorbance ranging from 350 to 710 nm using a spectrophotometer (Hitachi, Tokyo, Japan). Chlorophyll was quantitated according to Minocha et al. (2009).

For soluble proteins, either 0.25 ml (red spruce) or 0.5 ml (hardwoods) of extraction buffer (100 mM Tris-HCl, 20 mM MgCl₂, 10 mM NaHCO₃, 1 mM EDTA, and 10% (v/v) glycerol, pH 8.0) was added to 50 mg of thawed foliage. Samples were then frozen and thawed three times and the supernatant was used for protein analysis according to Bradford (1976).

2.6 Statistical analyses

Statistical analyses were conducted separately for each species to evaluate significant differences between the treated (WBW) and reference (EBW) watersheds. The data for each variable were analyzed using repeated measures ANOVA with one main factor: treatment with two levels (reference and N-treated) and one repeated measure factor for year of sampling (1998–2000). Tukey's test was used for treatment comparisons. Pearson correlation coefficients were used to assess the significance of correlations among the various parameters studied. Statistical analyses were done using SYSTAT (version 10.2) (SYSTAT, Richmond, CA, USA) and Microsoft Excel (version 2003); $P \leq 0.05$ (*) indicates significant treatment differences within each species. Dataset (not peer reviewed) for this manuscript are available in the Forest Service Research Data Archive (Minocha et al. 2018).

3 Results

In line with earlier reports on the effects of annual variations in site conditions (e.g., duration of growing season, extreme environmental events, and insect and/or pathogen invasions) on tree biology (Minocha et al. 2010, 2015), the present study revealed species-specific inter-annual variations in soluble nutrients and metabolites (Suppl. Tables 1 and 2). Collecting repeat observations over 3 years on the same trees and conducting repeat measure statistical analyses on these data enabled us to observe clear effects of N+S additions on nutritional and metabolic changes that occurred over and above those occurring due to inter-annual climatic variations (e.g., drought, disease and/or insect defoliation). In addition, another set of data were collected in 2013 to evaluate the longer-term (i.e., over two decades) impact of chronic N+S-additions on these parameters in foliage and sapwood.

3.1 Foliar soluble (5% PCA extractable) ions

Significantly lower concentrations of cellular Ca were observed in the foliage of American beech and one-year-old red spruce needles at WBW compared to EBW during 1998–2000 (Fig. 2A). However, by 2013, no changes in Ca concentrations were observed in foliage and sapwood of all three species (Fig. 2B and C). Magnesium was also lower in the leaves of N+S-treated American Beech and one-year-old red spruce needles during 1998–2000 (Fig. 2D). However, in 2013, Mg concentrations were significantly higher in one-year-

old red spruce needles and in its sapwood (Fig. 2E and F). In general, soluble K data did not show any specific trends over time (Fig. 2G, H, and I). American beech foliage from WBW had higher P during 1998–2000 (Fig. 2J). By 2013, with the exception of higher concentrations of P in sugar maple sapwood, no other changes were evident in the foliage or sapwood of other species with N+S-amendments at WBW (Fig. 2K and L). Aluminum was significantly higher in one-year-old red spruce needles at WBW in comparison with EBW at all times and in all tissues tested (Fig. 2M, N, and O). Manganese was significantly higher in the foliage of the two hardwoods species, American beech and sugar maple at WBW during 1998–2000 (Fig. 2P). However, in 2013, no difference was observed in Mn concentrations of the foliage or sapwood of either species (Fig. 2Q and R).

