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Research paper

Long-term trends of changes in pine and oak foliar nitrogen metabolism in response to chronic nitrogen amendments at Harvard Forest, MA

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We evaluated the long-term (1995–2008) trends in foliar and sapwood metabolism, soil solution chemistry and tree mortality rates in response to chronic nitrogen (N) additions to pine and hardwood stands at the Harvard Forest Long Term Ecological Research (LTER) site. Common stress-related metabolites like polyamines (PAs), free amino acids (AAs) and inorganic elements were analyzed for control, low N (LN, 50 kg NH_4NO_3 ha^{-1} $year^{-1}$) and high N (HN, 150 kg NH_4NO_3 ha^{-1} $year^{-1}$) treatments. In the pine stands, partitioning of excess N into foliar PAs and AAs increased with both N treatments until 2002. By 2005, several of these effects on N metabolites disappeared for HN, and by 2008 they were mostly observed for LN plot. A significant decline in foliar Ca and P was observed mostly with HN for a few years until 2005. However, sapwood data actually showed an increase in Ca, Mg and Mn and no change in PAs in the HN plot for 2008, while AAs data revealed trends that were generally similar to foliage for 2008. Concomitant with these changes, mortality data revealed a large number of dead trees in HN pine plots by 2002; the mortality rate started to decline by 2005. Oak trees in the hardwood plot did not exhibit any major changes in PAs, AAs, nutrients and mortality rate with LN treatment, indicating that oak trees were able to tolerate the yearly doses of 50 kg NH_4NO_3 ha^{-1} $year^{-1}$. However, HN trees suffered from physiological and nutritional stress along with increased mortality in 2008. In this case also, foliar data were supported by the sapwood data. Overall, both low and high N applications resulted in greater physiological stress to the pine trees than the oaks. In general, the time course of changes in metabolic data are in agreement with the published reports on changes in soil chemistry and microbial community structure, rates of soil carbon sequestration and production of woody biomass for this chronic N study. This correspondence of selected metabolites with other measures of forest functions suggests that the metabolite analyses are useful for long-term monitoring of the health of forest trees.

Keywords: biochemical responses, defense responses, nitrogen deposition.

Introduction

As a result of increased human activity in industrialized areas and fertilizer use over the past 50 years, atmospheric deposition of nitrogen (N) has increased substantially over natural biogenic inputs (Galloway et al. 2003). This continual input of N has seriously harmed terrestrial ecosystems due to the accumulation of excess N in the form of ammonia, which is further oxidized to N oxides by microbes. According to Nihlgård (1985) and van Breemen and van Dijk (1988) the accumulation of N in soil is a key factor driving the decline of forest trees. A review of N fertilization experiments carried out across different ecosystems revealed that although the growth of some individual tree species increased in the short term, overall growth and productivity declined in the long term (Pardo et al. 2011). This decline

in growth was attributed to changes in soil chemistry and nutrient imbalances in the foliage of trees (Aber et al. 1989, Schulze 1989, DeHayes et al. 1999, Bertills and Näsholm 2000, Högberg et al. 2006).

The impacts of N addition on soil carbon (C) are inconsistent and depend on the initial N concentration along with other site-specific factors which may either lead to more C sequestration with low-to-moderate N additions, or C losses with high N additions (Högberg et al. 2006, Högberg 2007, de Vries et al. 2009). As suggested by Smithwick et al. (2013), and also observed at Harvard Forest by Turlapati et al. (2013), the response to elevated N is spatially heterogeneous and extremely complex as it affects the tradeoffs between aboveground and belowground functions. It is often difficult to untangle the effects of excess N from other contributing factors including the form of N used, nutrient deficiencies, climate, land-use history, lithology, spatial water table patterns, microbial N uptake and plant species composition for any ecosystem (St Clair et al. 2009, Högberg et al. 2010, Pardo et al. 2011, Gillin et al. 2015). Comparative studies that span across species and environmental gradients that yield information at the cellular as well as the ecosystem level are critical for understanding and projecting whole-tree responses to altered biogeochemical cycling (Smithwick et al. 2013).

Besides accumulating soluble N in soil, the acids in pollution solubilize Ca^{2+} and aluminum (Al^{3+} from its bound form in the soil). Initially this increase in Ca^{2+} availability may facilitate growth provided the site is Ca deficient. However, an increase in Al^{3+} mobilization is known to block Ca^{2+} uptake by roots, and Al^{3+} also binds tightly to soil particles in turn displacing Ca^{2+} and other divalent cations from the soil; this would eventually result in leaching of Ca from the ground water to surface water bodies (e.g., lakes). Many forest soils all over the globe have been shown to be Ca depleted causing select tree species (Norway spruce, red spruce and sugar maple) to develop Ca deficiency (Bailey et al. 1996, Likens et al. 1998, Nykvist 2000, Thimonier et al. 2000, Kobe et al. 2002, Jönsson et al. 2003, Huntington 2005, Högberg et al. 2006, Minocha et al. 2014). Simultaneously, N deposition has reached a level that has either already caused or will cause significant harm to the functions and structure of many forests globally (van Breemen and van Dijk 1988, Nykvist 2000, Galloway et al. 2004, Pardo et al. 2011).

Nitrogen accumulation in soil due to long-term fertilization and/or atmospheric deposition is apparently a major driver of changes in species composition in forests of the northeastern USA (Zaccherio and Finzi 2007, Bobbink et al. 2010). Although general relationships between species composition and N inputs are known to some extent, the specific metabolic mechanisms by which individual species respond to elevated N are not understood. Homeostatic concentrations of metabolites are often species specific and changes in these metabolites vary with the duration and dose of the stress factor (Gezelius and Nasholm 1993, Högberg et al. 2006, Minocha et al. 2010, Minocha et al. 2013).

Polyamines (PAs) are low molecular weight aliphatic organic metabolites that are obligatory requirements for cell survival and growth (Kusano et al. 2007, Minocha et al. 2014). The commonly occurring PAs in plants are putrescine (Put), spermidine (Spd) and spermine (Spm), which are derived from the amino acid (AA) glutamate (Glu) via ornithine (Orn) and/or arginine (Arg), and from *S*-adenosylmethionine. A positive charge at physiological pH enables them to stabilize polyanionic macromolecules and cellular membranes (Wallace et al. 2003). In addition to being organic N storage compounds, PAs also conjugate with hydroxycinnamic acid, fatty acids or alkaloids in order to produce plant defense-related compounds (Flores and Filner 1985, Martin-Tanguy 1997, Ghosh 2000, Bagni and Tassoni 2001). The PA metabolic pathway is linked on one side to the primary N metabolism and on the other to C metabolism via intermediates such as Glu, Arg, Pro, γ-amino-butyric acid (GABA) and ethylene (Majumdar et al. 2013). Polyamines also interact with nucleic acids (Bachrach 2005), act as cofactors for activating plasma membrane cellular H^+ pumps (Garufi et al. 2007) and are involved in modulating the transport of Ca^{2+} and K^+ across root plasma membranes in a species-specific manner (Zepeda-Jazo et al. 2011).

