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Effects of excess Nitrogen deposition on Rubus spp. (raspberry) within a central Appalachian hardwood forest

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EFFECTS OF EXCESS NITROGEN DEPOSITION ON *RUBUS* SPP. (RASPBERRY) WITHIN A CENTRAL APPALACHIAN HARDWOOD FOREST

A thesis submitted to
the Graduate College of
Marshall University
In partial fulfillment of
the requirements for the degree of
Master of Science
in
Biological Sciences
By
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ABSTRACT

This thesis contributes to on-going research at Marshall University on effects of excess nitrogen (N) deposition on hardwood forests. Excess N can decrease plant biodiversity and enhance loss of nutrients (e.g., Ca^{++}). Preliminary results have suggested that excess N has increased cover of *Rubus* (blackberry) in the herbaceous layer. The purpose of this study was to quantify the response of *Rubus* to the N treatment, relating *Rubus* cover to species richness and using foliar analysis to examine effects on nutrient availability. It is expected that increased *Rubus* will decrease biodiversity by eliminating N-efficient species, and that added N will simultaneously increase foliar N and decrease foliar cations. Species richness decreased significantly with N-mediated increases in *Rubus* cover. Foliar tissue contained higher N and lower Ca^{++} from added N, suggesting loss via leaching with NO_3^-. Observed increases of *Rubus* cover under a closed canopy is novel, considering its intolerance of shade

CHAPTER 1

INTRODUCTION

N Deposition

Nitrogen (N) deposition is increasing globally due to increases in high-energy combustion of fossil fuels and use of N-based fertilizers for agriculture. Despite efforts to regulate anthropogenic increases of N deposition (Clean Air Act of 1990), rates are not expected to decrease in the near future. Bobbink et al. (2010) predicted that N deposition in the eastern United States would rise from 10-15 kg N ha^{-1} yr^{-1} in 2000 to 20-30 kg N ha^{-1} yr^{-1} in 2030. Impacts from anthropogenic increases of N on aquatic and terrestrial ecosystems continue to be an area of emerging ecological concern, particularly for the ability of these ecosystems to absorb and store N (Galloway et al. 2004, 2008; Phoenix et al., 2006; Barbour et al., 1999). Excess N deposition has the potential to saturate the capacity for N retention in a variety of forest ecosystems, including those of the eastern hardwood region. Nitrogen saturated soils in these regions can possibly alter nutrient processing rates, acidify soils, and lead to forest decline (Magill et al., 1997, 2004).

The Herbaceous Layer

Although the herbaceous layer in an eastern hardwood forest of the U.S. makes up ~0.1% of the aboveground biomass, it can represent up to 90% of plant biodiversity in this ecosystem (Gilliam 2007). Biodiversity in hardwood forest herbaceous layers is highly dependent on spatial and temporal heterogeneity in soil resources and light availability (Kold and Diekmann 2004; Bartels and Chen, 2010; Gilliam and Dick, 2010; Costanza et al., 2011; Gilliam and Roberts, 2014). Spatial and temporal variation in environmental conditions can increase biodiversity because heterogeneous ecosystems promote coexistence between more species than homogeneous ecosystems by altering the interspecific competition (Maestre et al. 2012; Hutchings et al. 2003).

The herbaceous layer is particularly sensitive to fluctuations in N deposition, where excess N deposition can alter species composition and decrease biodiversity (Gilliam et al. 2006). Impacts on forest ecosystems arise when rates of N deposition exceed biotic demand for N, known as N saturation (Aber et al. 1998). The atmosphere is comprised of 78% N_2 gas that is nonreactive with virtually all living organisms (Gilliam 2014a). Conversion from unreactive N to reactive N, particularly NO_3^- and NH_4^+, has increased due to increases in anthropogenic N deposition (Galloway et al., 2004, 2008). Consequently, NO_3^- is the dominant form of available N for plant uptake when soils of hardwood forest ecosystems become N-saturated. Aber et al. (1998) predicted that N saturation increases N availability over time resulting in decreased nitrogen limitation for biological function. Nitrogen-saturated soils can enhance growth of nitrophilic plant species, competitively displacing N-efficient plant species. In undisturbed hardwood forest ecosystems, N-efficient plant species out-compete high N-requiring plant species through uptake of enough inorganic N to keep soil concentrations below the level of high N-requiring plant species (Gilliam 2014a).

Excess NO_3^- in N saturated soils has the potential to leach from mineral soils while simultaneously promoting loss of nutrient cation (Ca^{++}, Mg^{++}, K^+). Leaching of base cations, resulting from excess soil NO_3^-, decreases soil pH (Gilliam and Adams, 2011). Highly acidic soils have low base cation availability from the result of H^+-enhanced nutrient cation leaching in N-saturated soils (Moore and Houle, 2013). In a study on environmental heterogeneity in North Carolina and South Carolina, Costanza et al. (2011) found mean pH as the best predictor for species richness on a local scale (plots) and low pH was associated with low species richness. Acidic soils promote mobility of Al^{+++} and other heavy metals that can inhibit root growth, promote senescence of foliar tissue, inhibit uptake of other nutrients, and promote forest decline (Godbold et al., 1988; Shortle and Smith, 1988; Pitelka and Raynal, 1989; Boudot et al., 1994; Nagajyoti et al., 2010).

N Homogeneity Hypothesis

Gilliam (2006) developed the *N homogeneity hypothesis*, describing negative effects of excess N deposition through making a connection between decreases in spatial heterogeneity of soil N dynamics and decreases in species diversity for impacted forests. Spatial heterogeneity in soil resources maintains high biodiversity in a variety of herbaceous communities (Bartels and

Chen, 2010; Gilliam and Dick, 2010; Costanza et al., 2011; Lu et al., 2012; Reinecke et al., 2014). Species composition depends highly on spatial and temporal variability of soil nutrients and particularly, soil N dynamics (Gilliam and Adams, 2001; Bengston et al. 2006; Gilliam and Dick, 2010). Spatial heterogeneity of essential nutrients in soils, such as N, can increase the number of species in a community through decreasing competitive interactions. Typically, spatial heterogeneity of N processing is high in hardwood forest soils due to many influences, such as spatial variability in decomposing litter (Gilliam 2006).

Nitrogen saturated soils resulting from excess N deposition alter N cycling by shifting from processing organic N to dominance in the mobility of NO_3^-. As mentioned above, NO_3^- in excess of plant requirements readily leaches from mineral soil while simultaneously inducing leaching of other nutrient cations. High NO_3^- mobility in N-saturated soils decreases availability of essential nutrients (Ca^{++}, Mg^{++}, K^+) for forest plants. Herbaceous responses to N-saturated soils that are directly influenced by increased NO_3^- typically include decreased richness, impacting overall biodiversity of affected forests. Therefore, N-saturated soils shifting the N cycle to mobility of NO_3^- can potentially homogenize N dynamics, essential nutrient availability, and species composition, ultimately decreasing biodiversity of impacted forest ecosystems.

N/Herbaceous Studies at Fernow Experimental Forest

In 1988, the USDA funded a project to apply an acidification treatment of $(NH_4)_2SO_4$ to a single watershed in Fernow Experimental Forest (FEF), Parsons, WV (Adams et al., 1993). This application of $(NH_4)_2SO_4$ when dissolved in solution becomes acidic by nature. The purpose of this treatment was to address responses at an ecosystem level to increasing acid deposition. It was known that acid deposition had potential to decrease productivity of forested ecosystems, but it was not known to what extent these decreases in productivity were linked to increasing acid deposition alone (Gilliam et al., 1994). The herbaceous layers of forest ecosystems are particularly sensitive to acid deposition, therefore, Gilliam et al. (1994) examined the responses of the herbaceous layer vegetation and soil nutrients to excess acid deposition. They concluded that the acid treatment had not shifted species composition or nutrient concentrations when comparing the treated watershed with reference watersheds; however, there was evidence of higher Al and Fe uptake on the treated watershed, suggesting possible toxicity problems for herbaceous species exposed to excess acid deposition.

The acidification study has now shifted focus toward excess N deposition, even though the same treatment is still being applied. Experimental N treatment to a single watershed at FEF has resulted in an increase in dominance of a rhizomatous clonal species, *Rubus* spp (hereafter, *Rubus*). Following 14 years of excess N treatment at FEF, *Rubus* cover increased from ~1% cover to ~13% cover from 1991 to 2003, while species richness decreased from ~13 species/plot to 10 species/plot from 1994 to 2003. A decrease in a previously dominant herbaceous species, *Viola* spp.¸ has also been associated with this increase in *Rubus* cover. Therefore, the positive response of *Rubus* to N treatment appears to provide a valuable signal for examining the effects of excess N deposition in eastern hardwood forests for this study.

***Rubus* Ecology**

Rubus is a genus within the Rosaceae family that is found throughout temperate zones in North America, Europe, and Asia. *Rubus* is considered a ruderal and clonal species, that often dominates early successional habitats of the eastern hardwood region (Graham et al., 2003; Patamsytë et al., 2005; Donoso and Nyland, 2006; Caplan and Yeakley, 2010 and 2013; Bajcz, 2014). Ruderal species thrive in conditions of disturbed areas (high light, high moisture, and high fertility) and are highly adaptable to changing conditions; these life history traits contribute to a large distribution of *Rubus* (Donoso and Nyland, 2006). Clonal species high adaptability to changing conditions is made possible through vegetative growth via a rhizome. A rhizome root system provides access to spatial variability in soil resources (nutrients and water) and persistence of roots through non-growing seasons. The lateral expanse of clonal species allows increased colonization of adjacent areas for access to spatial resource pools.

The majority of the eastern United States hardwood region is classified as second growth because much of the hardwood forests were harvested in the early 1900s. Harvesting timber promotes rapid vegetative expanse and germination of *Rubus* through increased light and high nutrient availability (particularly N) by opening the overstory canopy (Donoso and Nyland, 2006). *Rubus* is present across the eastern US region partially due to secondary succession after the majority of this hardwood region was harvested for timber in the late 1800s and early 1900s (Donoso and Nyland, 2006). *Rubus* becomes the dominant species within 2-3 years following clearcutting and peaks in vegetation by the fifth year (Donoso and Nyland, 2006). In the seventh year following clearcutting, tree saplings start to grow through the *Rubus* canopy, diminishing high-light conditions, and by the tenth year after clearcutting *Rubus* has almost completely died back (Donoso and Nyland, 2006). *Rubus* is now present under many closed canopies of mature hardwood forest in the eastern US, mainly due to natural disturbances in the form treefall gaps that maintain *Rubus* at ~1-2% cover (Donoso and Nyland, 2006).

Rubus dieback under a closed canopy is particularly due to intolerance of low light conditions (high light compensation point) (Richard and Messier 1996), but seeds can persist in a dormant state for over 100 years (Donoso and Nyland, 2006). Graber and Thompson (1978) found 95% and 90% germination in recovered *Rubus* seeds from soils of 38 year old and 95 year old clearcut stands, respectively. Persistence of *Rubus* seeds allows colonization of disturbed areas, where 40% canopy removal promotes *Rubus* seed germination (Donoso and Nyland, 2006). Further, Richard and Messier (1996) found no *Rubus* growth under a 7% photosynthetic photon flux density (PPFD), but *Rubus* was always present under a 25% PPFD. Therefore, secondary succession has established *Rubus* in eastern hardwood forests, but natural and anthropogenic disturbances have maintained *Rubus*.

When areas are disturbed, higher light conditions become available along with an influx of N to soil solution from decomposing litter for plant absorption. Nitrogen is a macronutrient that is required in relatively high amounts because of its importance in several key biomolecules, including protiens, nucleic acids, and chlorophyll. There are two available forms of N for plant absorption, NO_3^- and NH_4^+. Nitrate is readily available under disturbed conditions or when excess N deposition is present in a hardwood forest ecosystem. When NO_3^- is the prominent form of N available, *Rubus* seed germination and biomass increases (Claussen and Lenz, 1999; Donoso and Nyland, 2006). *Rubus* is considered a nitrophilous species, where high levels of N promote *Rubus* dominance over other species (Hedwall et al., 2011). Further supporting the nitrophilous nature of *Rubus*, Lautenshlager (1999) found *Rubus* biomass to increase when N was the only added nutrient. Therefore, it can be expected that excess N deposition will significantly increase *Rubus* cover when *Rubus* was previously established in early succession.