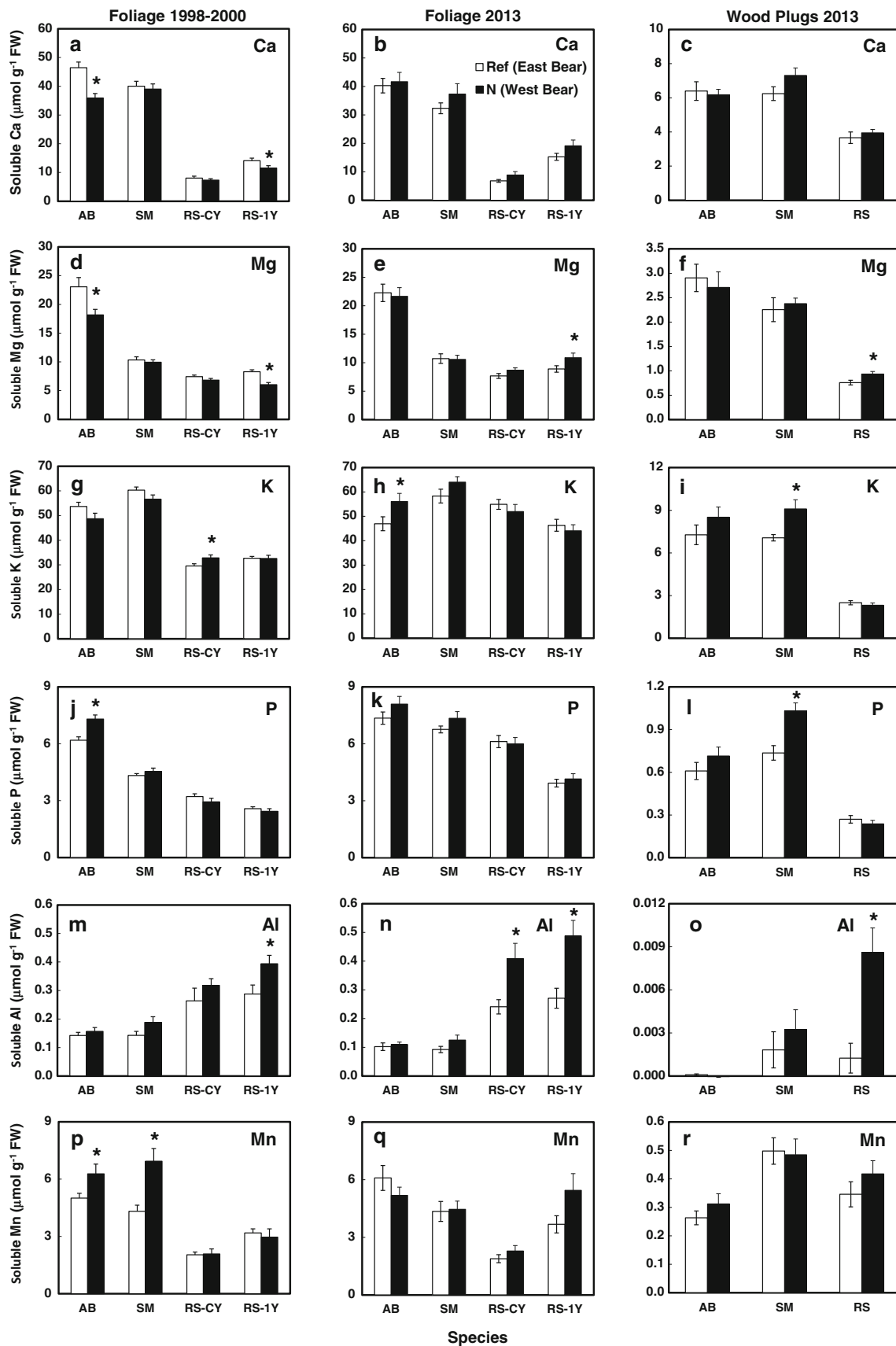
3.2 Foliar free polyamines and amino acids

With the exception of current-year red spruce needles, Put was significantly higher in the foliage of all species at WBW compared to EBW during 1998–2000 (Fig. 3A). By 2013, Put was still elevated in the foliage of American beech and in current-year red spruce needles without any change in sugar maple and one-year-old red spruce needles (Fig. 3B). One time sampling of sapwood in 2013 revealed no differences in Put concentrations among treatments (Fig. 3C).

Nitrogen additions at WBW significantly increased Spd concentrations in the foliage of sugar maple at all times tested (Fig. 3D and E); no changes in Spd were observed in sapwood of all three species (Fig. 3F).