Under conditions of chronic N exposure, metabolic changes that result in the increased production of PAs and AAs can be a mechanism to detoxify elevated levels of NH^{4+} ions in cells (Ericsson et al. 1993, 1995, Minocha et al. 2000, Bauer et al. 2004). Earlier studies have shown that long-term adjustments causing up-regulation of Put biosynthesis (e.g., via genetic manipulation or response to long-term abiotic stress) can lead to metabolic consequences that span far beyond the PA pathway; they include effects on several AAs, sugars, sugar alcohols, phytochelatins, organic acids and inorganic elements (Minocha and Long 2004*a*, Page et al. 2007, 2012, Mattoo et al. 2010, Mohapatra et al. 2010*a*, 2010*b*). Additional physiological effects of Put overproduction and accumulation also include changes in the oxidative state of cells and alterations in their C and N balance (Mohapatra et al. 2009, 2010*b*). The accumulation of AAs in plants in response to different forms of stress, including N fertilization of boreal and temperate forests, has also been reported previously (Näsholm et al. 1994, 1997, Ericsson et al. 1995, Kovács et al. 2012). Arginine and Put concentrations in foliage are among the known indicators of mineral nutrient imbalances caused by N deposition in both conifers and hardwoods (Ericsson et al. 1993, 1995, Näsholm et al. 1994, 1997, 2000, Wargo et al. 2002, Minocha et al. 2010, 2013). Whereas the inclusion of PAs and AAs in short-term studies has received ample attention in scientific literature, attempts to use them as indicators for stress monitoring in long-term studies are infrequent.

Data from long-term experiments collectively provide a unique insight into the ecological patterns and mechanisms that drive plant responses (Knapp et al. 2012). Such patterns are not always apparent from short-term studies. The present study was conducted at the Harvard Forest Long Term Ecological Research (LTER; http://harvardforest.fas.harvard.edu) site (Petersham, MA, USA), where long-term fertilization with ammonium nitrate (NH_4NO_3) has been ongoing since 1989 (Aber 1992, Aber et al. 1995). Previous reports from this experiment found that chronic N additions resulted in species-specific changes in the foliar biochemistry and metabolism that accompanied alterations in soil N cycling rates (Magill et al. 2000, 2004, Minocha et al. 2000, Bauer et al. 2004). In addition, these treatments affected soil respiration and microbial biomass (Frey et al. 2004, Wallenstein et al. 2006), along with bacterial community structure in the hardwood soils (Turlapati et al. 2013, 2015). The current report complements the earlier studies to evaluate long-term effects of N treatments on soil solution chemistry and species-specific foliar and sapwood biochemistry of pine and oak trees at this site.

Materials and methods

Site description, sample collection and analyses

The treatment plots used in this study are part of the Chronic Nitrogen Amendment Study at the Harvard Forest LTER site (42.5°N, 72°W). The site has a temperate climate with monthly temperatures ranging from −7 °C in January to 20 °C in July. Average annual precipitation is 110 cm (http://harvardforest.fas.harvard.edu). The site averages ~8 kg ha^{-1} $year^{-1}$ of total N deposition (Ollinger et al. 1993). As reported earlier by Magill et al. (2004), the land-use history of the pine and hardwood stands used in this study is very different. The pine site was pastured until 1920, and a red pine (*Pinus resinosa* Ait.) plantation was established on this site in 1926. Fire destroyed some of the trees in 1957. The hardwood stand has no agricultural land-use history but was substantially damaged by a hurricane in 1938, and was harvested for firewood during 1942–44.

In 1988, 30 × 30 m plots were established in each of two adjacent stands, red pine and a mixed deciduous. Starting in 1989, while one plot served as control, six equal doses of NH_4NO_3 were applied to Low N (LN, 50 kg N ha^{-1} $year^{-1}$) and High N (HN, 150 kg N ha^{-1} $year^{-1}$) plots of each stand from May through September each year.

Foliar samples of red pine and black and red oak (*Quercus velutina* Lam. ands; *Q. rubra* L., hereafter referred to only as oak) were collected in July or August 1995, 1996, 1997, 2002, 2005 and 2008 from mid to upper canopy of mature trees by shooting down small branches of asymptomatic trees with a shotgun. The number of trees sampled varied with year and tree species. Each year trees were selected randomly from each treatment plot. In general, with the exception of 2005 for pine and 1997 for oak where sample sizes were five, 10 or more trees were sampled for other years. More detailed information for sample size for each year is provided in the legend of each figure. All samples were processed as described in Minocha et al. (2000). Briefly, for each sample, a pool of ~500 mg fresh weight (FW) of foliage was collected from a single branch from each tree. Current year needles of pine were cut with scissors into 1–2 mm pieces, and 4 mm disks were punched from oak leaves with a paper punch carefully excluding major veins. The clippings were mixed and a sample (~200 mg FW) was placed in a pre-weighed 2 ml microfuge tube with 1 ml of 5% perchloric acid (PCA). All samples were immediately placed on ice for transport to the laboratory and stored at −20 °C until further analysis. The tubes with tissue sample and PCA were weighed, frozen and thawed three times and then centrifuged at 13,000*g* for 10 min. The resulting supernatant 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.

For analyses of PAs and AAs, the supernatant of the PCA extracted samples was subjected to dansylation and quantitation by HPLC (PerkinElmer Inc., Waltham, MA, USA) according to previously published protocols (Minocha et al. 2000, Minocha and Long 2004*b*). The reaction was terminated using l-asparagine (50 µl of 20 mg ml^{-1} in water) rather than alanine as described in the original method. In using this protocol the separation of Arg and threonine (Thr) was not always complete; for quantitation, the peak areas of these two AAs were added together to formulate a combined calibration curve.

Inorganic ions (soluble in 5% PCA) were quantified using a simultaneous axial Inductively Coupled Plasma emission spectrophotometer (Vista CCD, Varian Inc., Palo Alto, CA, USA) and Vista Pro software (Version 4.0), following appropriate dilutions with deionized water.

Sapwood plugs

In 2005 and 2008, sapwood plugs from actively growing clear (no stained or rotten wood) sapwood were extracted from each tree using a drill and an increment hammer. Samples were taken at breast height, avoiding areas with obvious injury to the stem. Three sapwood plugs for oak and four for pine were collected from different faces of each tree. A drill was used to remove the bark and the cambial layer. A dead blow hammer was then used to drive the increment hammer into the exposed sapwood. A plunger was used to extract the sapwood plug into labeled Ziploc bags. Plugs were ~1.5 cm in length. Prior to processing, each plug was cut into 4–6 equal segments. After removing any extraneous materials such as bark or discolored wood, samples (~600 mg FW) were transferred into pre-weighed and labeled microfuge tubes. Following the addition of 1 ml of 5% PCA, samples were stored on ice in the field and frozen at −20 °C in the laboratory until further analysis. Sapwood samples were processed for PA, AA and ion analysis as described above for foliage.

Soil solution chemistry

Details of the installation of Zero-Tension Lysimeters (ZTL) and soil solution sample collection are described in Minocha et al. (2000) and references therein. Briefly, five polyethylene ZTLs were installed per treatment plot. Soil solution was collected after major rain events. All five samples per plot were pooled prior to analyses. Over the course of each year, there were ~50 collections from each plot. Samples were transported on ice to the laboratory and filtered through pre-combusted Whatman GF/F glass fiber filters (Whatman Inc., Clifton, NJ, USA) within 36 h of collection before freezing. These solutions were analyzed for inorganic elements using ICP as described above.

Annual mortality data

Mortality numbers were calculated each year as percent of dead trees compared with the number of live trees at the beginning of the study in 1988. In order to keep the number of trees constant for mortality calculations for the entire duration of the study, those trees that were <5 cm diameter at breast height (DBH) in size at the beginning of the study (1988) but grew to >5 cm by summer 2008 were included in the total number of trees in inventory of 1988–2008.

Statistical analyses

Since the baseline cellular content of most metabolites was distinctly different for pine and oak, each species was analyzed separately. Each year's data was independently analyzed using one-way ANOVA to evaluate treatment effects within a specific year. Tukey's test was used for treatment comparisons. Analyses were done using SYSTAT Version 10.2 for Windows (SYSTAT, Richmond, CA, USA) and Microsoft Excel (Version 2010); $P \leq 0.05$ (*) indicates significant differences unless otherwise specified.