Purpose and Hypotheses

The purpose of this study was to assess the effects of excess N deposition on the herbaceous layer of a mixed hardwood forest. More specifically, the objectives of this study are to: 1) quantify *Rubus* response to N treatment from 1991 to 2014, 2) relate *Rubus* cover to species richness of the herbaceous layer during this period, and 3) determine effects of N additions on foliar nutrients of *Rubus.* Examining *Rubus* foliar nutrients will provide an indication of impacts that excess N deposition has on nutrient status in a hardwood forest ecosystem. Expected effects of excess N treatment are: 1) excess N deposition will increase *Rubus* cover, 2) increased *Rubus* cover will result in a decrease in biodiversity on the treatment watershed and 3) excess N treatment will increase foliar N of *Rubus* while decreasing foliar cation nutrients.

Study Site

This study was conducted at Fernow Experimental Forest (FEF), Tucker County, West Virginia (39^o 03' N, 79^o 49' W). Fernow Experimental Forest is a montane hardwood forest in the Allegheny Mountain portion of the unglaciated Allegheny Plateau, adjacent to the Monongahela National Forest. Mean annual precipitation for the FEF is approximately 1430 mm yr^{-1}, with higher precipitation occurring during the growing season and with increasing elevation (Gilliam and Adams, 1996). Wetfall N deposition is approximately 10 kg N ha^{-1} yr^{-1} and dry N deposition is approximately 2 kg N ha^{-1} yr^{-1} at FEF. Study site consists of coarse textured Inceptisols (loamy-skeletal, mixed mesic Typic Dystrochrept) of the Berks and Calvin series, sandy loams derived from sandstone (Gilliam et al., 2005).

There were three adjacent watersheds used in the study from FEF: Watershed 3 (WS3), Watershed 4 (WS4), and Watershed 7 (WS7). Watershed 4 served as a reference watershed representing a mixed-aged hardwood stand last cut between 1904 and 1911. Watershed 7 served as another reference watershed representing approximately 45-year-old even-aged hardwood stand that was treated with herbicide six years prior to being let go to grow in 1969. Watershed 3 served as the treatment watershed for this study, receiving three aerial applications beginning in 1988 of $(NH_4)_2SO_4$: 9 kg N ha^{-1} in spring, 17 kg N ha^{-1} in mid-summer, and 9 kg ha^{-1} in late fall, totaling 35 kg ha^{-1} yr^{-1}. WS3 represents a mixed-aged hardwood stand last cut in 1969, removing approximately 90% of tree basal area, except for three hectares of the shade area around the stream channel. Important overstory tree species found on WS3, WS4, and WS7 are *Acer pensylvanicum* L., *Acer saccharum, Betula lenta, Fagus grandifolia, Fraxinus americana* L., *Liriodendron tulipifera, Prunus serotina, Quercus prinus* L., *Quercus rubra, Robinia pseudocacia* L., *and Sassafras albidum* (Nutt.) Nees (Gilliam et al. 1995).

CHAPTER 2

HERB-LAYER STUDY

Introduction

Although herbaceous layers of the eastern hardwood region are often over looked due to the low contribution they provide to overall aboveground biomass, they make up ~90 % of biodiversity in eastern hardwood forests (Gilliam 2007). Herbaceous layer biodiversity is an important component of the ongoing study at the FEF examining effects of excess N deposition on an eastern hardwood forest ecosystem. Excess N deposition has the potential to alter herbaceous communities by decreasing biodiversity. This study compared herb-layer composition between an N treated watershed (WS3) and a reference watershed (WS4) to interpret impacts from excess N deposition on herb-layer composition in the eastern hardwood region.

Material and Methods

Field Sampling

The herbaceous layer was sampled during the peak of the growing season, beginning of July, in seven circular 0.04-ha sampling plots on each of WS3 and WS4 totaling 14 sampling plots on an on-going basis from 1991 to 2014. Each sampling plot center was permanently marked in 1991, and Dr. William J. Peterjohn, West Virginia University, had since established each sampling plot center using GPS coordinates. All vascular plants ≤1m in height were identified and estimated for cover (%) in five 1-m^2 random subplots located in each sampling plot for WS3 and WS4. Subplots were located using a stratified-random polar coordinates method to avoid over sampling the center of plots (Gaiser 1951).

Mineral soil was collected on an on-going basis from 1993 to 2014 for WS3 and WS4 by hand trowel at five points within the sampling plots for each watershed to a depth of 5 cm using methods described in Gilliam et al. (1996). The five mineral soil samples for each sample plot were bulked together and mixed thoroughly to yield a single composite sample that was separated into two polyethylene bags. One bag was incubated *in situ* by burying at a depth of 5 cm below the O horizon for approximately 30 d increments throughout the growing season and the other bag was immediately taken to the laboratory for extraction of NO_3^- and NH_4^+. Bags immediately taken back to the lab and incubated bags were extracted with 1M KCl and analyzed for levels of NO_3^- and NH_4^+ with an AutoAnalyzer 3.

Data Analysis

Data were compared between WS3 (treatment) and WS4 (reference) to examine temporal patterns for mean cover (total cover and *Rubus* cover) and species richness during the peak of the growing season (early to mid-July of each year) (Gilliam et al., 2006). Means were calculated from data collected from the seven plots on each watershed for each year of sampling. Means for *Rubus* cover, total cover, richness, and *Rubus* cover relative to total cover were compared between watersheds and among years of sampling with repeated measures of analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was used to compare total cover and *Rubus* cover yearly means from 1991-present in WS3 and WS4. Mean daily precipitation from April to July was used as the covariate for ANCOVA to determine if excess N deposition affected herbaceous and *Rubus* cover. Generalized linear regression was used for net nitrification versus *Rubus* cover yearly means from 1994-present. Net nitrification was calculated as incubated soil NO_3^- concentrations minus initial soil NO_3^- concentrations.

Detrended correspondence analysis (DCA) was used to assess annual changes in herb layer species composition from 1991-2014. Detrended correspondence analysis was run on total cover data for every species combined, followed by calculating centroids for seven plots/watershed/year. The top 10 weighted species calculated by running DCA were used to assess the influence these species had on annual change in herb layer composition on WS3 and WS4. Pearson rank correlations were used for annual means of DCA axes data, % total cover, % *Rubus* cover, and species richness (species/plot) for WS3 and WS4, separately.

Results and Discussion

Temporal Change in Total Cover

Total % cover was not significantly different among years for WS3 and WS4 for 1991, 1992, and 1994 (Gilliam et al., 2006). Total herb-layer cover significantly increased ($P \leq 0.05$) on

WS3 and WS4 after 1994 (Fig 2.1). Total cover was consistently higher on WS3 compared to WS4, but was only significant ($P \leq 0.05$) for 2009 and 2010 (Figure 2.1). Nitrogen increases total cover because it is presumably responsible for increasing leaf area by increasing cell size and number (Chapin 1980). Although the difference between WS3 and WS4 total cover is not significantly different for all years from 1994-present, consistently higher total cover on WS3 compared to WS4 can be considered an effect of excess N deposition.

The paralleled response among WS3 and WS4 for total cover from 1994-present is a unique feature worth explaining. Precipitation influences total cover of herbaceous communities, where increased precipitation is associated with higher total cover (Anderson et al., 1969). Linear regression for annual means of precipitation versus annual means of total cover indicated significance for watersheds ($P \leq 0.0746$) and an insignificant interaction ($P \leq 0.4645$) between the slopes for WS3 and WS4 (Table 2.1). This lack of significant interaction between slopes allows for comparison of watersheds using precipitation as a covariate in ANCOVA. The model for ANCOVA was significant ($P \leq 0.0821$) for total cover (Table 2.2), therefore, WS3 has on average 11.6% more total cover ($P \leq 0.0694$) than WS4 (Table 2.3). Significantly higher cover on WS3 compared to WS4 is a result of N treatment, where excess N deposition is shifting limiting factors through alleviating N limitations.

Temporal Change in Species Richness

Species richness was not significantly different ($P \leq 0.05$) between watersheds from 1991-1994 (Gilliam et al., 2006). Species richness did not significantly change ($P \geq 0.05$) for the sampling period on WS3 during N treatment either, but there was a decrease from ~13 species/plot in 1994 to ~11 species/plot in 2014 (Fig 2.3). During this time period, species richness on WS4 significantly increased ($P \leq 0.05$) from ~11 species/plot to 15 species/plot, making species richness on WS3 lower than WS4 (Fig 2.3). Species richness normally increases as hardwood forests mature (e.g. Jacquemyn et al., 2001). Therefore, the significantly lower species richness on WS3 due to a temporal decrease may be an effect of excess N deposition.

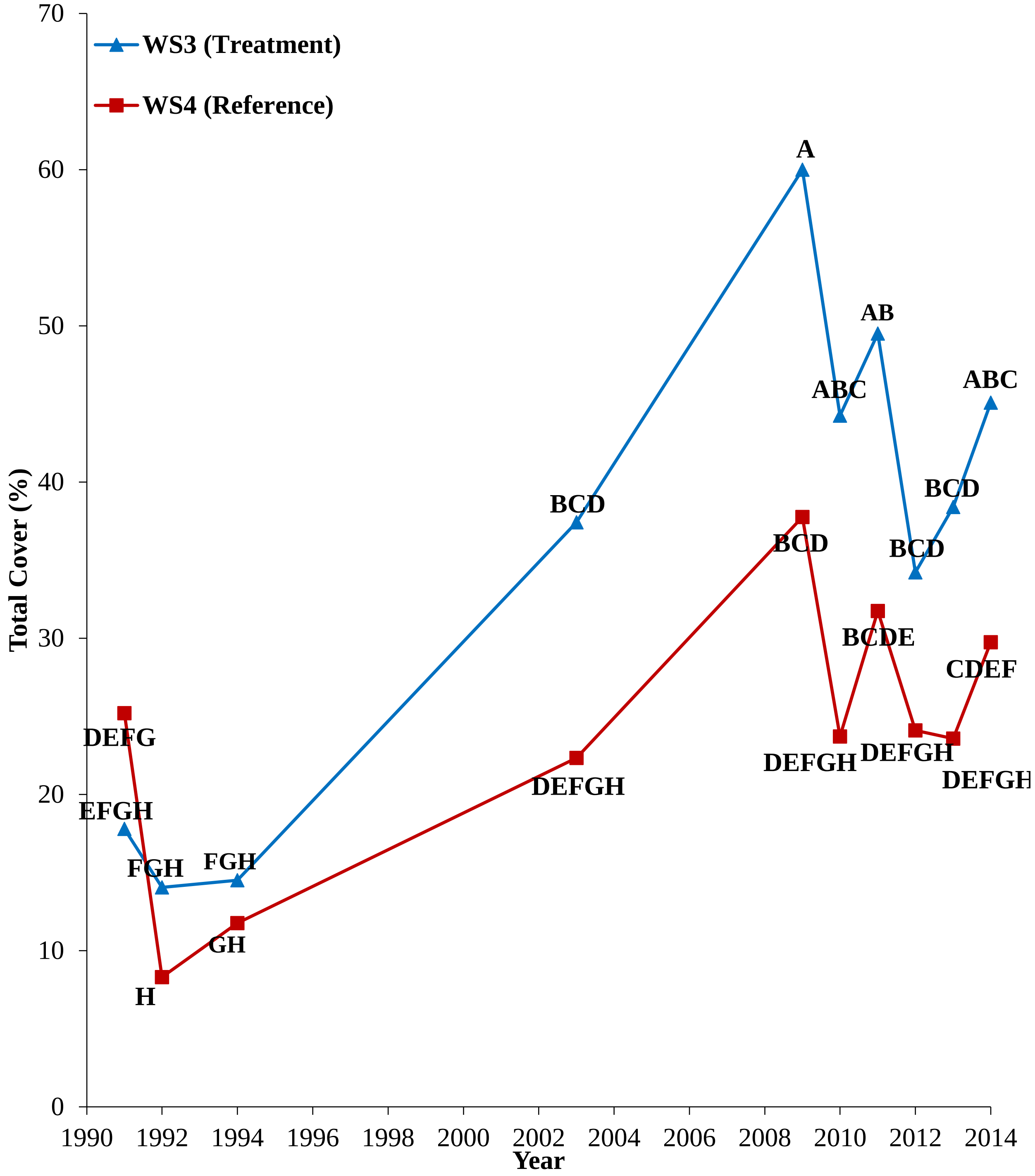

Figure 2.1: Repeated measures of ANOVA for annual means from July sampling for percent total cover for WS3 (treatment) and WS4 (reference) from 1991-present. Means with the same letter are not significantly different ($p \leq 0.05$) between watersheds and among years.