Glutamic acid (Glu) was higher at all times tested in the foliage and sapwood of sugar maple at WBW (Fig. 4A, B, and C). In 2013, Glu was higher in American beech foliage at WBW (Fig. 4B). During 1999–2000, Pro was higher in all three species except for current-year red spruce needles (Fig. 4D). In 2013, this trend continued only for sugar maple foliage and sapwood (Fig. 4E and F). Fertilization with N+S increased GABA in all three species during 1999–2000 (Fig. 4G). In 2013, GABA remained high in American beech sapwood and in both tissues of sugar maple (Fig. 4H and I).

Due to changes in the HPLC system, some AAs could only be quantified either during 1999–2000 or 2013. Foliar aspartic acid (Asp, 1998–2000 only) was significantly higher in N+S-treated sugar maples (Fig. 4J). Alanine (Ala, 2013 only) was higher in foliage and sapwood of sugar maple and foliage of American beech (Fig. 4K and L).



◀ **Fig. 2** Concentrations of soluble elements in foliage and sapwood of American beech (AB), sugar maple (SM), and red spruce (RS) growing at East Bear Watershed (EBW) and West Bear Watershed (WBW) of BBWM. Data are mean \pm SE of the same 20 trees/species subjected to repeat sampling each year in 1998, 1999, and 2000. In 2013, 15 trees/species were sampled for leaves and 10 trees/species for sapwood cores. Current-year (CY) and one-year-old (1Y) needles were sampled from red spruce trees. * indicates significant differences between the foliage of the same species for WBW and EBW ($P \leq 0.05$)

3.3 Total chlorophyll and soluble proteins (2013)

In 2013, the concentration of total chlorophyll was significantly higher in sugar maple leaves and one-year-old red spruce needles at WBW (Fig. 5). Soluble proteins were significantly lower only in sugar maple (Fig. 6).

4 Discussion

Long-term whole ecosystem scale experiments provide an opportunity to examine the response of cellular metabolites over time with a goal to identify a suite of select stress-induced indicator metabolites. Most of these indicator metabolites have specific functions in normal growth and development of plants under ambient conditions. However,

under stress from a single or multiple sources cells make appropriate adjustments in the concentrations of these metabolites in order to recover from stress and could thus become a useful tool for monitoring stress from nutrient imbalance. While some of these metabolites initiate a cascade of signaling pathways, e.g., abscisic acid (Vishwakarma et al. 2017), others participate in signal transduction and activate each other, e.g., hydrogen peroxide and PAs (Gupta et al. 2016). These early detectable changes in indicator metabolites act as a warning signal for upcoming potential disruptions in both C and N cycling in cells (Bhatnagar et al. 2001; Handa et al. 2018; Majumdar et al. 2016; Minocha et al. 2014; Mohapatra et al. 2009). During periods of stress, these metabolites rebalance, and the plants attain a homeostatic state that is often different from that maintained under normal growth conditions. This defense strategy comes at a price in terms of its effect on growth rates. These metabolic changes are partially or fully reversible depending upon whether the stress is removed from one or all factors as was observed at two Ca-supplemented sites (Minocha et al. 2010; Wargo et al. 2002). If unfavorable conditions persist, even these responses may not be sufficient for survival; this is what was observed at Harvard Forest, MA, USA, where red pine and red maple trees sustained high mortality when exposed to 2–6 times higher NH_4 as compared to BBWM (Minocha et al. 2015).