Results

The response of pine trees to nitrogen treatments

Although foliage for PA and ion analyses was collected six times (1995–97, 2002, 2005 and 2008), for AA analyses it was collected only three times (2002, 2005 and 2008). The sapwood plugs were collected only twice (2005 and 2008). As reported by Magill et al. (2004), by the end of the 2002 growing season 56% of the pine trees in the HN-treated plot had died, thereafter a smaller number of pine trees were sampled from the HN treatment plot.

Pine foliage Putrescine content in the foliage was significantly higher for both LN and HN treatments vs the control for all years with the exception of HN in 2005 (Figure 1a). Spermidine content in the foliage was also higher in N-treated trees, but not in 2005 and not consistently between the two N treatments (Figure 1b). With the exception of HN in 2008, foliar Glu and Gln levels were higher for LN and HN for all 3 years (Figure 2a and b). The combined content of Arg + Thr increased with HN in 2002 and with LN in 2005 (Figure 2c). Alanine increased with LN in 2005 (Figure 2d). Except for HN in 2008, GABA increased with both N treatments in all 3 years (Figure 2e). No treatment effects were observed for Pro for all 3 years (Figure 2f). Among

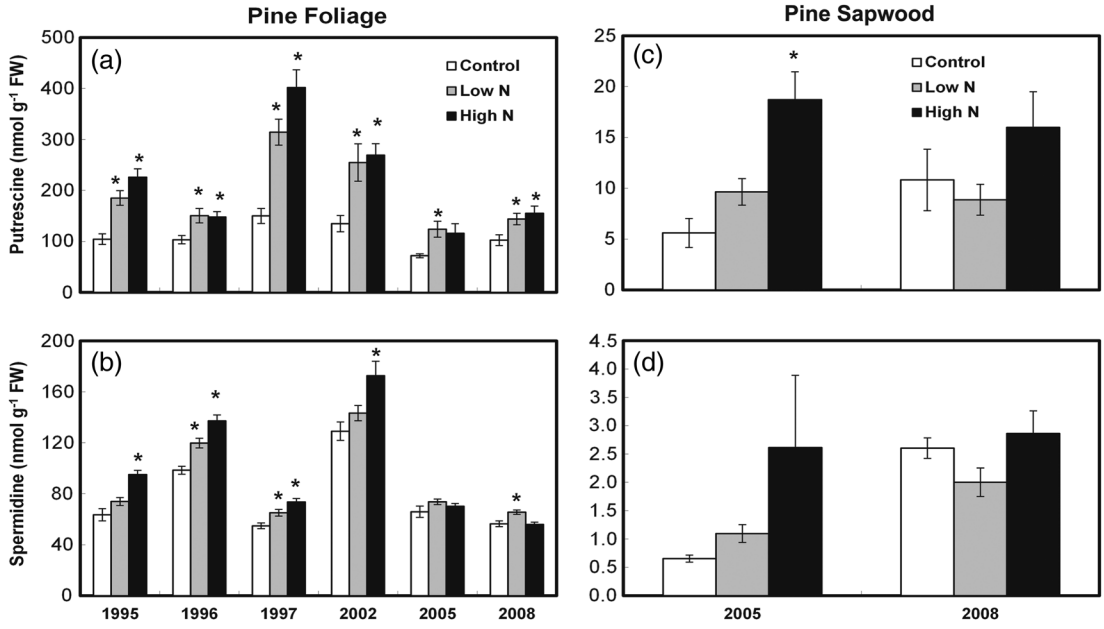

Figure 1. Effect of chronic N additions on foliar (a and b) and sapwood (c and d) polyamines: putrescine and spermidine in pine. Data presented for each treatment for foliage are mean ± SE where $n = 20$ from 1995 to 1997 (with the exception of HN treatments in 2002 where $n = 10$); from 2002 to 2008 $n = 10$ and for 2005 $n = 5$. For sapwood samples $n = 10$ for both 2005 and 2008. Asterisks denote significant differences from the control treatment at $P \leq 0.05$.

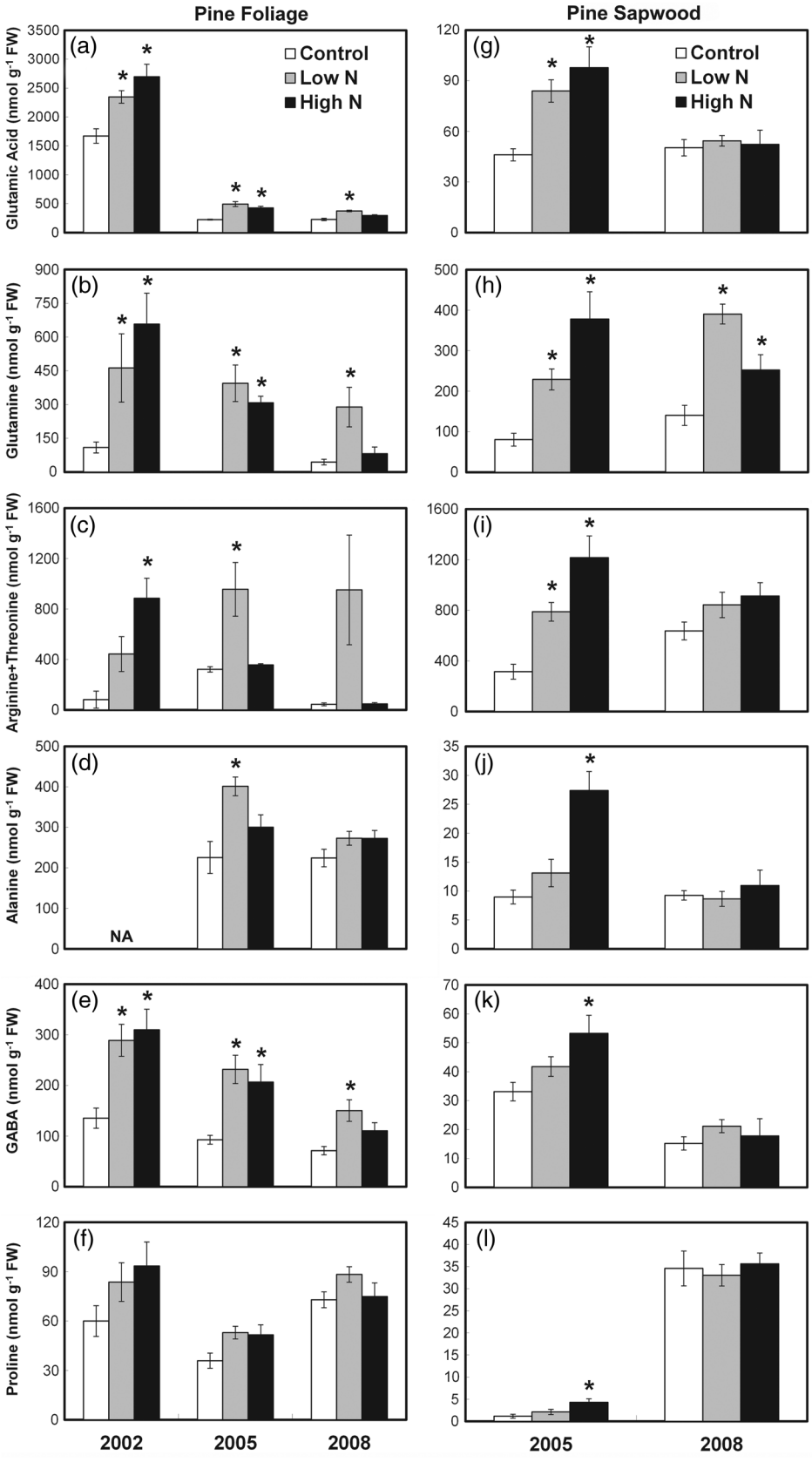

Figure 2. Effect of chronic N additions on foliar (a–f) and sapwood (g–l) amino acids, respectively, in pine: glutamic acid (a and g), glutamine (b and h), arginine + threonine (c and i), alanine (d and j), GABA (e and k) and proline (f and l). For additional details refer to the legend of Figure 1. NA stands for alanine not available in 2002.

inorganic elements, Ca was lower for HN only in 1996 and for both treatments in 2005 (Figure 3a).