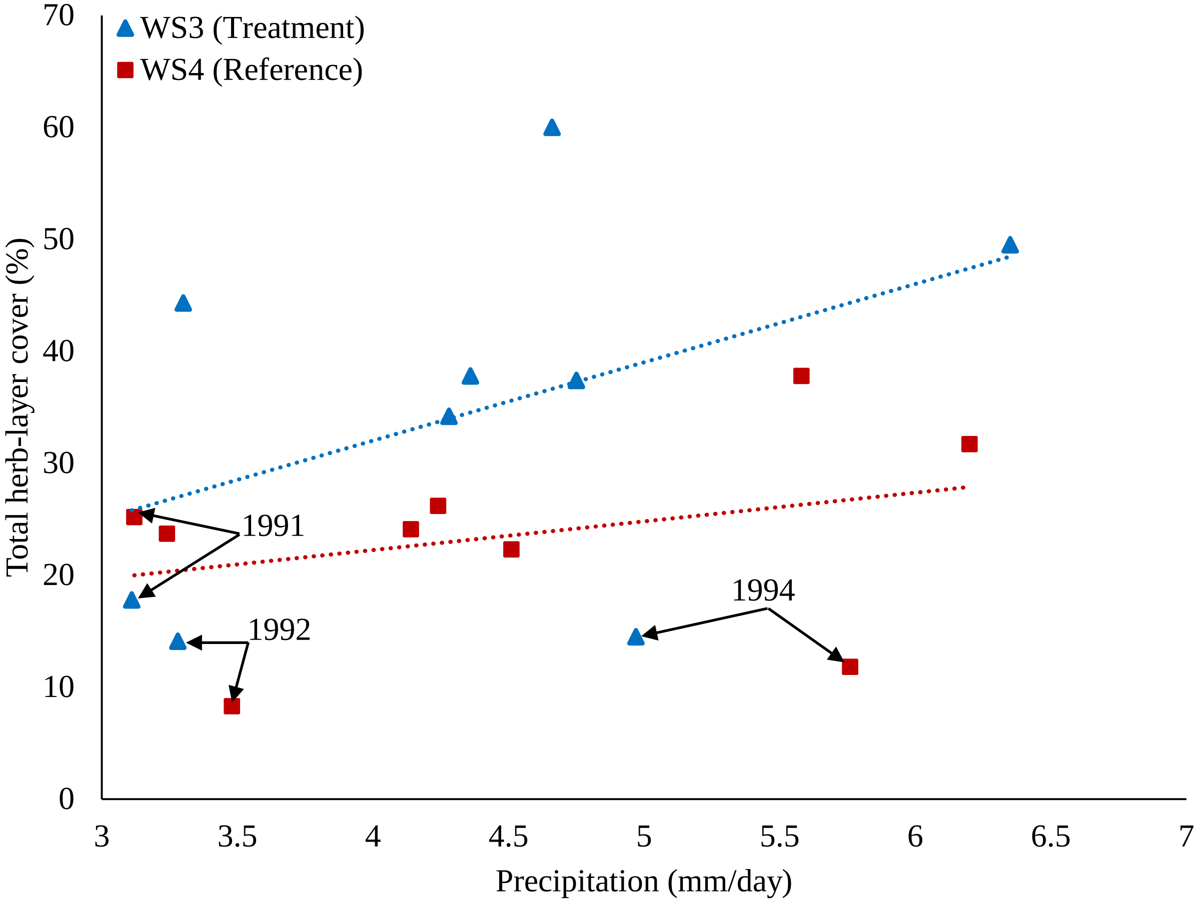

Figure 2.2: Generalized linear regression of annual daily means for precipitation from April to July versus yearly means for total % herb-layer cover from July sampling for WS3 and WS4 from 1991, 1992, 1994, 2003, and 2009-2013 at Fernow Experimental Forest, West Virginia. Lines represent mean linear regression: WS3: $R^2 = 0.1977$, $y = 6.9939x + 4.0464$, $P \leq 0.2305$; WS4: $R^2 = 0.1048$, $y = 2.5664x + 11.972$, $P \leq 0.3955$.

Table 2.1: Generalized linear model for annual daily means of precipitation from April to July versus mean annual total % herb-layer cover from July sampling for WS3 and WS4 at Fernow Experimental Forest, West Virginia.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Model	3	1019.633484	339.877828	2.11	0.1451
Precipitation	1	330.1011505	330.1011505	2.05	0.1744
Watershed	1	598.3895496	598.3895496	3.71	0.0746
Precipitation*Watershed	1	91.1427843	91.1427843	0.57	0.4645

Table 2.2: Analysis of covariance sums of squares for annual daily means of precipitation from April to July versus mean annual total % herb-layer cover from July sampling for WS3 and WS4 at Fernow Experimental Forest, West Virginia.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Model	2	928.490700	464.245350	2.97	0.0821
Precipitation	1	330.1011505	330.1011505	2.11	0.1670
Watershed	1	598.3895496	598.3895496	3.82	0.0694

Table 2.3: Summary of analysis of covariance estimates for annual daily means of precipitation from April to July versus mean annual total % herb-layer cover from July sampling for WS3 and WS4 at Fernow Experimental Forest, West Virginia.

Parameter	Estimate	Standard Error	t Value	Pr > |t|
Intercept	3.08838150	13.56829200	0.23	0.8230
Precipitation	4.55188891	2.88561324	1.58	0.1355
WS3	11.55642062	5.91042363	1.96	0.0694
WS4	0.00000000	.	.	.

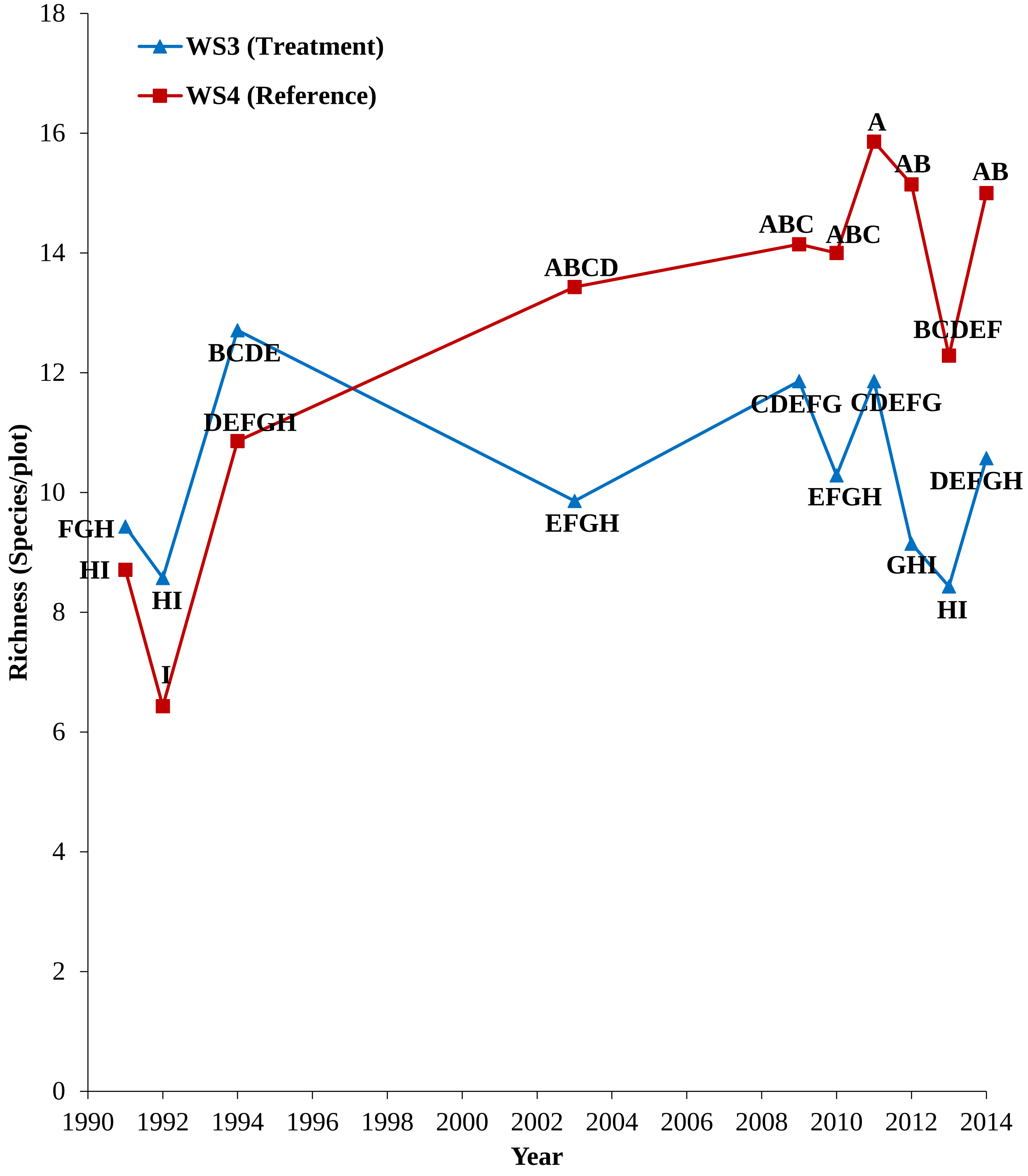

Figure 2.3: Repeated measures of ANOVA for annual means for species richness from July sampling on WS3 (treatment) and WS4 (reference) from 1991-present. Means with the same letter are not significantly different ($p \leq 0.05$) between watersheds and among years.

In hardwood forest ecosystems with high integrity, N-efficient plant species typically out-compete high N-requiring plant species through uptake of enough inorganic N to keep soil concentrations below the level required by nitrophilic plant species (Gilliam 2014b). It was not until after 1994 that the soil of WS3 was determined to be N-saturated from excess N deposition (Gilliam et al., 1995). Nitrogen-saturated soils as a result of excess N deposition are known to cause decreases in species richness (Bobbink et al. 1998, 2010), where the less prevalent nitrophilic species out-compete the higher number of N-efficient species for available N (Gilliam 2014b).

Temporal Change in Rubus Cover

Following five years of treatment to WS3 (1989-1994), no significant differences ($p>0.05$) were found in *Rubus* cover among years or in comparison to reference WS4 (Fig 2.4). Gilliam et al. (2006) indicated *Rubus* importance increasing based on relative cover on WS3 but slightly decreasing in importance on WS4 during this same time period. Preliminary results indicated an increase of *Rubus* presence in the herb-layer when total cover is increasing on WS3 along with a decrease in number of species/plot. This is supported by a significant increase ($P\leq0.05$) in *Rubus* cover relative to total cover from 1991-2014 (Fig 2.5). There have been few studies that have examined effects of experimental N additions on *Rubus*, but some studies have found increased *Rubus* germination, luxury consumption, and increased biomass from excess N deposition (Grader and Thompson 1978; Jobidon 1993; Claussen and Lenz 1999; Lautenshlager 1999; Kula et al. 2012; Bajcz 2014; McDonnell et al. 2014).

Rubus cover on WS3 significantly increased from ~2% cover in 1994 to ~12% cover in 2003 (Fig 2.4). Although *Rubus* growth is favored by conditions that are typical of early succession (e.g. high levels of light, nutrients, and moisture), the ~45 year old closed canopy of WS3 exhibits conditions typical of later succession (e.g low light and moisture). Donoso and Nyland (2006) concluded via literature review that *Rubus* becomes dominant in the first 2-3 years after clearcutting, peaks between 3-5 years, and after 10 years most of the *Rubus* has died off because tree species start developing the overstory stratum. *Rubus* on WS7 followed a similar pattern after clearcutting and 6 years of herbicide treatment. *Rubus* growth peaked at 37% ground cover after 5 years of growth and after 10 years declined to 7% ground cover (Kochenderfer and Wendel, 1983).