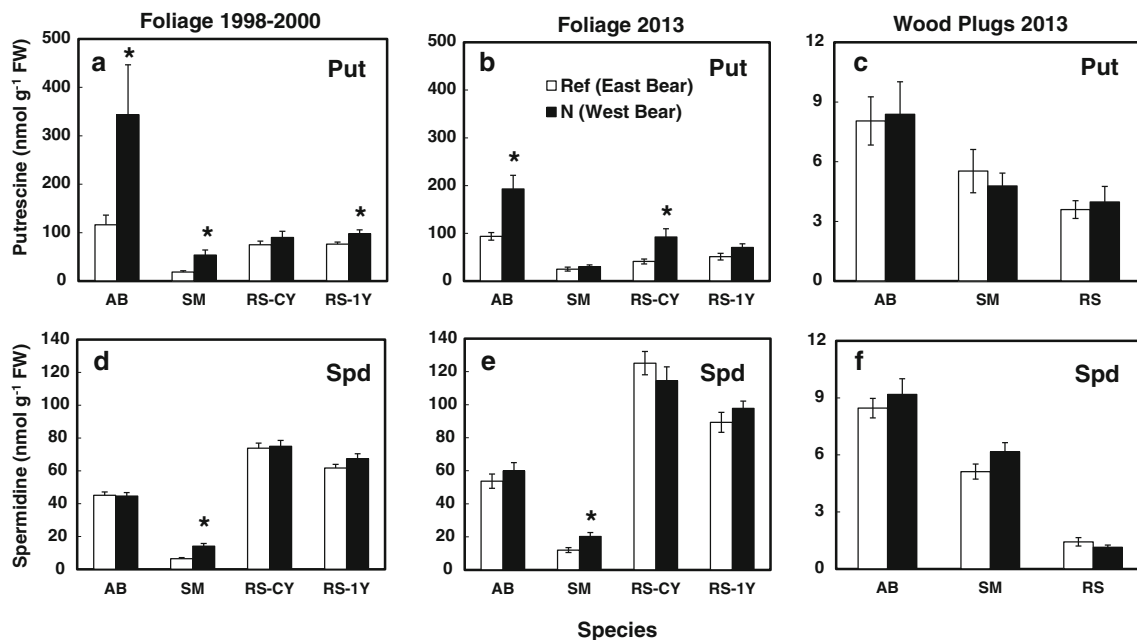


Fig. 3 Concentrations of free putrescine and spermidine in foliage and sapwood of American beech (AB), sugar maple (SM), and red spruce (RS) growing at East Bear Watershed (EBW) and West Bear Watershed (WBW) of BBWM. Data are mean \pm SE of the same 20 trees/species subjected to repeat sampling each year in 1998, 1999, and 2000. In 2013,

15 trees/species were sampled for leaves and 10 trees/species for sapwood cores. Current-year (CY) and one-year-old (1Y) needles were sampled from red spruce trees. * indicates significant differences between the foliage of the same species for WBW and EBW ($P \leq 0.05$)