Magnesium did not change with either of the N treatments for all the years (Figure 3b). Manganese content was lower for HN in 1996 and for LN in 2005 (Figure 3c). Phosphorous concentration was significantly lower for HN in 1996, 1997 and 2002 (Figure 3d). Aluminum concentration increased with LN in 1996 and decreased with HN in 2002 (Figure 3e). Overall, no changes were observed for the years 1995 and 2008.

Pine sapwood plugs The concentrations of all metabolites and inorganic elements were much lower in sapwood plugs as compared with foliage. Putrescine was higher with HN in 2005 but not in 2008 (Figure 1c). Spermidine did not change with N addition (Figure 1d). Most AAs were higher in either one or both N treatments in 2005 (Figure 2g–l). In 2008, only Gln was higher with LN and HN (Figure 2h). Proline concentration was higher for all three treatments in 2008 vs 2005 (Figure 2l). Among inorganic elements, Ca was high with HN in 2008 (Figure 3f). Magnesium was higher with LN in 2005 and with HN in 2008 (Figure 3g). Manganese was higher with LN in 2005 and with both N treatments in 2008 (Figure 3h). Whereas P was lower with HN in 2005, it was lower with LN in 2008 (Figure 3i). Aluminum concentration did not change with treatment and overall was lower in 2008 (Figure 3j).

Pine mortality Mortality of trees in the LN plot was ~10% higher than control for all the years except for 2003 (Table 1). For HN, mortality was on the rise until 2003 after which a smaller proportion of the trees died in this plot relative to control. For the last measurement period of 2006–08, mortality in HN plot was only 4.3% relative to 12.6% for control.

Pine stand soil solution chemistry (ZTL) Pine stand soil solution chemistry (ZTL) showed that Ca was higher with HN in 2002 and with LN in 2005 (Figure 4a). Magnesium was higher in HN in 1997 and 2002 (Figure 4b). Manganese was higher with both LN and HN for 1997, 2002 and 2005 with the exception of LN in 1997 (Figure 4c). Both N treatments in 1996, 2002 and 2005, and HN only in 1995 caused an increase in P (Figure 4d). Aluminum was higher with both treatments in 1995, 2002 and 2005 and only with HN in 1997 (Figure 4e).

The response of oak trees to nitrogen treatments

Oak foliage There were no significant metabolic effects of N amendments on pines in the LN plot. Putrescine concentration was significantly high for HN in all years except for 1997 (Figure 5a), but Spd was higher in HN only for 2002 and 2008 (Figure 5b). The amino acid Glu increased with HN only in 2008 (Figure 6a).Glutamine and Arg + Thr + Gly (peaks inseparable by HPLC) were higher in HN in 2002 and 2008 (Figure 6b and c), alanine was high for HN in 2005 and 2008 (Figure 6d). In all 3 years, GABA, Pro (Figure 6e and f) and Ser (data not shown) were higher in HN-treated foliage. Among inorganic elements, Ca was lower in HN in all years except 2002; with LN Ca was lower only in 2005 and 2008 (Figure 7a). Magnesium was lower in HN in 2008 (Figure 7b). Manganese concentrations were lower in both LN and HN for all years except for 2002 (Figure 7c). Phosphorus was lower only with LN in 2002 (Figure 7d). Aluminum decreased with LN and HN in 2002 (Figure 7e).

Oak sapwood plugs Putrescine was high with both N treatments in 2005, but in response to HN only in 2008 (Figure 5c). Spermidine increased only with HN in 2005 (Figure 5d). Except for Glu and Gln in 2008, all AAs were higher in HN in 2005 and 2008 (Figure 6g–l). However, there was no response to LN treatment for any of the years. Among the inorganic elements, Ca was significantly lower with HN both in 2005 and 2008 (Figure 7f). Both treatments decreased Mn in sapwood plugs in 2005 and 2008 (Figure 7h); however, Mg and P were not affected by chronic N treatments (Figure 7g and i). Aluminum was higher only with HN in 2008; overall Al concentrations were very low in 2008 (Figure 7j).

Oak mortality The rate of mortality of LN trees was overall lower than that of control plot trees for all years (Table 1). The rate of mortality gradually increased from 2003 to 2008 in HN treatment and was ~15% higher than that of control in 2008 (Table 1).

Oak stand soil solution chemistry (ZTL) Oak stand soil solution chemistry (ZTL) showed that Ca, Mg, P and Al were higher in soil solution from HN-treated plots in 2002 (Figure 4f, g, i and j), Mn was unaffected (Figure 4h), and Al was also higher in HN in 1996 and 2005 (Figure 4j).

Discussion

Inter-annual variations

The present study revealed pronounced inter-annual variations in all metabolites thus indicating changes in growth conditions at this site from year to year. These yearly changes in climate may or may not have affected all treatment plots equally because of possible interactions between treatments and yearly climate. Recently, such uneven interactions have been reported for a Ca supplementation study at Hubbard Brook experimental Forest, NH, USA (Green et al. 2013). Inter-annual variations in site chemistry, related metabolite concentration and growth rates are a common occurrence in many studies including the Harvard Forest site due to annual variations in local site climate (Minocha et al. 2000, 2010, Wargo et al. 2002, Magill et al. 2004, Urbanski et al. 2007, Cho et al. 2010). These differences are caused by factors including but not limited to daily temperature and rainfall leading to changes