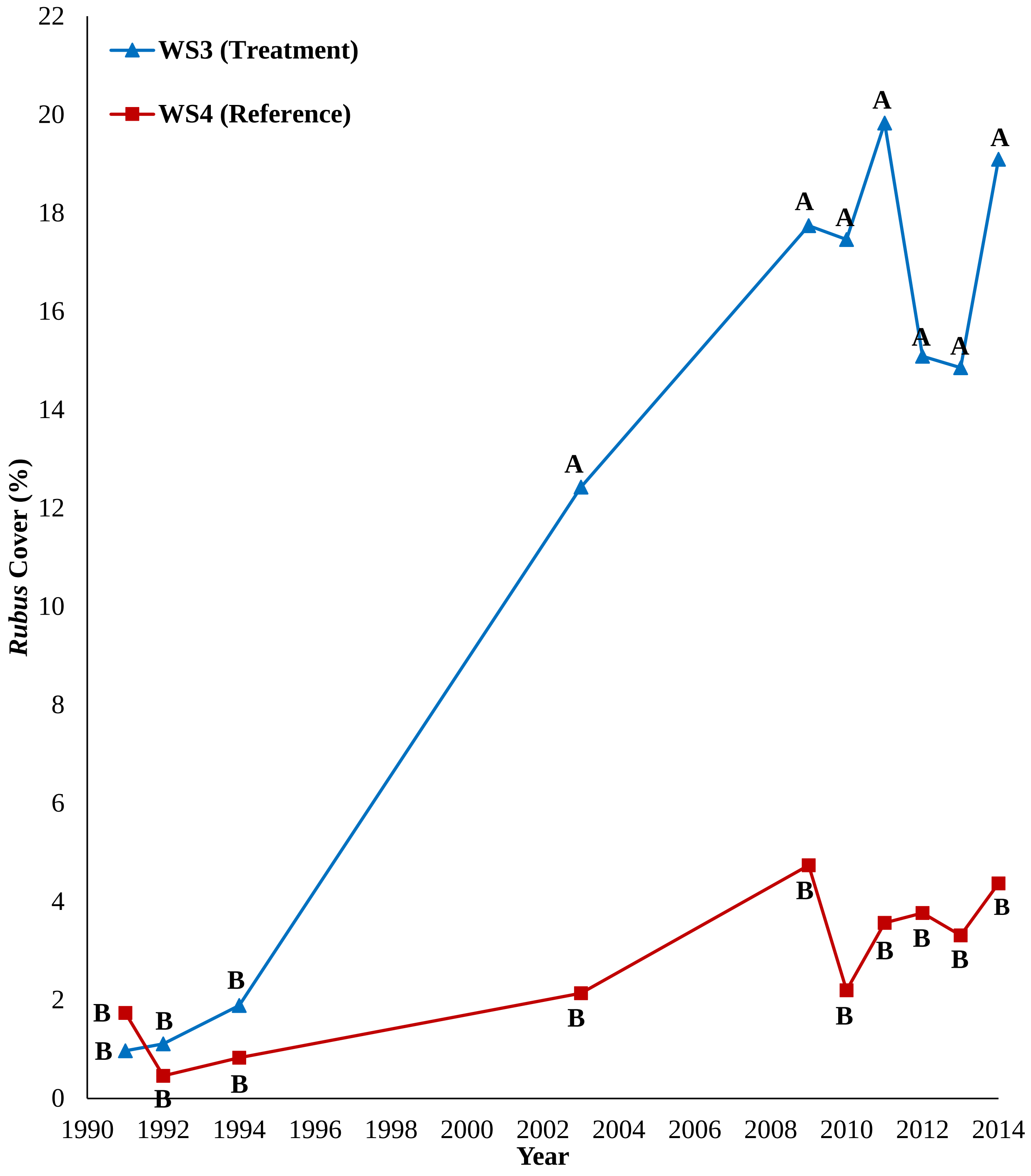

Figure 2.4: Repeated measures of ANOVA for annual means for percent *Rubus* cover from July sampling for WS3 (treatment) and WS4 (reference) from 1991-present. Means with the same letter are not significantly different ($p \leq 0.05$) between watersheds and among years.

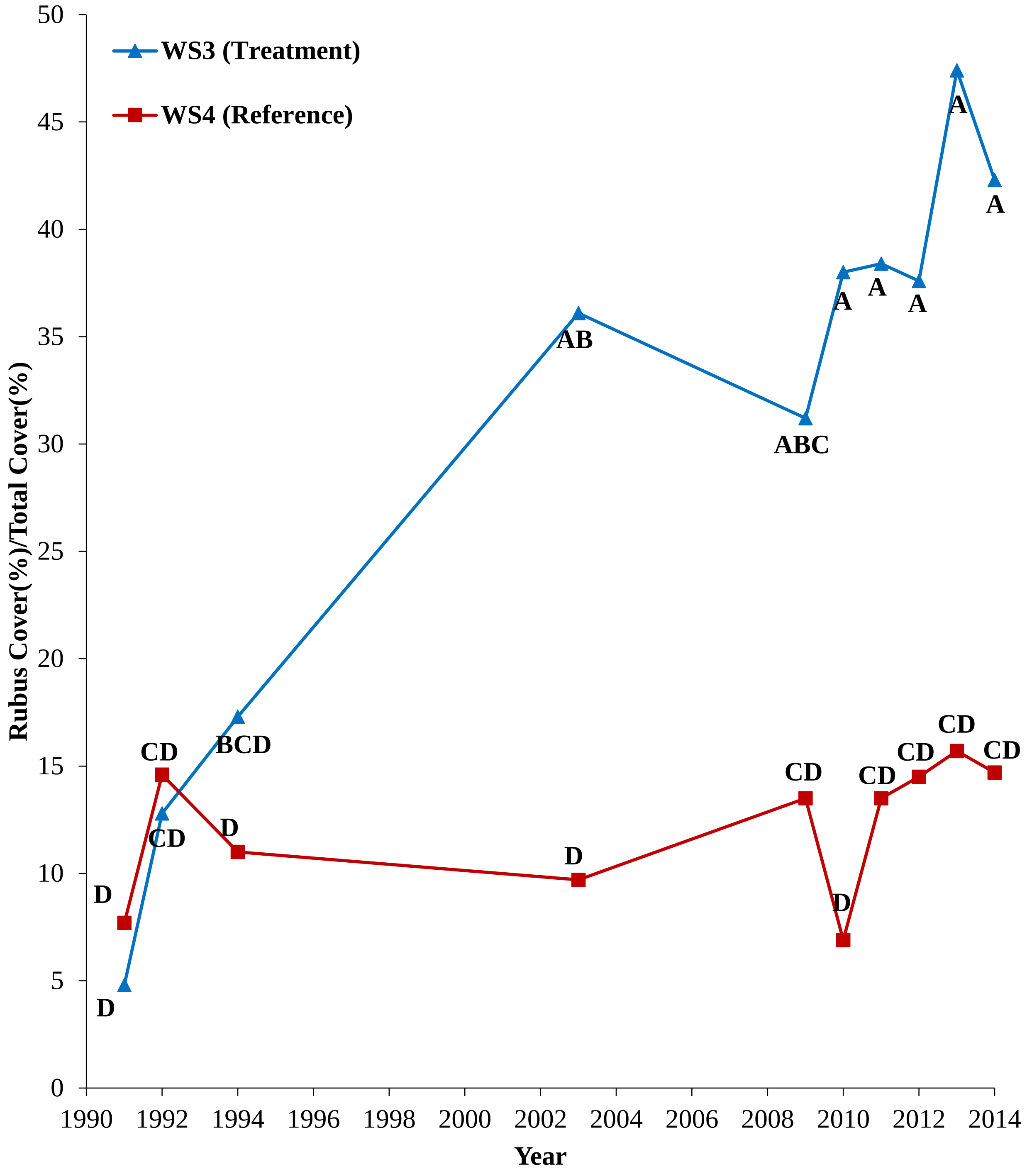

Figure 2.5: Repeated measures of ANOVA for annual means for percent *Rubus* cover relative to percent total cover from July sampling for WS3 (treatment) and WS4 (reference) from 1991-present. Means with the same letter are not significantly different ($p \leq 0.05$) between watersheds and among years.

Rubus cover on WS3 significantly increased after five years of N treatment (Fig 2.4). Emergence of an overstory stratum in early successional habitats provides limited light transmittance to the understory strata resulting in the die-back of many dominant species in early successional habitats. *Rubus* is able to persist belowground in later successional habitats because seeds are able to stay dormant for up to 100 years in mineral soil until disturbance conditions promote germination (Donoso and Nyland 2006). Therefore, excess N deposition could be promoting favorable conditions for germination of dormant *Rubus* seeds from the early successional period and allowing *Rubus* to overcome high light requirements, in turn allowing *Rubus* to become the dominant species presently.

Rubus is considered a ruderal species, characterized by the ability to adapt to changing conditions in the quality of habitat. Claussen and Lenz (1999) found higher dry weight of *Rubus* when *Rubus* was present in ecosystems treated with NO_3^- as opposed to NH_4^+, wherein *Rubus* was highly adaptable to changing soil conditions from this treatment. *Rubus* is also considered a nitrophilous, requiring high-N availability, where luxury uptake of N allows *Rubus* to outcompete many species in early successional stages. Typically N is limited in hardwood ecosystems, wherein N-efficient species absorb enough N to prevent nitrophilous species from expanding, explaining low *Rubus* cover prior to and during the first five years of N treatment to WS3 (Fig 2.4). When excess N is available, nitrophilous species can out-compete N-efficient species for resources due to luxury uptake of N. Studies such as Lautenshlager (1999) found an increase in *Rubus* biomass when N was the only added nutrient to a mature forest. There are fewer nitrophilous compared to N-efficient species in hardwood forest ecosystems (Gilliam 2014a), explaining an increase in *Rubus* cover relative to total cover from ~5% in 1991 to ~43% in 2014 (Fig 2.5) and the decrease of ~3 species/plot on WS3 from 1994-2014 (Fig 2.2). A decrease in species richness did not occur until there was a significant increase ($P \leq 0.05$) in *Rubus* cover from 1994-2003 (Fig 2.4), further suggesting the competitive advantage of *Rubus* arising from excess N availability.

Rubus cover on WS3 responded more ($y = 3.6495x - 4.14$) compared to WS4 ($y = 0.4741x + 0.4362$) to increasing precipitation (Fig 2.6). There was a significant difference ($P \leq 0.003$) between watersheds for *Rubus* cover in the generalized linear model and the interaction between precipitation and watershed is not significant ($P \leq 0.234$), meaning the slopes

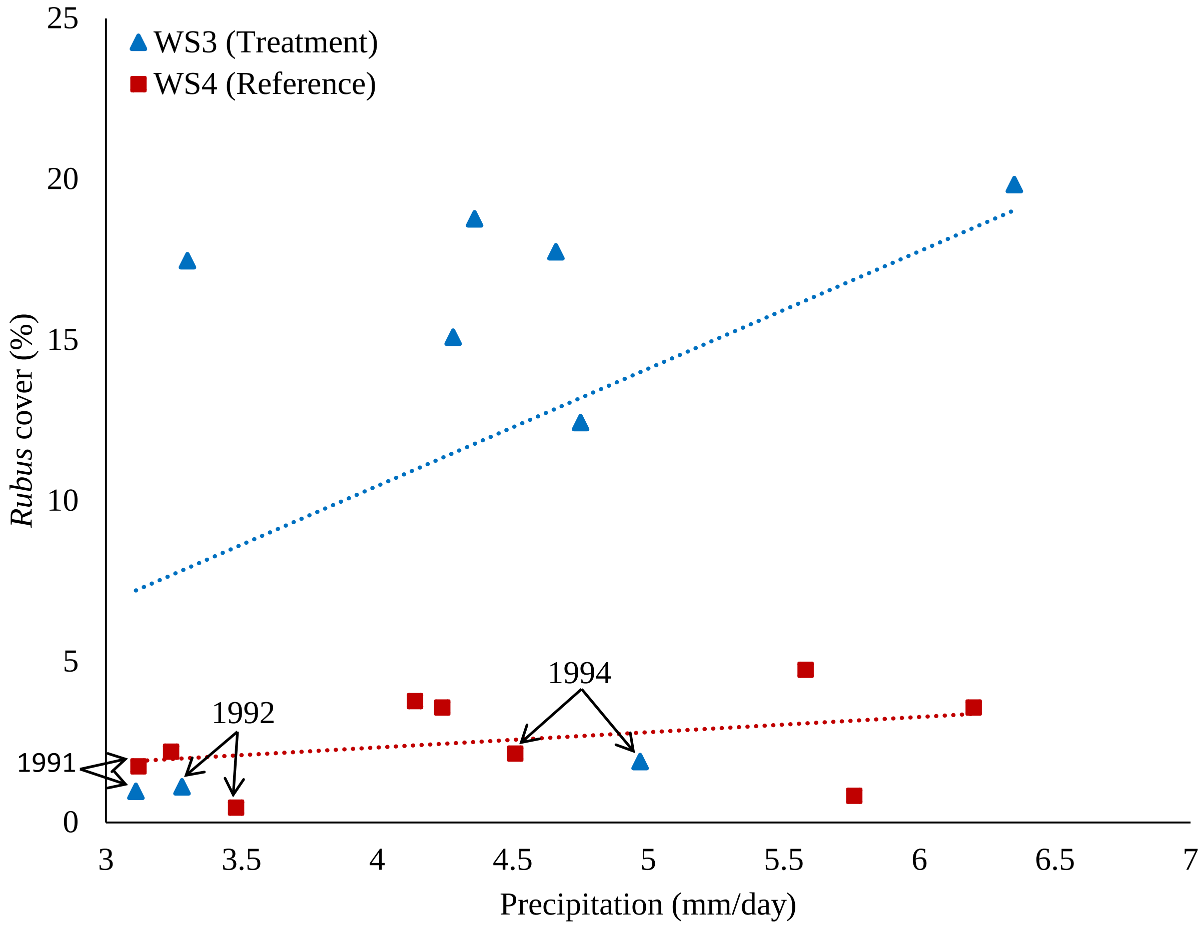

Figure 2.6: Generalized linear regression of annual daily means for precipitation from April to July versus yearly means for *Rubus* % cover from July sampling for WS3 and WS4 from 1991, 1992, 1994, 2003, and 2009-2013 at Fernow Experimental Forest, West Virginia. Lines represent mean linear regression: WS3: $R^2 = 0.2152$, $y = 3.6495x – 4.14$, $p \leq 0.2082$; WS4: $R^2 = 0.1401$, $y = 0.4741x + 0.4362$, $p \leq 0.3210$.