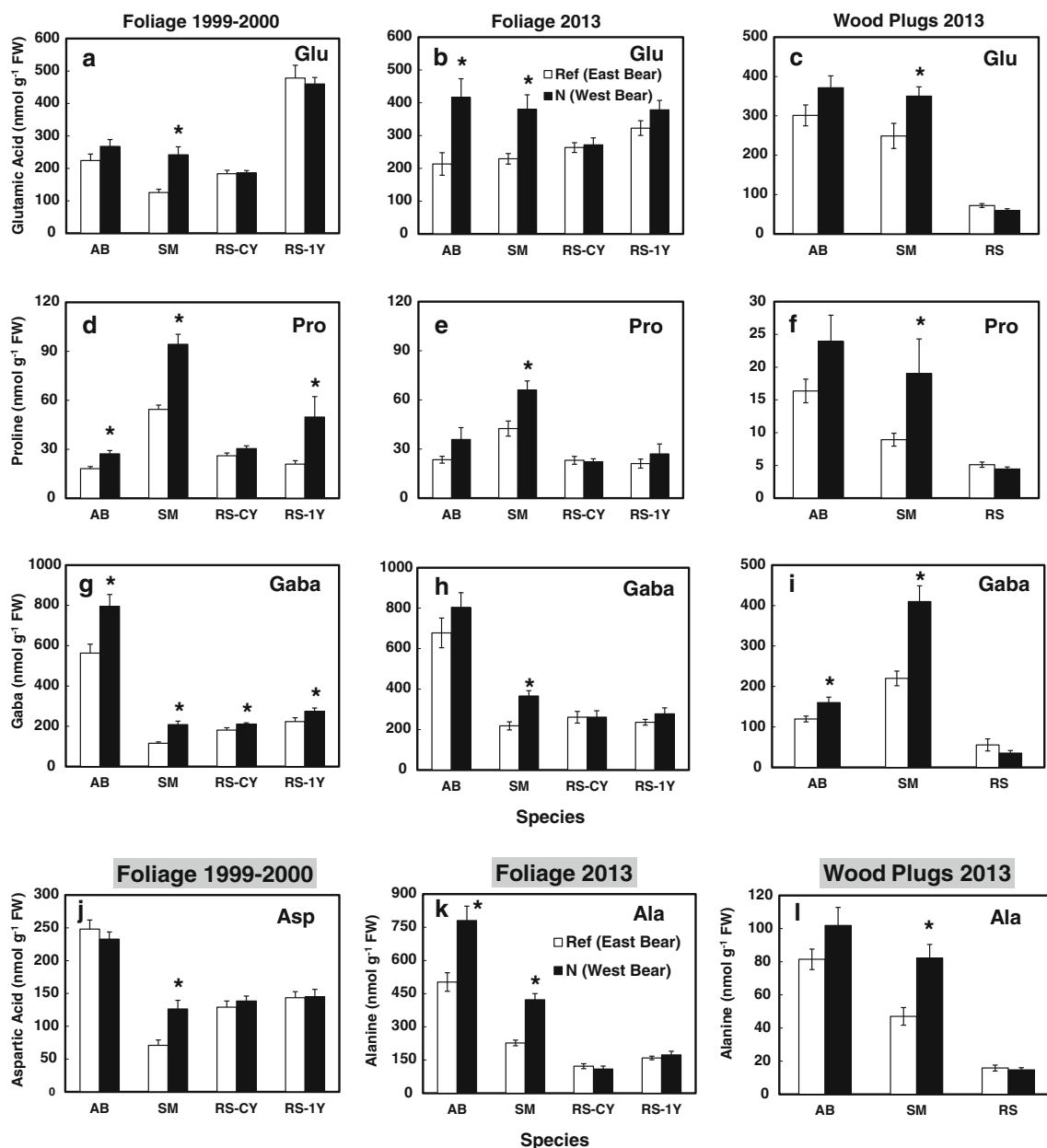


Fig. 4 Concentrations of free amino acids in foliage and sapwood of American beech (AB), sugar maple (SM), and red spruce (RS) growing at East Bear Watershed (EBW) and West Bear Watershed (WBW) of BBWM. Due to changes in HPLC methodology after 2000, Asp could only be quantified during 1999–2000, and Ala in 2013. Data are mean \pm SE of the same 20 trees/species subjected to repeat sampling each year in

1998, 1999, and 2000. In 2013, 15 trees/species were sampled for leaves and 10 trees/species for sapwood cores. Current-year (CY) and one-year-old (1Y) needles were sampled from red spruce trees. * indicates significant differences between the foliage of the same species for WBW and EBW ($P \leq 0.05$)

4.1 Effects of chronic N+S supplementation on soluble nutrients and metabolites were species-specific and changed with length of treatment

Putrescine accumulation in plants in response to various types of environmental stress has been extensively reviewed (Alcázar et al. 2006; Masson et al. 2017; Minocha et al. 2014; Wuddineh et al. 2018). Nitrogen addition/deposition-induced deficiency in total or soluble (analyzed from the same

dilute acid extract used for soluble/free metabolite analyses) foliar Ca is known to modulate polyamine metabolism in the foliage of several tree species (Minocha et al. 2010; Minocha et al. 1997, 2015; Schaberg et al. 2011; Wargo et al. 2002). In these studies, only under Ca deficient conditions, an inverse relationship was observed between the cellular contents of Put and Ca, leading to the suggestion that Put may serve as a substitute for Ca regardless of plant/soil N status. At BBWM, decreases observed in foliar soluble Ca and Mg in