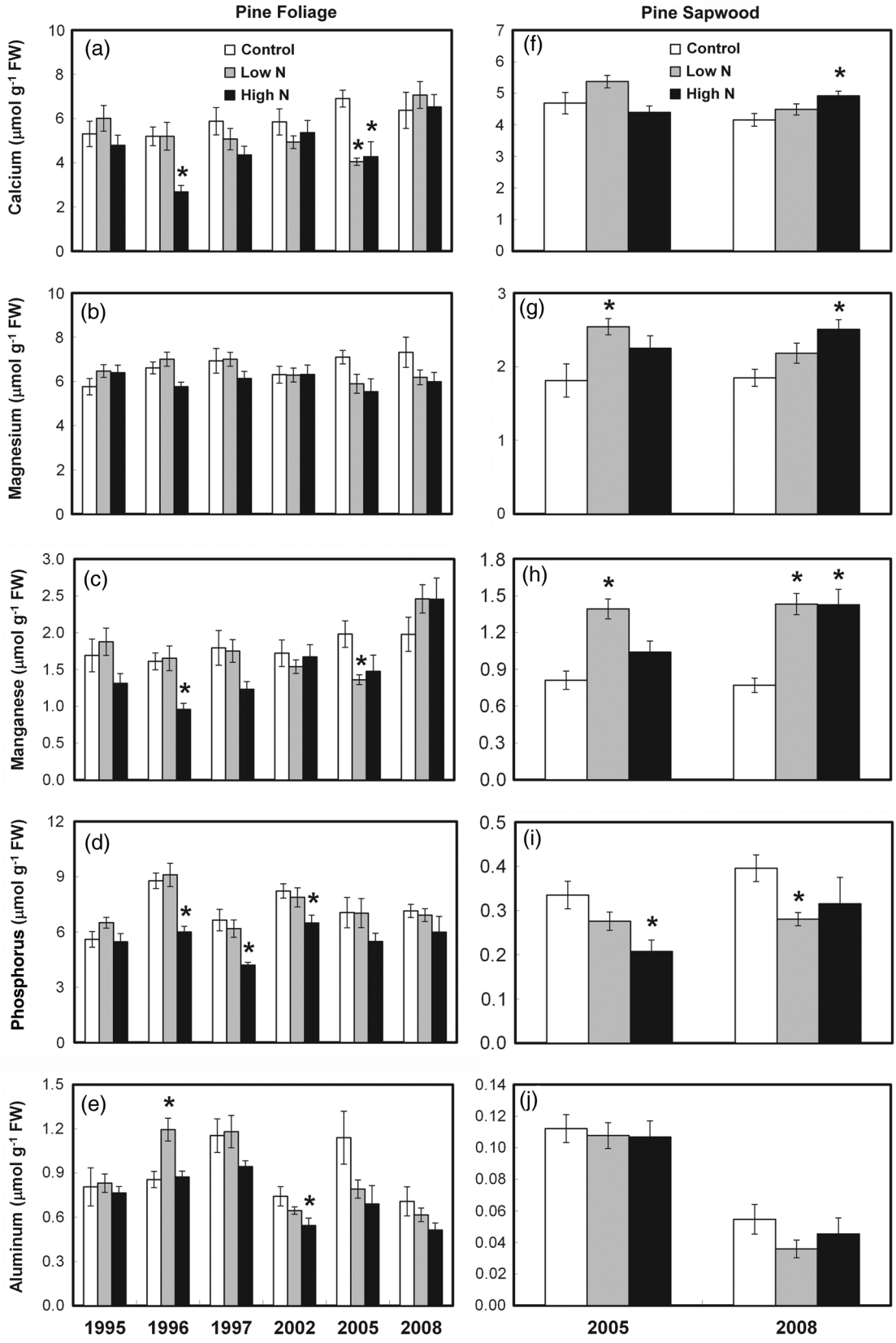

Figure 3. Effect of chronic N additions on soluble inorganic elements in foliage and wood, respectively, in pine: calcium (a and f), magnesium (b and g), manganese (c and h), phosphorus (d and i) and aluminum (e and j). For additional details refer to the legend of Figure 1.

Table 1. Mortality data for pine and oak from 1988 to 2008. Mortality numbers were calculated each year as percent dead trees compared with the number of live trees at the beginning of the study. Trees that were <5 cm DBH in size at the beginning of the study (1988) but grew to >5 cm by summer 2008 were included in the total number of trees in inventory of 1988–2008. The numbers shown in parentheses next to treatments represent the total number of trees used for mortality study.

% Mortality								
DBH measured	October 1988	November 1990	November 1993	February 1997	March 2000	April 2003	April 2006	October 2008
Pine (*n*)								
Control (87)	0.0	0.0	2.3	10.3	21.8	26.4	36.8	49.4
Low N (117)	0.0	0.0	1.7	21.4	33.3	41.9	47.9	57.3
High N (94)	0.0	0.0	3.2	11.7	35.1	68.1	84.0	88.3
Oak (*n*)								
Control (97)	0.0	1.0	7.2	18.6	22.7	25.8	29.9	36.1
Low N (72)	0.0	1.4	6.9	12.5	16.7	22.2	25.0	29.2
High N (105)	0.0	0.0	5.7	12.4	27.6	37.1	46.7	50.5

in duration of growing season, extreme environmental events and insect and/or pathogen invasions. The dominant stress inducing factor which may vary between experimental plots (experimental forest) or field sites (regional study) and is consistently present year after year (e.g., N in present study) becomes the main cause of significant metabolic changes that are over and above the inter-annual variations and are easily detectable. These significant changes have the potential to be used as metabolic markers for stress detection. Since all biological processes are dynamic and often interconnected within cells, the direct effects of one or more stress factors on one component of this network end up indirectly affecting other connected pathways in a positive or negative direction. That is why a suite of non-specific metabolic markers (that concurrently respond to more than one stress factor), and not just a single marker, would be valuable for field studies where trees at a given study site are often exposed to multiple stressors (e.g., acidic deposition, N, Al etc.).

Effects of chronic N additions

In spite of their importance, so far there have been limited studies on the long-term metabolic changes (spanning over decades) in foliage of forest trees in response to multi-year N exposure. Additionally, the effects of stress (including chronic N exposure) on sapwood metabolism have been rarely reported. Besides the complexities of inter-annual variations, significant changes in metabolite concentrations as well as foliar and soil solution chemistry were observed for both stands although the extent of effect on the type of parameter (metabolites, foliar and soil chemistry) varied both annually and over the period of treatments. The impact of the physiological stress due to higher N was more pronounced for pine as compared with oaks, which although not tested directly in this study may also be related to differences between the species and the land-use histories of the two sites. Changes in the concentrations of select metabolites, including Put, were used as markers for assessment of the current health status where chronic N addition was the dominant stress (Minocha et al. 2000 and in present study). Putrescine in foliage was also found to be a reliable marker of Ca deficiency in a prior study conducted at several sites across the Northeastern USA with varying levels of soil Ca deficiency and Al toxicity (Minocha et al. 1997). In this study, the stress from these factors varied to a greater extent than the normal range of inter-annual variation among the sites. Specific details for each stand are briefly described here.

Pine stand

Increased Put content in red pine needles in the LN- and HN-amended vs the control plots signifies that these trees continued to be under physiological stress since our last report (Minocha et al. 2000). The N-amended trees apparently stored the excess N in the form of organic compounds in order to remove ammonia from their immediate environment. The effects of N treatments on Put in pines were much more pronounced in 1995, 1997 and 2002 relative to other years. Such patterns of change were not as evident for control plots, suggesting that chronic N additions affected how the trees respond to annual environmental conditions, such as the drought that occurred in 1995 at this site. Previously, Ca addition at Hubbard Brook Experimental Forest, NH, USA has been shown to change evapotranspiration rates (Green et al. 2013 and references therein), which may cause other indirect effects (e.g., drought). The intensity of such effects obviously varies with local climate and species type; e.g., the pines responded differently from oaks described later. The lack of prominent changes in Spd is consistent with observations in other systems where in spite of wide variations in cellular Put levels, Spd fluctuates within a much narrower range regardless of tissue type or species because it is a tightly regulated metabolite in cells (Bhatnagar et al. 2001, Minocha et al. 2010, Majumdar et al. 2013 and references therein; Minocha et al. 2014).