Table 2.4: Summary of generalized linear model for annual daily means of precipitation from April to July versus mean annual *Rubus* cover from July sampling for WS3 and WS4 at Fernow Experimental Forest, West Virginia.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Model	3	490.6243236	163.5414412	5.41	0.011
Precipitation	1	48.148994	48.148994	1.59	0.228
Watershed	1	395.5955611	395.5955611	13.08	0.003
Precipitation*Watershed	1	46.8797685	46.8797685	1.55	0.234

Table 2.5: Summary of ANCOVA sums of squares for annual daily means of precipitation from April to July versus mean annual *Rubus* cover from July sampling for WS3 and WS4 at Fernow Experimental Forest, West Virginia.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Model	2	443.7445551	221.8722775	7.08	0.0068
Precipitation	1	48.1489940	48.1489940	1.54	0.2343
Watershed	1	395.5955611	395.5955611	12.62	0.0029

Table 2.6: Summary of ANCOVA estimates for annual daily means of precipitation from April to July versus mean annual *Rubus* cover from July sampling for WS3 and WS4 at Fernow Experimental Forest, West Virginia.

Parameter	Estimate	Standard Error	t Value	Pr > |t|
Intercept	-5.9353	6.07200039	-0.98	0.3438
Precipitation	1.89813	1.29135227	1.47	0.1623
Watershed WS3	9.3963	2.64499722	3.55	0.0029
Watershed WS4	0	.	.	.

can be considered parallel (Table 2.4). *Rubus* cover for 1991, 1992, and 1994 are similar to *Rubus* cover on WS4, during the same time period that was considered to be prior to the herbaceous layer responding to excess N additions to WS3 (Fig 2.6). After 1994, *Rubus* cover significantly increased ($P \leq 0.05$) and because the interaction statement was not significant ($P \leq 0.234$), ANCOVA was used to examine the average difference between WS3 and WS4 for *Rubus* cover using precipitation as a covariate (Table 2.4). The model ($P \leq 0.0068$) and watershed source ($P \leq 0.0029$) were significant based on ANCOVA sums of squares (Table 2.5). On average, WS3 had ~9.4% more *Rubus* cover ($P \leq 0.0029$) than WS4 from 1991-2013 (Table 2.6). This response of *Rubus* cover versus precipitation on WS3 suggests that excess N additions are shifting limiting factors by alleviating N-limitations, in turn allowing ~ 42% *Rubus* cover relative to % total cover in 2014 (Fig 2.5).

Disturbed habitats that are associated with *Rubus* dominance are typically high in soil NO_3^- (Donoso and Nyland 2006). Magill et al. (2000) found similar soil NO_3^- concentrations after 9 years of 50 kg N ha^{-1} yr^{-1} additions and 3 years of 150 kg N ha^{-1} yr^{-1}additions, where the three fold difference in N additions is consistent with the three fold difference in the amount of time to reach similar soil NO_3^- concentrations. Magill et al. (2000) also experienced an eight year delayed response of the study site to 50 kg N ha^{-1} yr^{-1} additions, similar to the delayed response for five years from 35 kg N ha^{-1} yr^{-1} additions to WS3 at FEF. A delayed response is most likely the amount of time for soils to become N-saturated from excess N deposition, at which point NO_3^- is probably responsible for altering herb-layer dynamics. With respect to N treatment on WS3, altering of herb-layer dynamics has resulted from maintenance of significantly higher *Rubus* cover from 2003-present (Fig 2.4).

Jobidon (1993) found a significant increase in germination of *Rubus* during a second consecutive year of 112, 224, and 336 kg-N ha^{-1} treatments. Although these treatments in the Jobidon (1993) study were higher than the 35 kg-N ha^{-1} treatment to WS3, the cumulative amount of excess N deposition from two years of 112 kg-N ha^{-1} treatment was 224 kg-N ha^{-1} compared to the 175 kg-N ha^{-1} after 5 years of treatment at FEF. This is important information because it was not until after 1994 that *Rubus* significantly increased in cover, when cumulative amounts were higher than the study by Jobidon (1993). Between 1994 and 2003, *Rubus* cover increased from ~2% cover to ~12.5% (Fig 2.3). Delayed responses to N additions at FEF and increasing response from consecutive years of excess N deposition provide evidence that soil N saturation from cumulative loads of N deposition dictate *Rubus* cover under closed canopies. More specifically, *Rubus* cover increases when excess N deposition promotes N-saturated soils, where mobility of NO_3^- dominates the N cycle.

Gilliam et al. (2001) found lower variability in soil water NO_3^- and nitrification in N saturated soils at FEF, leading Gilliam (2006) to develop the *N Homogeneity Hypothesis*, which predicts that excess N deposition decreases both spatial heterogeneity of soil N dynamics and herb layer biodiversity. *Rubus* has responded to N additions on WS3 by increasing in cover, in turn contributing to a decrease in biodiversity and possibly homogenization of soil N dynamics. Eilts et al. (2011) suggests that clonal species, such as *Rubus*, can contribute to decreases in resource heterogeneity, soil fertility, and species diversity due to rapid lateral expanse of rhizomes. This same effect on diversity from rapid lateral expanse could be responsible for *Rubus* cover significantly increased from 1994 to 2003 (Figure 2.3) and species richness on WS3 decreased and has remained significantly ($P \leq 0.05$) lower than species richness on reference WS4, with the exception of 2009 (Fig 2.2).

The failure to detect a change in *Rubus* from 2003-present on WS3 suggests that *Rubus* may have reached a threshold for response to this N treatment (Fig 2.1). Excess N deposition has maintained levels of NO_3^- in the soils on WS3 through N saturation and homogenizing spatial N dynamics (Gilliam 2006) contributing to a significant increase in *Rubus* cover from 1994-2003, but after 2003 *Rubus* has not significantly changed in cover. Using nitrification as a predictor for *Rubus* cover on WS3 reveals that before *Rubus* cover significantly increased ($P \leq 0.05$) after 1994, average annual nitrification was higher and associated with lower *Rubus* cover (Fig 2.7). Consistent annual excess N deposition has maintained N-saturated soils from 2009-2013 on WS3, resulting in homogenization of nitrification and higher *Rubus* cover compared to WS4 (Fig 2.7). Soils on WS4 were N-saturated after 1994 and show similar mean annual nitrification to WS3 from 2009-2013 (Fig 2.7). Generalized linear model for nitrification and *Rubus* cover is significant ($P<0.0001$), therefore *Rubus* cover is significantly different between watersheds ($P<0.0001$) and nitrification ($P \leq 0.0028$) is a predictor of *Rubus* cover for 1994 and 2009-2013 (Table 2.7). Therefore, the difference in *Rubus* cover between WS3 and WS4 is the result of N additions to an ecosystem when soils are N-saturated.

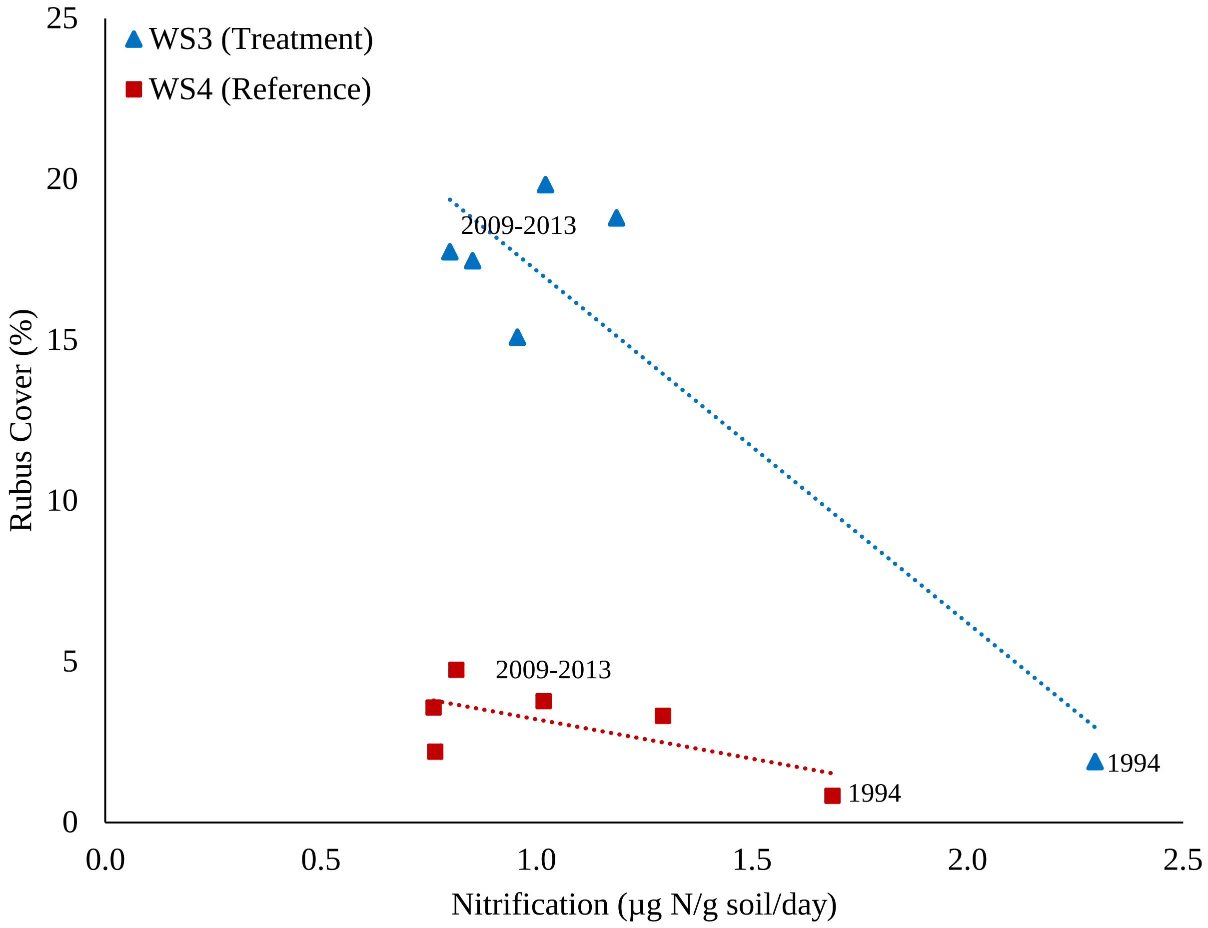

Figure 2.7: Generalized linear regression of annual means for nitrification versus annual means for *Rubus* % cover from July sampling for WS3 and WS4 from 1994 and 2009-2013 at Fernow Experimental Forest, West Virginia. Lines represent mean linear regression: WS3: $R^2 = 0.8475$, $y = -10.96x + 28.122$, $p \leq 0092$; WS4: $R^2 = 0.4484$, $y = -2.4511x + 5.6601$, $P \leq 0.1457$.

Table 2.7: Summary of generalized linear model for mean annual nitrification versus mean annual *Rubus* cover from July sampling for WS3 and WS4 at Fernow Experimental Forest, West Virginia.

Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
Model	3	630.0469848	210.0156616	42.96	<.0001
Nitrification	1	88.0133018	88.0133018	18.01	0.0028
Watershed	1	507.6705699	507.6705699	103.86	<.0001
Nitrification*Watershed	1	34.3631131	34.3631131	7.03	0.0292

Herb-Layer Composition

Beta diversity is defined as variation of species composition among sites within a geographic area and is often used to address conservation of biodiversity in a functioning ecosystem (Legendre et al. 2005). Beta diversity was directly assessed in this study between watersheds (WS3 and WS4) and among years (1991-2014) through the use of DCA (Gauch 1982). Changes in species composition are represented in every "unit" and every "unit" is the average standard deviation of species turnover (Gauch 1982). Thus, the distance in ordinal space between points is a measure of dissimilarity of species composition for WS3 and WS4 among years.