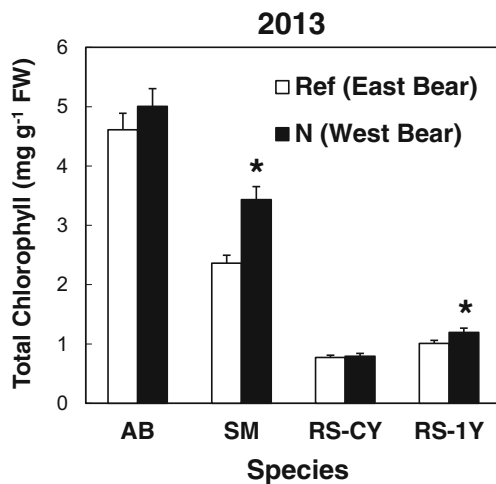


Fig. 5 Concentrations of total chlorophyll in American beech (AB), sugar maple (SM), and red spruce (RS) growing at East Bear Watershed (EBW) and West Bear Watershed (WBW) of BBWM. Data are mean \pm SE of 15 trees for each species collected in 2013. Current year (CY) and one-year-old (1Y) needles were sampled from red spruce trees. * indicates significant differences between the foliage of the same species for WBW and EBW ($P \leq 0.05$)

American beech and red spruce foliage from 1998 to 2000 are in line with earlier reports on total Ca and Mg by Elvir et al. (2005). A simultaneous increase in concentrations of these ions in soil solution and stream water indicated that soil leaching was the main cause of their decline in foliage (Fatemi et al. 2012; Fernandez et al. 2003). An increase in Put in these species may be at least partially associated with the observed decrease in soluble Ca during 1998–2000; the remainder could be attributed to the conversion of toxic-free ammonia into N-rich metabolites including Put. Alternatively,

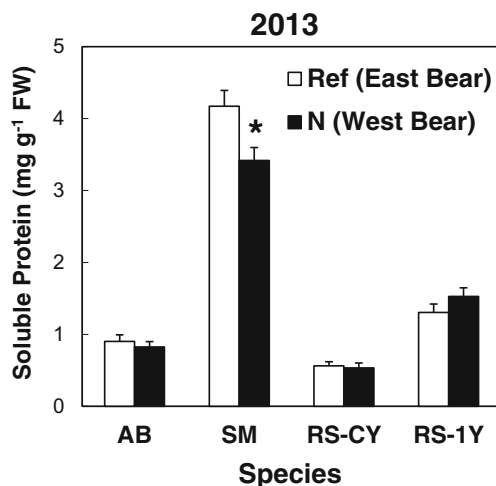


Fig. 6 Concentrations of total soluble proteins in American beech (AB), sugar maple (SM) and red spruce (RS) growing at East Bear Watershed (EBW) and West Bear Watershed (WBW) of BBWM. Data are mean \pm SE of 15 trees for each species collected in 2013. Current year (CY) and one-year-old (1Y) needles were sampled from red spruce trees. * indicates significant differences between the foliage of the same species for WBW and EBW ($P \leq 0.05$)

the increase in Put in all three species was solely a result of the detoxification of ammonia assuming the decrease seen in Ca and Mg for American beech and red spruce was within the tolerance range for deficiencies in Ca and Mg for each of the two species. Regardless of the cellular Ca sufficiency level, trees accumulate N metabolites to detoxify excess ammonia (Minocha et al. 2000, 2015), which explains why Put was still elevated in 2013 for American beech and red spruce even when there were no longer differences in foliar Ca indicating that these species had reached a new homeostatic state.

Whereas Put is known to modulate stress in most trees, Spd is a plant growth regulator and is synthesized from Put (Fig. 1). Relative to Put, Spd concentrations are generally more tightly regulated within cells and thus fluctuate within a very tight range even under conditions of Ca deficiency or N saturation (Minocha et al. 2010, 2015). The observed lack of change in Spd in red spruce and American beech is consistent with the previously reported observations by our group.

Of the three species, sugar maple was the first to show a short-lived decline in foliar Ca in 1993 in response to N+S additions; Ca levels recovered by 1998 (Elvir et al. 2010). An increase in Put and Spd without an accompanying change in Ca or Mg (seen during 1998–2000) in sugar maple at WBW was interpreted as a positive response to the need to store excess N present in the foliage as reported by Elvir et al. (2005). In 2013, higher levels of Spd and total chlorophyll, and a decrease in total soluble proteins again suggest better growth conditions for sugar maple. Sugar maple had probably been growing under N-limited conditions at BBWM, and thus benefited from N+S additions. Our data are supported by trends in basal area increment and photosynthesis measurements reported by Elvir et al. (2010).