In general, the amount of N partitioned into foliar AAs was highest in 2002 when compared with other years; with the exceptions of Pro, all AAs were higher in response to N treatments. In

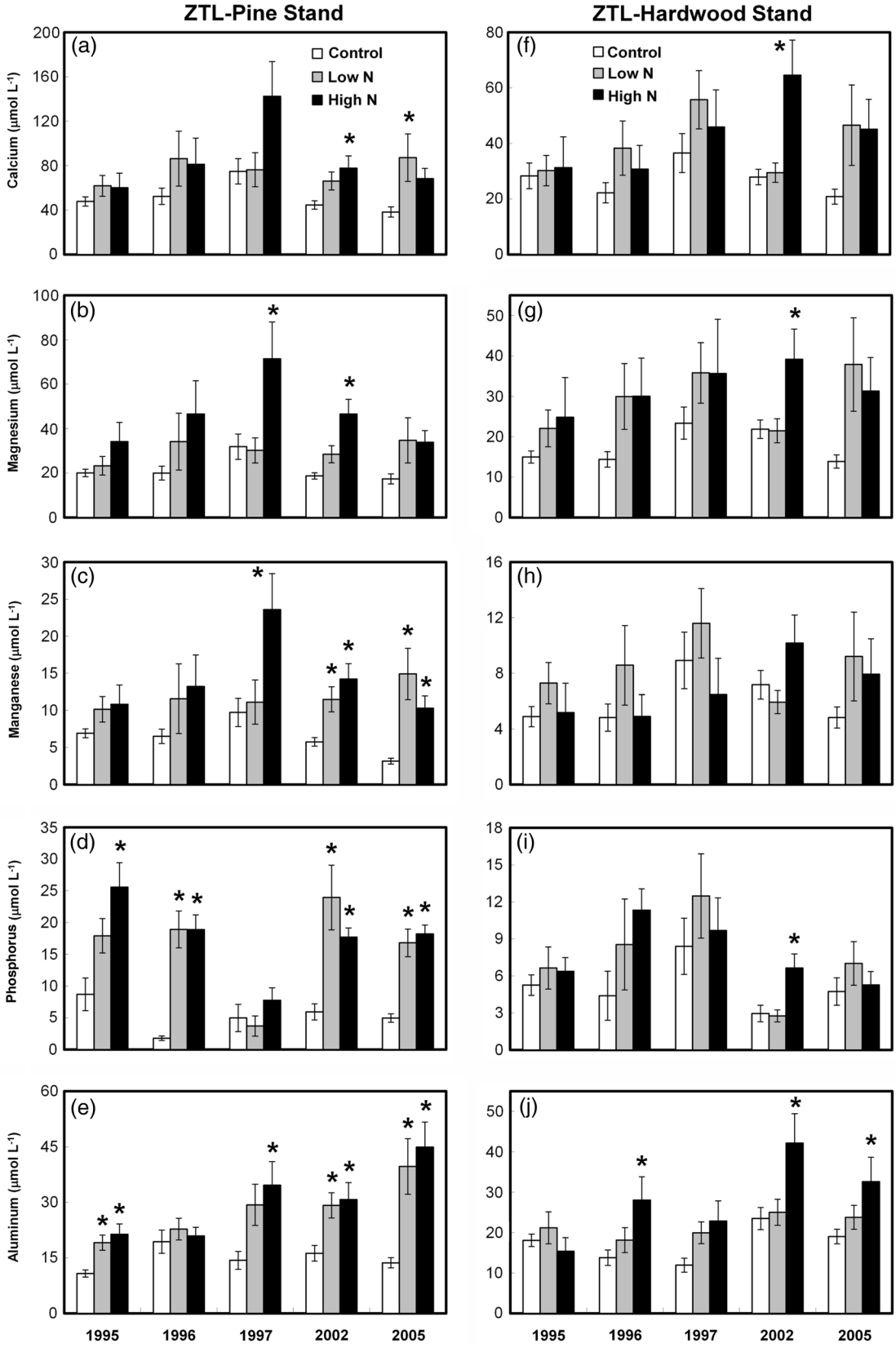

Figure 4. Effect of chronic N additions on ZTL soil solution inorganic elements in pine and hardwood stands, respectively: calcium (a and f), magnesium (b and g), manganese (c and h), phosphorus (d and i) and aluminum (e and j). Data are mean ± SE of 15–20 samples collected per treatment from 1995 to 1997, and 25–50 for 2002 and 2005. The asterisks denote significant differences from the control treatment at $P \leq 0.05$.

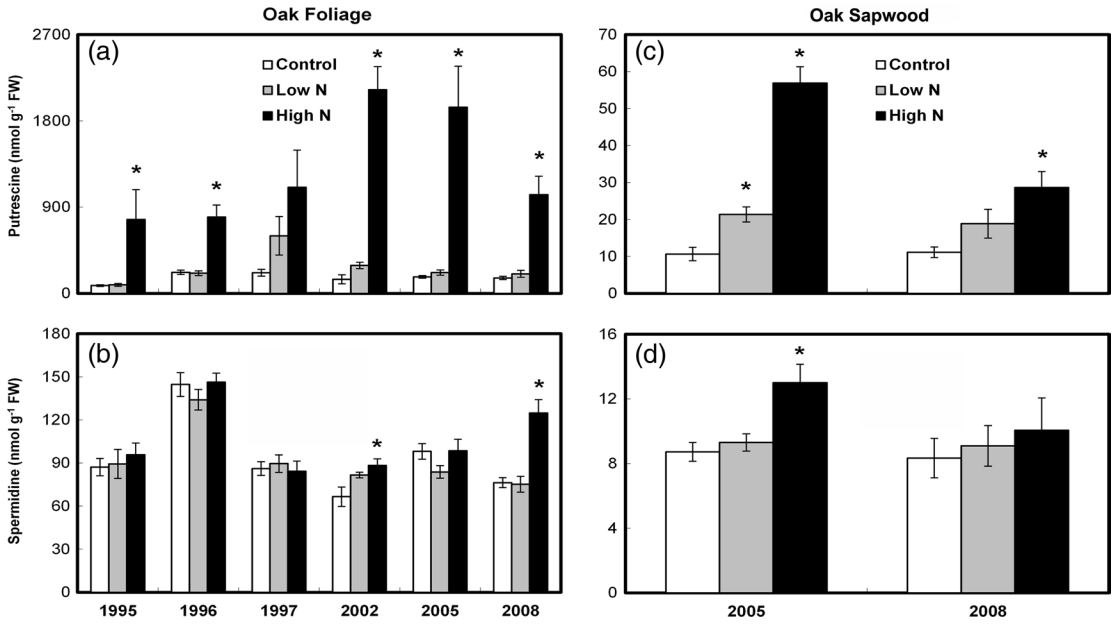

Figure 5. Effect of chronic N additions on foliar (a and b) and sapwood (c and d): putrescine and spermidine in oak. Data presented for each treatment for foliage are mean ± SE where $n = 10$ from 1995 to 2008, with one exception of 1997 where $n = 5$. Data presented for each treatment for sapwood are mean ± SE, $n = 10$. The asterisks denote significant differences from the control treatment at $P \leq 0.05$.

2005, most effects were seen only with LN, and by 2008 there was no longer an effect of HN on AAs in the pines. These results are consistent with those of Bauer et al. (2004), who also reported an accumulation of AAs with HN in the pine stand at Harvard Forest in 2000. Previous studies have shown that the metabolite GABA acts as a stress signaling compound that indicates a C : N imbalance (Bouche and Fromm 2004, Roberts 2007). In the present study, GABA accumulated in the pine foliage, which could be indicative of an imbalance in C : N. Glutamic acid, Put (both are the direct precursors for GABA synthesis) and Gln, the very first product from N assimilation in cells, showed increases similar to GABA.

Accompanying these metabolic changes was a decrease in foliar Ca and P mostly in response to HN and only for some years. The ZTL soil solution data, which showed an increase in these two elements for both N treatments, suggest that they were being lost to groundwater from N-treated plots until 2005, the date of the last ZTL collection. It is hard to tell whether this intermittent decrease in nutrients did act as an additional stress factor that lead to the accumulation of N metabolites since the threshold concentrations of Ca in this species are not known.

Environmental stress does not affect sapwood metabolism in the same manner as it does the foliage. A possible explanation is that while foliage is directly exposed to environmental stress factors, wood is relatively protected from it. Whereas foliar stress metabolism reflects current health status, the sapwood metabolism on the other hand may reveal the net effects of this N enrichment on sapwood N storage and nutritional status; however, hardly any metabolic work has been done with sapwood. A decrease in cumulative wood biomass with chronic N application was previously reported at Harvard Forest (Magill et al. 2004). In general, the concentrations of PAs, AAs and inorganic ions in sapwood were extremely low relative to foliage; thus the stress signal is sometimes harder to detect. The increase in Put in sapwood in 2005 indicated the presence of excess N in this tissue. In general, not enough is known at present about sapwood metabolism and thus no major conclusions can be drawn from these data.