Herb layer composition was similar between WS3 and WS4, despite their difference in stand age (65 years), for 1991, 1992, and 1994 (Fig. 2.8), confirming results from Gilliam et al. (2006). From 1994-2003 WS3 changed ~1.2 units along DCA axis 2, the largest change in herb layer composition on both WS3 and WS4 (Fig 2.8). From 2003-2014 the variation among WS3-year centroids was ~0.3 along DCA axis 1 and ~0.2 along DCA axis 2 (Fig 2.8). During this time period variation among WS4-year centroids was ~0.2 along DCA axis 1 and ~0.3 along DCA axis 2 (Fig 2.8). Therefore, it can be concluded that excess N deposition altered herb layer composition on WS3 from 1994 to the present.

Rubus cover significantly ($P \leq 0.05$) increased from 1994-2003 on WS3, but did not significantly ($P \leq 0.05$) change from 2003-2014, remaining significantly ($P \leq 0.05$) higher compared to WS4 (Fig 2.4). Herb layer composition on WS3 from 2003-2014 was associated more with *Rubus* compared to WS4 from 1991-2014 (Fig 2.9). *Rubus* was significantly correlated with DCA axis 2 ($r = 0.94$, $P \leq 0.0001$) and total cover ($r = 0.94$, $P \leq 0.0002$) on WS3 (Table 2.8). These results suggest that the change in herb layer composition from 1994-2003 on WS3 was due to a significant increase in %*Rubus* cover from excess N deposition.

Similar to WS3, *Rubus* was significantly correlated DCA axis 1 ($r = -0.83$, $P \leq 0.0053$) and total cover ($r = 0.8953$, $P \leq 0.0011$) on WS4 (Table 2.9). These results indicate that *Rubus* significantly influenced herb layer composition through increasing total cover on both WS3 and WS4. In contrast to WS3, total cover was significantly correlated with DCA axis 1 ($r = -0.6705$, $P \leq 0.0481$) and species richness was significantly correlated with DCA axis 1 ($r = -0.6809$, $P \leq 0.0435$) and total cover ($r = 0.6993$, $P \leq 0.0360$) on WS4 (Table 2.9), suggesting that total cover and species richness are also influencing herb layer composition. Similar to results by

Brunet et al. (1998), *Rubus* responds to excess N deposition through increasing % cover, in turn significantly influencing herbaceous composition on WS3 in this study.

Although spatial heterogeneity of soil nutrients has been shown to influence herbaceous species composition of hardwood forests ecosystems (Gilliam and Dick 2010), herb species themselves can alter spatial patterns of soil nutrient availability (Gilliam and Dick 2010), part of the *circulus vitiosus* stated by Jenny et al. (1969) to acknowledge the dilemma of distinguishing between effects of plants on soil versus the effects of soil on plants. Wedin and Tilman (1990) found that some grass species affected net N mineralization and nitrification over a three year period by causing a ~10-fold difference in rates. Data from this study suggests that N additions affect both herbaceous species composition and N dynamics, but there is also the reciprocating effect of herbaceous species composition on spatial heterogeneity of soil N dynamics. The reciprocating effect in this study is particularly influenced by excess N deposition altering herb layer composition through shifting the N cycle towards high NO_3^- mobility and significantly ($P \leq 0.05$) increasing % *Rubus* cover relative to % total cover from ~5% to ~42% on WS3 (Fig 2.5).

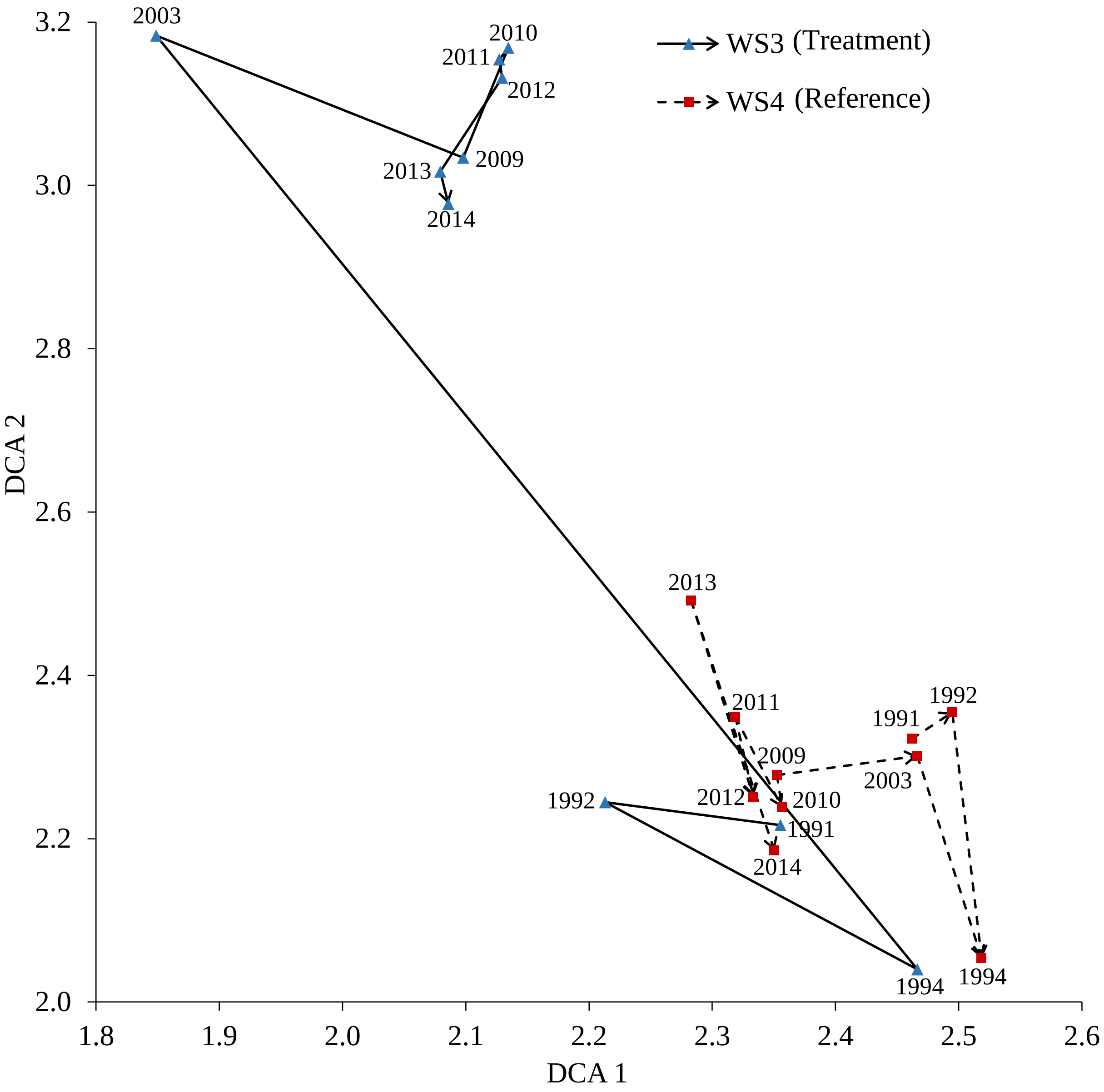

Figure 2.8: Detrended correspondence analysis of herbaceous layer species composition for WS3 (Treatment) and WS4 (Reference) at Fernow Experimental forest, WV. Each point represents a mean centroid for seven sample plots per watershed per year from 1991-2014.

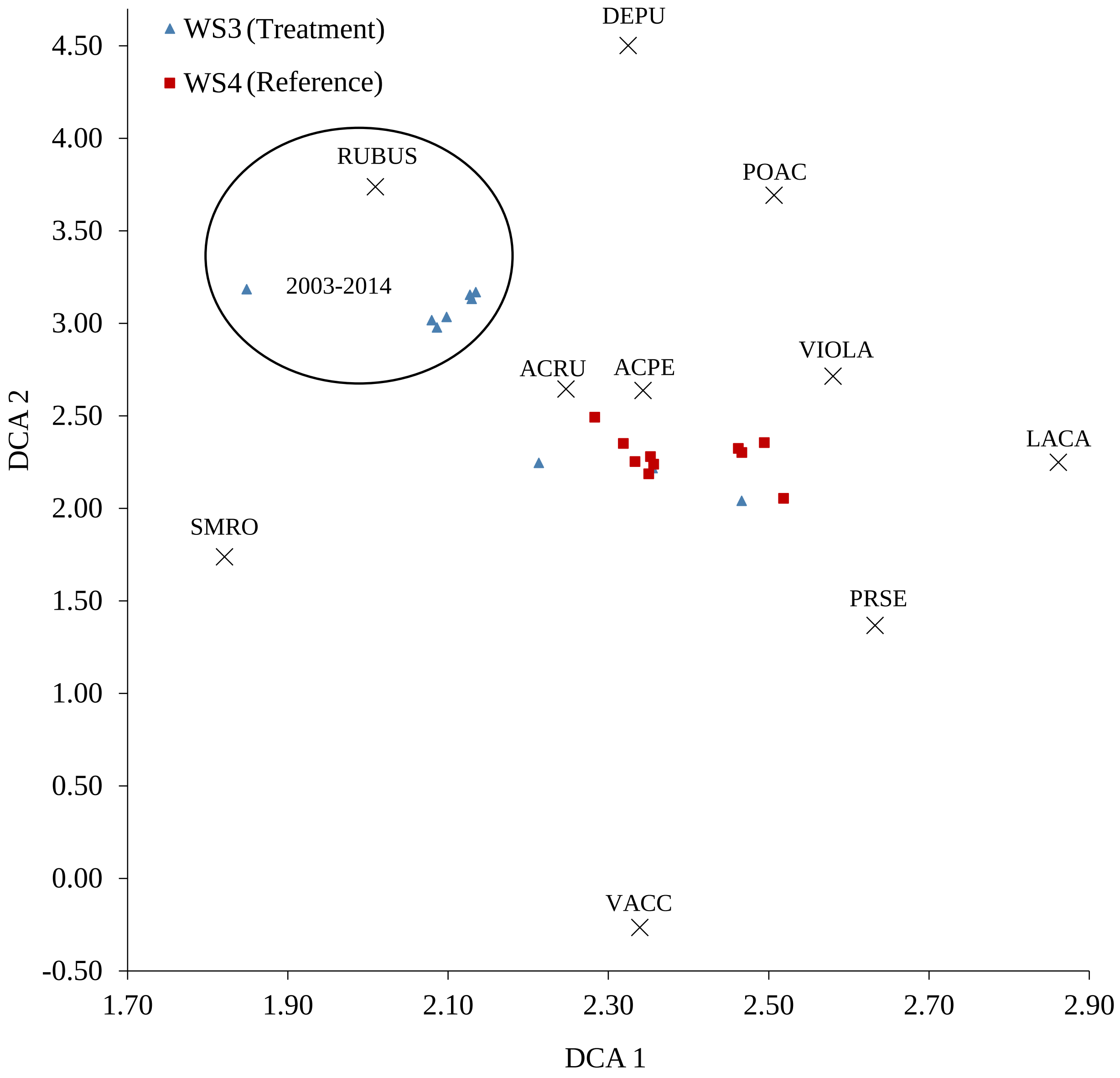

Figure 2.9: Detrended correspondence analysis of herbaceous layer species composition for WS3 (Treatment) and WS4 (Reference) at Fernow Experimental forest, WV. Each point represents a mean centroid for seven sample plots per watershed per year from 1991-2014. “X” represents the location in ordination space for the prominent herb layer species: RUBUS = *Rubus* spp.; ACPE = *Acer pensylvanicum*; ACRU = *Acer rubrum*; Viola = *Viola* spp.; PRSE = *Prunus serotine*; SMRO = *Smilax rotundifolia*; DEPU = *Dennstaedtia punctiloba*; POAC = *Polystichum acrostichoides*; LACA = *Laportea canadensis*; VACC = *Vaccinium* spp..

Table 2.8: Pearson rank correlation matrix of yearly means for DCA axis 1 values, DCA axis 2 values, % total cover, richness (species/plot), and % *Rubus* cover from 1991-2014 for treatment WS3. * indicates significant correlation (P<0.05) and ** indicates significant correlation (P<0.01).