Nitrogen and S addition to soil causes the mobilization of Mn and Al, both of which can be toxic to plants as they induce deficiencies in Ca and Mg, and decrease photosynthesis and yield (Millaleo et al. 2010; Shortle and Smith 1988). An increase in foliar Al, seen only in case of red spruce at WBW, was probably not in the toxic range because no Al-induced Ca deficiency was observed in 2013. In support of these observations, Elvir et al. (2010) also did not observe any significant changes in red spruce basal area increment analyzed in 2003 and 2006. Earlier it had been reported that a decline in sugar maple growth at the Alleghany Plateau (PA, USA) was associated with an increase in Mn (Long et al. 1997; Wargo et al. 2002). At least fourfold higher concentrations of soluble Mn were reported in unlimed as compared to limed plots, and this increase was associated with a decrease in soluble Ca accompanied by an increase in Put, and sugar maple decline (Wargo et al. 2002). At BBWM, while soluble Mn accumulated in the foliage of both N-treated hardwoods in 1998–2000, these differences did not persist in either species in 2013. Elvir et al. (2005) reported an increase in foliar total Mn in hardwoods for 1998–2000 that was no longer there in

2002. However, the observation that sugar maple showed no Ca deficiency or other stress symptoms at WBW indicates that there were no apparent toxic negative effects of the increase in Mn on growth during 1998–2000. Neither the tolerance limits nor the effects of a short-term increase in Mn on the growth of American beech are known.

The increase in foliar N is known to cause shifts in the internal distribution (partitioning) of N within a leaf thus leading to changes in the concentration of several free AAs, especially Arg, under N saturation conditions (Bauer et al. 2004; Ericsson et al. 1993, 1995; Näsholm et al. 1997). Similar to nutrients and PAs, AAs also accumulate in a species-specific manner under the same growth conditions (Huhn and Schulz 1996; Minocha et al. 2010, 2013).

Ammonium from N fertilization/deposition first gets incorporated into Glu which along with Asp, is the precursor for most other AAs that are needed for protein synthesis and many other secondary metabolites; syntheses of both PAS and AAs require TCA cycle intermediates (Fig. 1). Elevated levels of Asp (analyzed only in 1999–2000) and Glu in the foliage and sapwood of sugar maple indicated a major shift in both C and N metabolism in response to N+S addition.

Both GABA and Pro are known to accumulate in response to an increase in cold, drought, salinity, hypoxia, and a decrease in pH; all of which also cause Put accumulation (Minocha et al. 2014; Sharma and Verslues 2010 and references therein). An overproduction of Pro in plants has been shown to impart stress tolerance via a multitude of pathways ranging from acting as an osmolyte to maintain cell turgor or osmotic balance, to acting as a signal molecule under drought conditions (Hayat et al. 2012). At BBWM, an increase in foliar GABA and Pro was seen in N-treated trees of all species after the initial decade of treatments; but by 2013, these changes persisted only in sugar maple. Like Pro, GABA also plays significant roles in maintaining N and C balance in plants, in addition to its protective role in stress (Bouche and Fromm 2004). A recent report suggested that Pro, Ala, Arg, GABA, and succinate are involved in N salvage/recycling and redistribution and their accumulation indicates a disequilibrium in C and N in green microalga *Chlorella* (Chen et al. 2017). At Harvard Forest also, an accumulation of the abovementioned salvage AAs were also observed in foliage and sapwood of pine and oak in response to chronic N additions (Minocha et al. 2015). Species-specific accumulation of Pro, Ala, GABA, and Glu, during 1998–2000, indicated the storage of nontoxic usable forms of cellular N that helps reestablish C and N equilibrium by using carbon from photosynthesis and glycolysis as suggested by Chen et al. (2017). Altogether, such data show that the storage of excess N in the form of a few salvaged AAs is a phenomenon not exclusive to any one species or kingdom. The lack of accumulation of these AAs or nutrient deficiencies in red

spruce by 2013 under same conditions could mean that C and N metabolism had reached a new homeostatic state.