By 2005, the intensity of N effects on most foliar metabolites and nutrients had declined in the HN plot. We hypothesize that since a large number of pine trees in the HN plot died by 2002 (Magill et al. 2004), the surviving trees that we sampled thereafter were either benefiting from the release of nutrients from dead trees and/or were inherently more resistant to chronic N exposure. Concentrations of Ca or Mg in ZTL soil solution varied by treatment in some years, but concentrations did not change dramatically in 2005, meaning that soils in the HN pine plot had already reached N saturation by this time and were not able to retain any more N. Since, in general, under Ca limiting conditions, addition of Ca has a positive relationship with wood production in trees (Demarty et al. 1984, Halman et al. 2013), the observed increase in Ca and Mg concentrations in sapwood with HN in 2008 may be indicative of a positive response in trees that survived and acclimated to high N. These arguments are supported by mortality data from this stand revealing that the rate of mortality was very low during the period of 2006–08 for high N (Table 1).

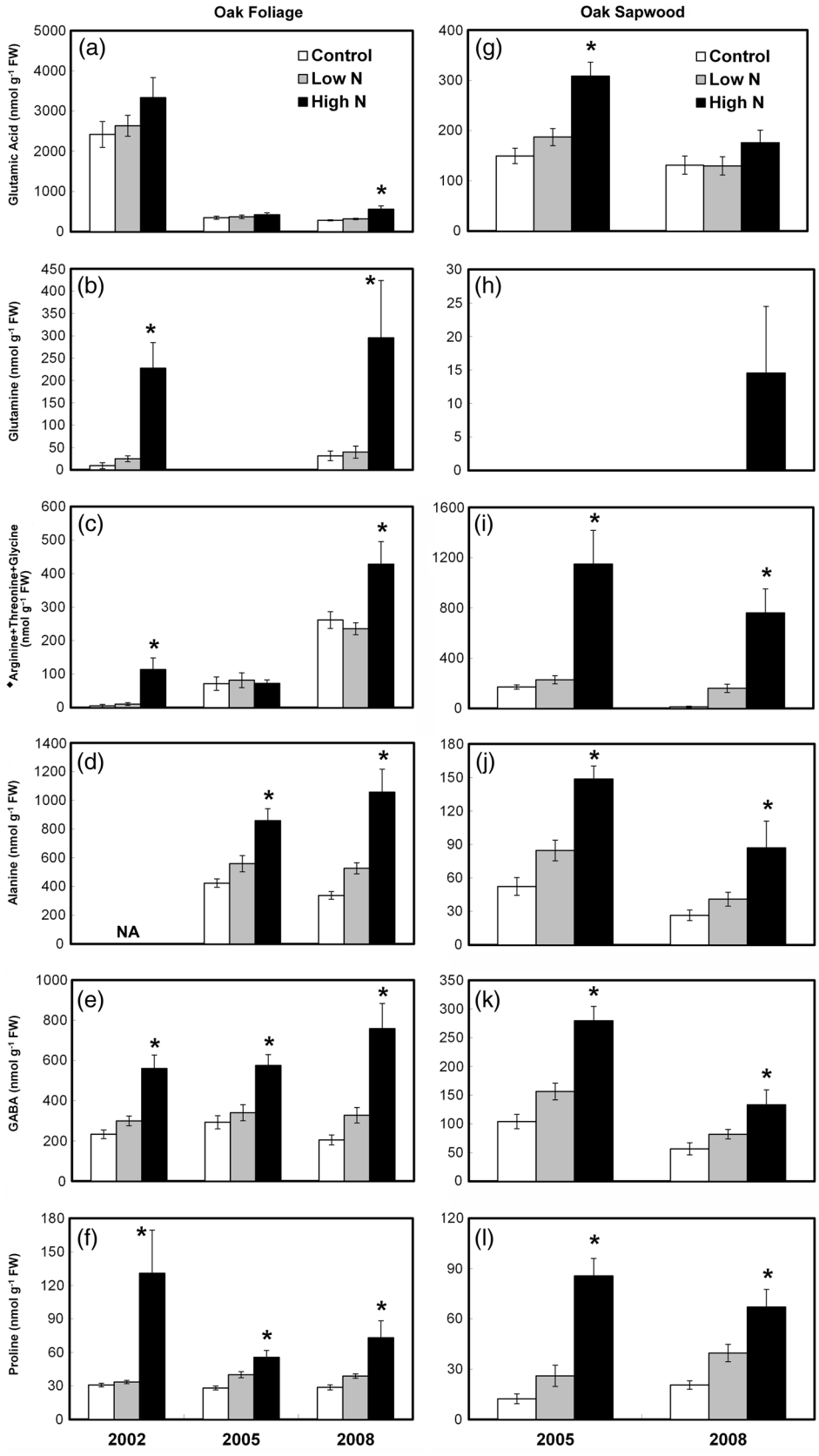

Figure 6. Effect of chronic N additions on foliar and sapwood amino acids, respectively, in oak: glutamic acid (a and g), glutamine (b and h), arginine + threonine + glycine (c and i), alanine (d and j), GABA (e and k) and proline (f and l). NA stands for alanine not available in 2002. For additional details refer to the legend of Figure 5.