	DCA axis 1	**DCA axis 2**	**Total cover**	**Richness**	***Rubus* cover**
DCA axis 1	1.0000				
DCA axis 2	-0.8301 **	1.0000			
Total cover	-0.6175	0.8531 **	1.0000		
Richness	0.3050	-0.0702	0.2805	1.0000	
***Rubus* cover**	-0.6489	0.9441 **	0.9361 **	0.1622	1.0000

Table 2.9: Pearson rank correlation matrix of yearly means for DCA axis 1 values, DCA axis 2 values, % total cover, richness (species/plot), and % *Rubus* cover from 1991-2014 for reference WS4. * indicates significant correlation (P<0.05) and ** indicates significant correlation (P<0.01).

	DCA axis 1	**DCA axis 2**	**Total cover**	**Richness**	***Rubus* cover**
DCA axis 1	1.0000				
DCA axis 2	-0.5427	1.0000			
Total cover	-0.6705 *	0.2711	1.0000		
Richness	-0.6809 *	-0.0372	0.6993 *	1.0000	
***Rubus* Cover**	-0.8332 **	0.3145	0.8953 **	0.7818 *	1.0000

CHAPTER 3

RUBUS FOLIAR NUTRIENT ANALYSIS

Introduction

Investigations of foliar nutrient concentrations can provide insight to nutrient dynamics of hardwood forest ecosystems. Most hardwood forests exhibit nutrient, especially N, limitations and respond to increased nutrient availability through increased growth, wherein fertile habitats have been associated with higher growth rates, leaf production, and root biomass (Chapin 1980). For this study a single dominant species—*Rubus*—is utilized to further our understanding of the effects of excess N deposition on nutrient cycling of eastern hardwood forests.

Impacts on nutrient dynamics can arise when rates of N deposition exceed the biotic demand for N, known as N saturation (Aber et al., 1998). Soil nutrient availability can influence foliar nutrient concentrations in herb-layer species, with soil nutrient availability varying spatially and temporally in response to many factors, especially soil N dynamics (Gilliam et al. 2001, Gilliam and Dick 2010). The N cycle in N saturated soils is dominated by NO_3^-, and excess NO_3^- has the potential to leach from mineral soil while simultaneously promoting the loss of nutrient cations (Ca^{++}, Mg^{++}, K^+). Excess N deposition can homogenize nutrient dynamics through dominance of NO_3^- mobility in mineral soils. Gilliam and Adams (2011) determined that variation of soil water NO_3^- decreases as mean soil water NO_3^- increases. Heterogeneity of essential nutrients in soils is positively correlated with species richness and understory species richness is positively correlated with overstory species richness (Gilliam 2014a). Therefore, homogeneity of essential nutrients can decrease herbaceous species diversity in impacted forest ecosystems.

Soils of WS3 and WS4 at FEF are N-saturated, with symptoms of N saturation including elevated levels of stream NO_3^-, high levels of nutrient cations in stream water, significantly ($P \leq 0.05$) lower levels of foliar Ca in tree and herb-layer species, high rates of net nitrification, low season variability of stream NO_3^-, and high discharge of streams NO_3^- following N treatment (WS3) (Peterjohn et al., 1996; Gilliam et al., 1996; Gilliam et al., 2001). Gilliam et al.

(1996) addressed effects of excess N deposition on nutrient availability by using a single herb-layer species, *Viola rotundifolia,* a species present on every plot of WS3 and WS4. Foliar tissue of *Viola rotundifolia* contained significantly ($P \leq 0.05$) higher levels of N accompanied by significantly ($P \leq 0.05$) lower levels of Ca and Mg on WS3 at FEF from excess N deposition (Gilliam et al., 1996). In 2014, *Rubus* cover accounted ~42% of the total cover on WS3 (Fig 2.5) and *Viola rotundifolia* was no longer found on every plot in WS3 and WS4 at FEF. Therefore, *Rubus* can be used as an indicator of nutrient status at FEF. Indeed, excess N deposition has decreased species richness (Fig 2.3) and increased *Rubus* cover from ~1% in 1991 to ~19% in 2014 (Fig 2.4).

Stream, soil, and foliar chemistry have led to the conclusion that rates of N deposition are altering N status in northeastern forests of the US (Aber et al., 2003). Studies are needed to address the severity of altering N status on nutrient dynamics, especially in regions where N deposition is already altering N status. Garten (1976, 1978) concluded from consistent P:N, Ca:Mg, and Mg:K ratios through a correlation matrix of 110 plant species that correlated nutrients within plants suggest similar biochemical functions. Garten (1978) further established sets of nutrients and corresponding functions as: 1) nucleic acid-protein (P, N, Cu, S, and Fe), 2) structural/photosynthetic (Mg, Ca, and Mn), and 3) enzymatic (Mn, K, and Mg). Herbaceous plants are particularly sensitive to soil nutrient availability and can provide more insight to soil nutrient dynamics. Gilliam and Adams (1995) found similar correlations between the same nutrients in soil and foliar tissue among young and mature hardwood stands at FEF, establishing a link for addressing nutrient dynamics at FEF from excess N deposition.

In Chapter 2, WS3 and WS4 were the only two watersheds used for the herb layer study from 1991-2014. Even though the difference in stand age of WS3 and WS4 is ~55 years, there were no significant differences between watersheds found prior to initiation of N treatment to WS3 (Gilliam and Turrill 1993). Incorporating another reference watershed (WS7) of similar stand age (~45 years) to WS3 addresses the differences in nutrient dynamics between watersheds that can arise from stand age (Small and McCarthy 2005). The purpose of this chapter is to use nutrient levels in *Rubus* foliar tissue as an indication of nutrient status in the herbaceous layer on N-treated (WS3) and untreated watersheds (WS4 and WS7) to address the growing concern on effects of excess N deposition on soil nutrient availability.

Materials and Methods

Field Sampling

Foliar material was collected from *Rubus* within or near seven circular 0.04-ha sample plots in WS3, WS4, and WS7, with the exception of one plot on each of WS4 and WS7 whereon no *Rubus* was found. *Rubus* leaves were collected in the beginning of July (selected as peak of growing season) for the years 2013 and 2014. Each sample of foliar material was placed in a polyethylene bag and stored in a cooler before returning to the Weeds and Dirt Laboratory at Marshall University. *Rubus* foliar tissue was placed in paper bags and dried for 24 hours at 25 C in an oven. Dried samples were ground to pass a 1-mm sieve and at least 2 g were placed into polyethylene bags and shipped to University of Maine Analytical Lab for determination of N, P, K, Ca, Mg, Al, B, Cu, Fe, Mn, and Zn levels. Data consisted of foliar nutrient concentrations for seven plots on WS3 and six plots on WS4 and WS7 for 2013 and 2014.

Data Analysis

Nutrient levels in dried samples were compared between 2013 and 2014 collections using ANOVA ($\alpha = 0.05$) to determine if there were any significant differences between years of collection. There were no differences found between 2013 and 2014, therefore, nutrient data were compiled and the means for each nutrient were compared across watersheds using ANOVA ($\alpha = 0.05$). Spearman rank correlations for these means were also used to determine correlated nutrients for each watershed separately.

Results and Discussion

Macronutrients

Elevated levels of foliar N are characteristic of N saturated hardwood ecosystems (Aber et al., 1998). Therefore, untreated watersheds (WS4 and WS7) are expected to show no significant differences for concentrations of foliar N, because soils of each watershed are N saturated. The N treated watershed (WS3) should show a higher concentration of foliar N compared to the untreated watersheds due to excess N deposition. There was no significant difference ($P \leq 0.05$) for N concentrations between WS4 and WS7 (Fig 3.1). Nitrogen was significantly higher ($P \leq 0.05$) on WS3 compared to WS7, but not significantly higher than WS4 (Fig 3.1).

Failure to detect differences in *Rubus* foliar N between WS3 and WS4 may be due to luxury uptake of NO_3^- by *Rubus* in N-saturated soils on WS4. When soils are N-saturated, nitrifying bacteria oxidize more NH_4^- to NO_3^-, therefore, excess N deposition to WS3 is resulting in more NO_3^- than that of WS4. More NO_3^- in mineral soil of WS3 compared to WS4 is the result of more N being added, explaining higher N in *Rubus* foliar tissue on WS3 (Fig 3.1). Luxury consumption of NO_3^- in *Rubus* foliar material on WS4 could be due to enough NO_3^- from the soil to maintain vegetative growth but not germination of new dormant seeds, hence why there is a gradual, not significant ($P \leq 0.05$), increase in *Rubus* cover on WS4 (Fig 2.4). Additionally, stream NO_3^- concentrations significantly declined on WS3 following a peak stream NO_3^- concentrations in 1998 (Gilliam and Adams 2011). This decline in stream NO_3^- concentrations could be influenced by the significant increase in *Rubus* cover (Fig 2.4) on WS3 and responsible for the insignificant difference among WS3 and WS4 for foliar N concentrations.

Foliar N concentrations are proportional to photosynthetic rates in leaves (Chapin 1980), suggesting that higher N concentrations in *Rubus* foliar tissue can explain higher *Rubus* cover on WS3 compared to WS4 under low light conditions from the intact canopies. Nitrogen also contributes to increases in cell numbers and cell size (Chapin 1980), where significantly higher ($P \leq 0.05$) *Rubus* cover on WS3 can also be attributed to significantly higher *Rubus* foliar N concentrations (Fig 3.1). Even though soils of WS4 are considered N-saturated like WS3 (Gilliam et al. 1996), lack of excess N deposition from a treatment contributes to less N mobility compared to WS3. *Rubus* cover on WS3 is significantly ($P \leq 0.05$) higher than WS4 from 2003 to 2014, but does not significantly increase ($P \leq 0.05$) during this time period suggesting that light may be the limiting factor and not N on WS3 (Fig 2.4).

Nutrient limitations, especially P, can arise when excess N deposition alleviates the N limitation that is common in most hardwood forest ecosystems (Vitousek et al. 2010). Gress et al. (2007) found greater root ingrowth to microsites when fertilized with P and increased phosphomonoesterase (root-associated P enzyme) activity when N additions increased at FEF. Gress et al. (2007) concluded from these data that as N availability increases at FEF, P typically becomes more limiting. There were no significant ($P \leq 0.05$) differences across watersheds for P concentrations (Fig 3.2), but this does not mean that these watersheds are not P limited. Given that soils of WS3, WS4, and WS7 are N-saturated at FEF, suggests that there is no longer an N limitation and possibly a P limitation for this ecosystem. Additionally, Vitousek et al. (2010)