In sapwood compared to foliage, the concentrations of nutrients and metabolites were generally several folds lower and treatment differences were relatively few. It is worth pointing out all AAs accumulated mostly in sugar maple sapwood. It can thus be argued that most excess N is assimilated into various metabolites in the foliar tissue by using photosynthetic energy, which then move towards the main stem in the sap and get diluted along the way.

In the present study, large sample size per treatment and repeat observations from the same trees from 1998 to 2000 possibly significantly reduced any errors introduced from microsite variability among sampled trees within each watershed and any erroneous observations possibly caused by unusual climate of a single year.

4.2 Dynamic nature of foliar responses

The lack of information on the tolerance limits for nutritional requirements for each species makes it difficult to determine whether any of these species suffered from nutrient deficiencies during 1998–2000 at BBWM. Linder (1995) reported that the optimal nutrient concentration is generally considered more valid when calculated as a portion of N present in Norway spruce foliar tissue. Species-specific nutritional requirements (Finzi et al. 1998) factor into the observed differences in metabolic response of each species to N additions. However, in the absence of information on critical N loads for each species and for the BBWM site, metabolite indicators in conjunction with soluble ions, all of which quantified from the same extracts using a simple freeze-thaw method (Minocha et al. 1994), could be quite helpful in assessment of stress from nutrient imbalance in trees. As we know, epigenetic changes (changes in gene expressions without a change in DNA sequence) can cause variations at the individual tree level within a species as well as among species in response to changes in the environment (Bräutigam et al. 2013). The ability to modulate metabolic responses to a continuously changing environment gives trees the needed plasticity for survival from short- and long-term stress. Raj et al. (2011) showed that in poplar, previous environmental history has a lasting impact on a tree's capacity to respond to a current environmental stimulus. Taken together, these factors in combination may contribute to the species-specific modulation of metabolic responses to chronic N+S over time as observed at BBWM. Initially, all species make short-term adjustments to N+S treatment by upregulating N metabolism to detoxify their immediate environment from free ammonia. Additional N-rich metabolites could either be used for growth and/or stored as specific AAs to be used later to balance cellular C or N (as seen with sugar maple);

and each species adjusts accordingly to a new homeostatic state. At BBWM, the data indicate that sugar maple was N-limited and used the added N+S to modulate both C and N metabolism. Although red spruce and American beech recovered from initial nutrient deficiencies, both species continued to expend energy to detoxify tissues exposed to ammonia. Beech at this site may be responding to cumulative stress from both N+S additions and beech bark disease.

5 Conclusions

Nutritional imbalances observed during 1998–2000 in red spruce and American beech in response to N+S additions at the Bear Brook Watershed in Maine were no longer evident by 2013. Nitrogen-containing metabolites accumulated in a species-specific manner in both the leaves and the sapwood, perhaps to detoxify excess ammonia. While sugar maple was N-limited and metabolically benefitted from continued N+S additions, spruce and birch were not N-limited and adjusted to a new homeostatic state over time via storing excess N as polyamines and specific amino acids. Based on this study and previous similar research (Minocha et al. 2015), we conclude that even in the absence of knowledge about individual species tolerance limits for nutrients and critical N loads for the site, metabolic monitoring using a suite of biochemical parameters could become a very useful tool in assessing stress from nutrient imbalances. Biological processes are dynamic and local environmental changes or experimentally applied treatments will continue to modulate the tree responses with time.

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Author contributions Rakesh Minocha designed and wrote the manuscript with assistance from Swathi Turlapati (postdoctoral fellow) who also helped process some data. Stephanie Long (senior technician) helped with field collections and analyses, processing of all metabolic data. Ivan Fernandez helped with manuscript writing and editing and field collections of 2013.

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Compliance with ethical standards

Conflict of interest The authors of this work do not have any conflict of interest statement.

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