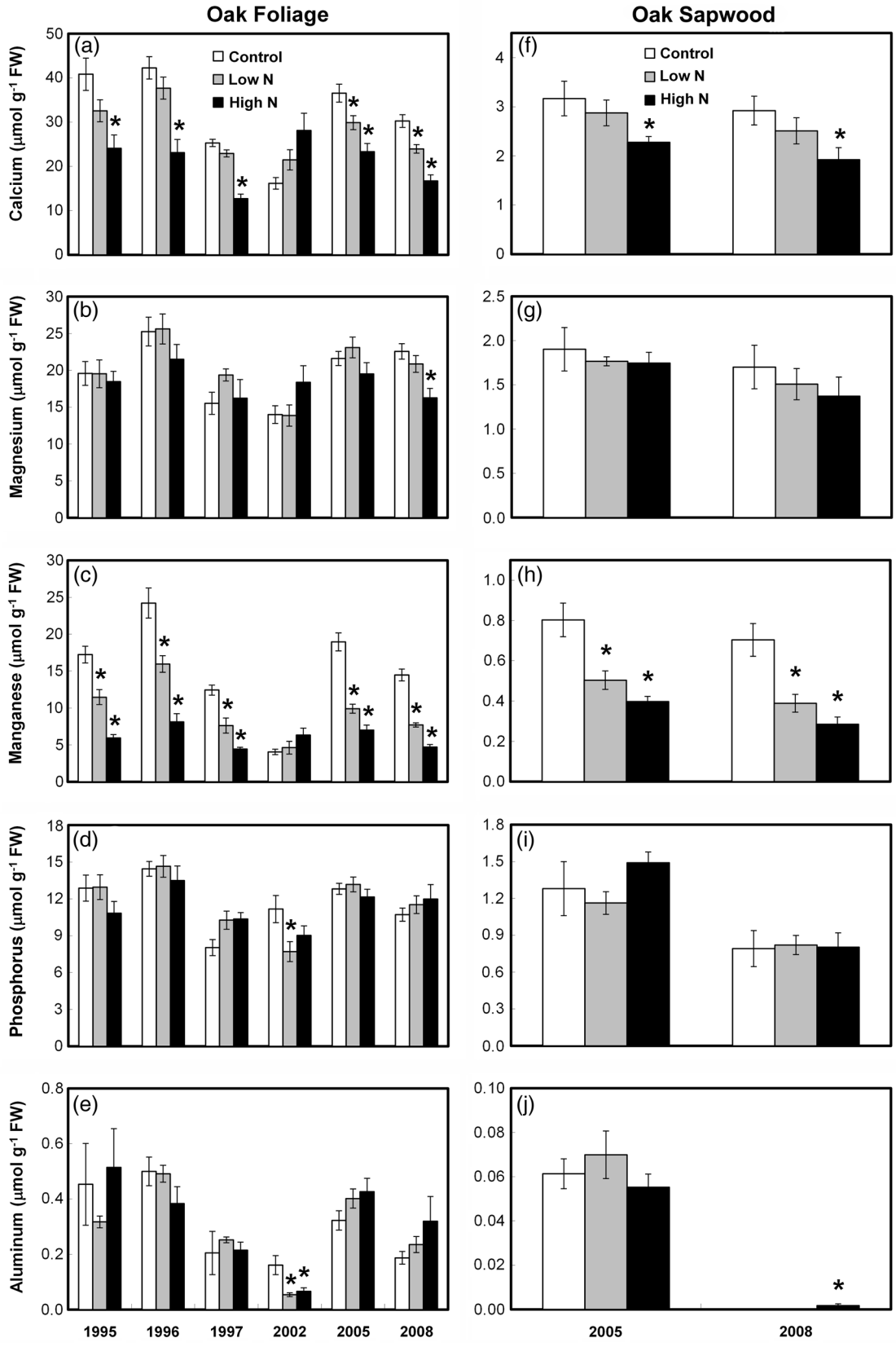

Figure 7. Effect of chronic N additions on soluble inorganic elements in foliage and sapwood, respectively, in oak: calcium (a and f), magnesium (b and g), manganese (c and h), phosphorus (d and i) and aluminum (e and j). For additional details refer to the legend of Figure 5.

Hardwood stand

In contrast to pine, it appears that oaks were able to tolerate the NH_4NO_3 treatments for over a decade. In fact, oaks growing in the LN plot showed little or no changes in N metabolites over the entire period of study. This finding was further corroborated by the mortality data over the treatment period and the findings of Magill et al. (2004) that LN treatment actually had beneficial effects on the health of oak trees. Previously, we reported higher soil bacterial diversity with both LN and HN treatments from the same plots, with the LN treatment being more affected (Turlapati et al. 2013). A direct link between greater bacterial diversity observed in 2009 and higher tree productivity observed by Magill et al. (2004) in the LN plot is plausible, but the mechanisms are unknown. Within the HN plot, in contrast, the accumulations of Put, Spd and most AAs in both foliage and sapwood by 2008 suggest that N in the HN plot had accumulated to a level either close to or higher than the tolerance limits of oaks.

Critical load of any element is known to change with growth conditions and thus a range is often used when referring to this concept. Lack of information on the Ca sufficiency range for oak makes it harder to evaluate the effects of consistently lower concentration of foliar and sapwood Ca itself in HN trees on the increase in their Put content, and on tree health. In addition, chronic N amendments also increase PAs and AAs in order to store excess N. If it were an issue at this site, Ca deficiency (possibly caused from indirect effects of this treatment) would have additive effects on metabolism that would be hard to single out. The same explanation holds for the pine stand. There was a high degree of variability in ZTL soil solution chemistry for the hardwood stand. While a trend towards an increase in nutrient leaching with N amendments was observed, it was significant only in 2002. By 2009, soil chemistry data of hardwood plots also exhibited no change in most inorganic ions with N treatments compared with control (Turlapati et al. 2013). However, 2009 soil samples did have significantly lower concentrations of PAs and AAs in N treatment plots (Frey et al. 2014). Therefore, soil nutrition does not seem to be the driving force behind these metabolic changes. Rather, it is being driven by N accumulation, which was beneficial for over a decade especially for oak in the LN plot (Magill et al. 2004). Energy costs of the synthesis of extra stress-related metabolites are likely to be part of the reason for the long-term decline in growth and productivity in the HN plot since 2003 (Ferrenberg et al. 2015). A concomitant decrease in Ca and Mn with an increase in oak mortality may indicate that Ca and/or Mn deficiency may be responsible for this mortality.

Relationship of metabolic markers and growth

Although indicative of current health, metabolic data are not capable of directly predicting growth rates or mortality of trees. There are several reasons for this including the following two. First, these data are often presented by physiologists on a per gram weight basis, thus neglecting the contribution of total foliar and root biomass to stand-level conditions. This leads to a lack of connection between net resources used/needed for protection from stress vs growth. Secondly, most stress-related metabolites serve multiple functions within cells besides managing stress, and their presence is required concurrently for the production of other metabolites. Often the metabolites that are involved in stress responses are also needed for growth and development (e.g., the three common PAs, essential AAs, carbohydrates and organic acids). For example, PAs are needed for critical biological processes like DNA replication, cell division and embryo development, etc. (Minocha et al. 1991, 2004). The use of such metabolites in one metabolic pathway sometimes limits their use in others; their partitioning may vary with time and conditions of growth. Based on the data presented here, we suggest that eventually, the need for stress protection supersedes growth processes and in turn leads to poor health and mortality, which happens at different rates for individual trees within the same plot depending upon many factors, e.g., genetic variability, tree age and pathogen infections, etc. These complexities make it harder to relate such markers directly with growth. Variations in annual growth rates for hardwoods at the Harvard Forest site have been reported (Urbanski et al. 2007) and as expected, there does not appear to be a direct correlation between these growth data and the metabolic markers for the hardwood control plot.

The fact that metabolic changes in response to environmental stressors are detectable and measurable prior to the appearance of visual symptoms in the trees (e.g., yellowing of needles, crown thinning and mortality as seen in the HN plots at Harvard Forest) makes them suitable for developing metabolic monitoring tool kits for early stress detection and management strategies. These findings are substantiated by soil solution and foliar nutrient data. Future studies should focus on understanding how feedback mechanisms between belowground (microbial) and aboveground responses ultimately control tree growth and forest productivity, and on enhancing our understanding of site-to-site variability in the response of various ecosystems to similar N amendments.

Conclusions

The results presented here demonstrate that regardless of the inter-annual variations in foliar biochemistry of pines and oaks (perhaps due to short-term changes in climatic conditions), both low and high N applications resulted in greater indices of physiological stress to the pines than the oaks. This trend appears to have stopped for pine after 2005 in the case of the HN treatment where the remaining trees were not showing any signs of stress. On the other hand, for oaks, until 2002 no negative effects on metabolic markers were seen with LN treatment. In fact, the LN treatment caused a substantial increase in live wood

biomass, probably because these stands were initially N-limited (Magill et al. 2004). In contrast, the metabolic data from foliage and sapwood clearly revealed a negative impact of HN on the trees and suggest that the HN hardwood plot may continue experiencing this slow decline in productivity if conditions of excess N persist. In the absence of knowledge for Ca and Mn sufficiency levels for oaks at Harvard Forest, a concomitant decrease in Ca and Mn with an increase in oak mortality may indicate that Ca and/or Mn deficiency is partially responsible for this mortality.

In the present study, while the trends in ion chemistry of the foliage and sapwood for oak were similar, for pines they were not. Environmental stress does not affect the metabolism of sapwood in the same manner as it does for foliage because sapwood is relatively protected. Whereas foliar stress metabolism reflects the current health status, sapwood metabolism may reveal the net effects of N enrichment on sapwood N storage and nutritional status.

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Conflict of interest

None declared.

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