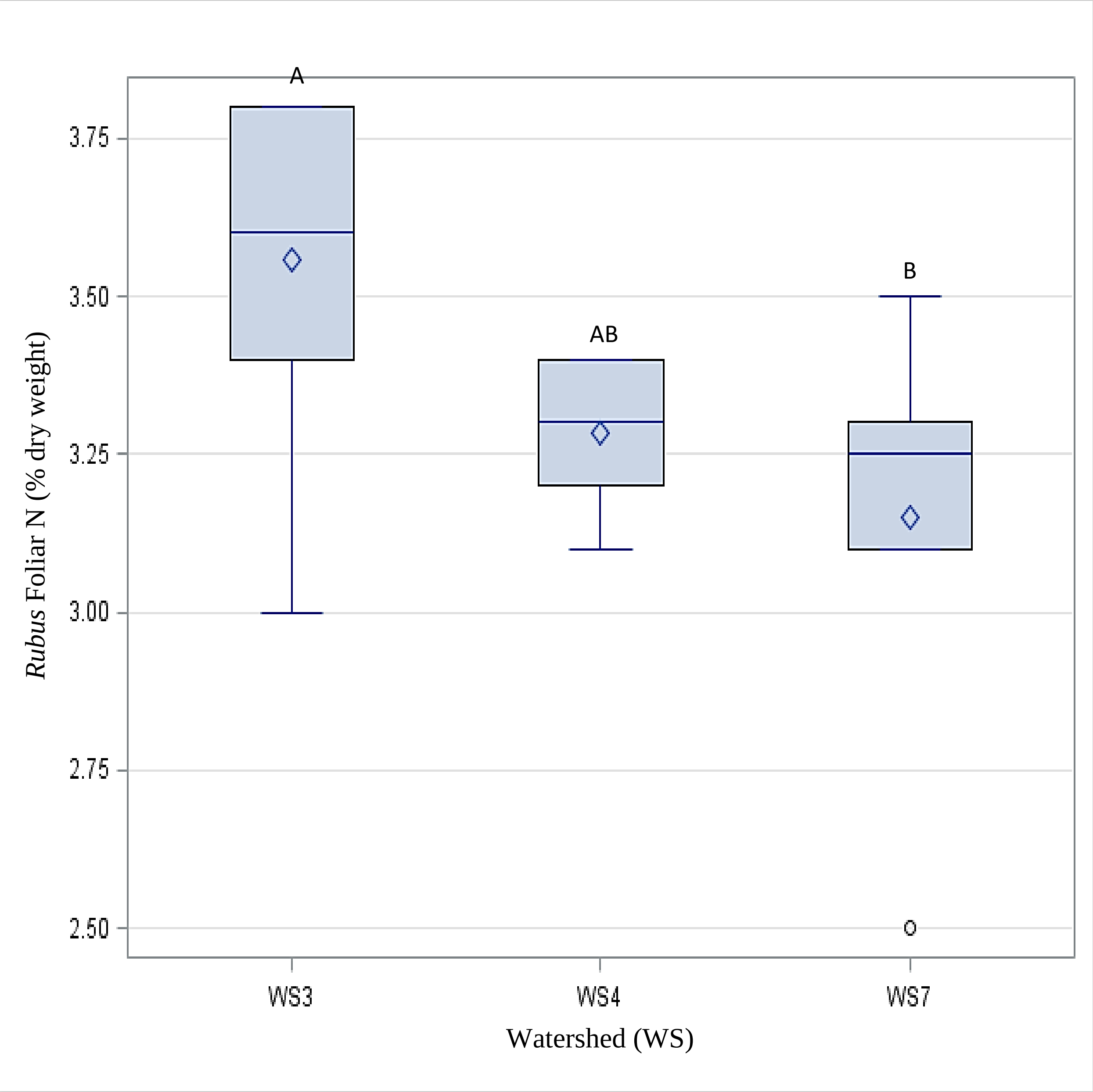

Figure 3.1: Mean % dry weight of *Rubus* foliar nitrogen (N) during 2013 and 2014 growing seasons combined for each watershed separately. Different letters represent a significant difference ($p<0.05$) between WS3 (Treatment), WS4 (reference), and WS7 (reference).

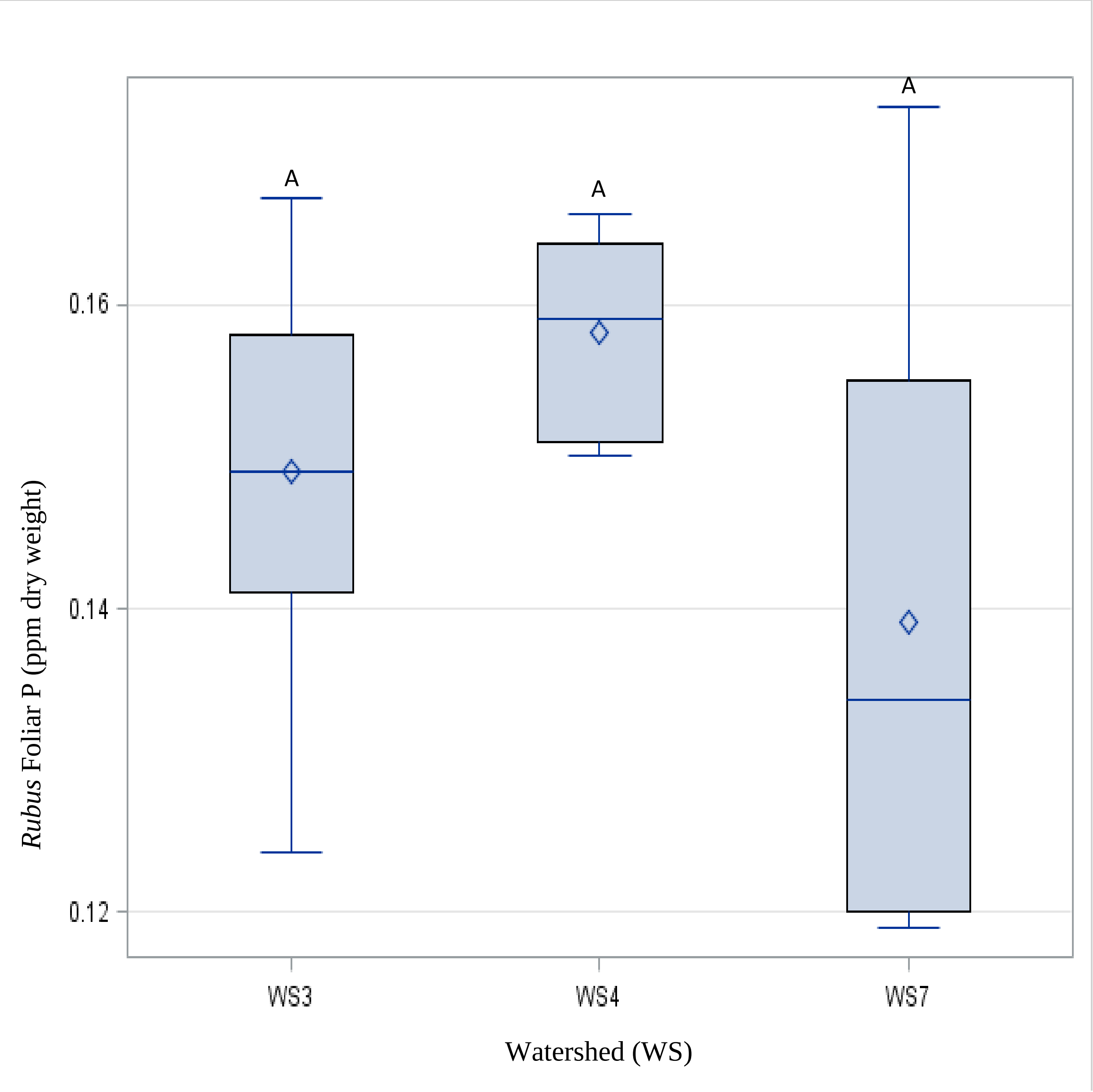

Figure 3.2: Mean % dry weight of *Rubus* foliar Phosphorus (P) during 2013 and 2014 growing seasons combined for each watershed separately. Different letters represent a significant difference ($p<0.05$) between WS3 (Treatment), WS4 (reference), and WS7 (reference).

outlined P limitation in weathered/older soils due to depletion of P from circulation within the ecosystem. Therefore, the insignificant differences for P across watersheds is most likely a combination of P limitation in all watersheds from a lack of P in parent material of weathered/older soils associated with the Appalachian region and N saturated soils from high N deposition.

The N cycle of N-saturated soils is dominated by high mobility of NO_3^- and results in the leaching of NO_3^- from mineral soil while promoting loss of essential base cations (Ca^{++}, Mg^{++}, K^+). Chapin (1980) found Ca and Mg deficiencies associated with increased NO_3^- mobility, explaining significantly lower ($P \leq 0.05$) Ca on WS3 compared to WS4 and WS7 (Fig 3.3). The significantly lower Ca on WS3 is consistent with results for *Viola rotundifolia* from Gilliam et al. (1996). Foliar Mg did not vary significantly ($P \leq 0.05$) across watersheds at FEF (Fig 3.4). These findings for foliar Mg are not consistent with Gilliam et al. (1996), where there was significantly lower Mg on WS3 compared to WS4 and WS7 in *Viola rotundifolia*. This suggests that *Rubus* may respond less to Mg availability than *Viola rotundifolia* under excess N deposition to a hardwood ecosystem.

Potassium (K) was significantly higher on WS3 compared to WS7, but WS4 was not significantly lower than WS3 or significantly higher than WS7 (Fig 3.4). The outlier for WS3 could be influencing this insignificant difference compared to WS4 by shifting the mean to be similar to the mean of WS4 (Fig 3.4). These results are also consistent with K concentrations in *Viola rotundifolia* from Gilliam et al. (1996), where *Viola rotundifolia* contained significantly higher ($p \leq 0.05$) levels of K on WS3 compared to WS7 and WS4 was not significantly lower ($p \leq 0.05$) than WS3 or significantly higher ($p \leq 0.05$) than WS7. Diffusion rates for K in plant tissue are typically faster than most nutrients with the exception of NO_3^- (Chapin 1980). Excessive uptake of cations, especially K, occurs when passive uptake of N is predominately in the form of NO_3^- as opposed to NH_4^+, where NH_4^+ uptake typically involves competition with base cations (Waring and Schlesinger 1985). Therefore, the significantly higher *Rubus* foliar K concentrations from increased NO_3^- uptake on WS3 compared to WS7 is due to excess N deposition, but the insignificant difference for K on WS3 and WS4 is due to similar mobility of NO_3^- from N saturated soils.

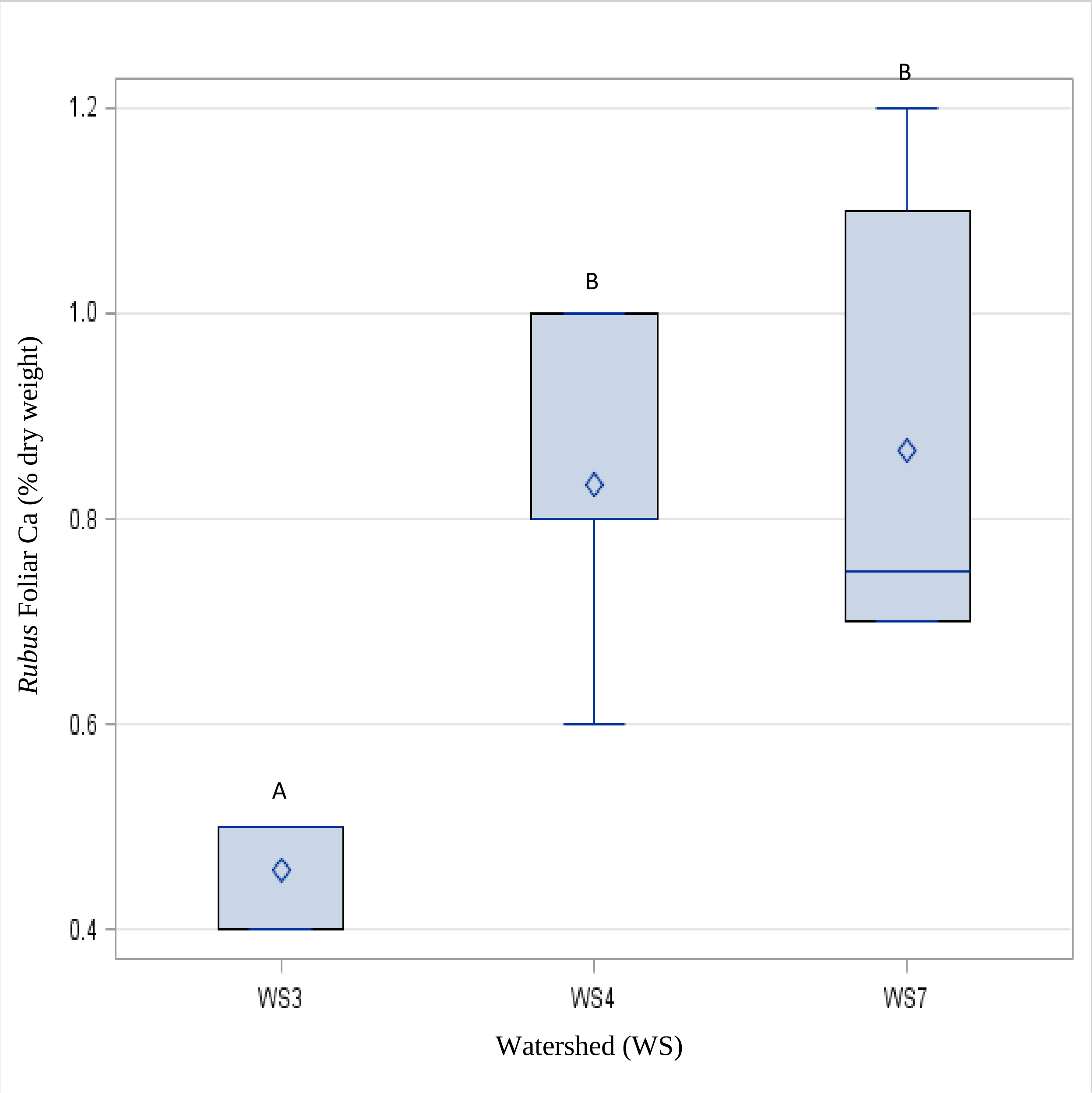

Figure 3.3: Mean % dry weight of *Rubus* foliar calcium (Ca) during 2013 and 2014 growing seasons combined for each watershed separately. Different letters represent a significant difference ($p<0.05$) between WS3 (Treatment), WS4 (reference), and WS7 (reference).

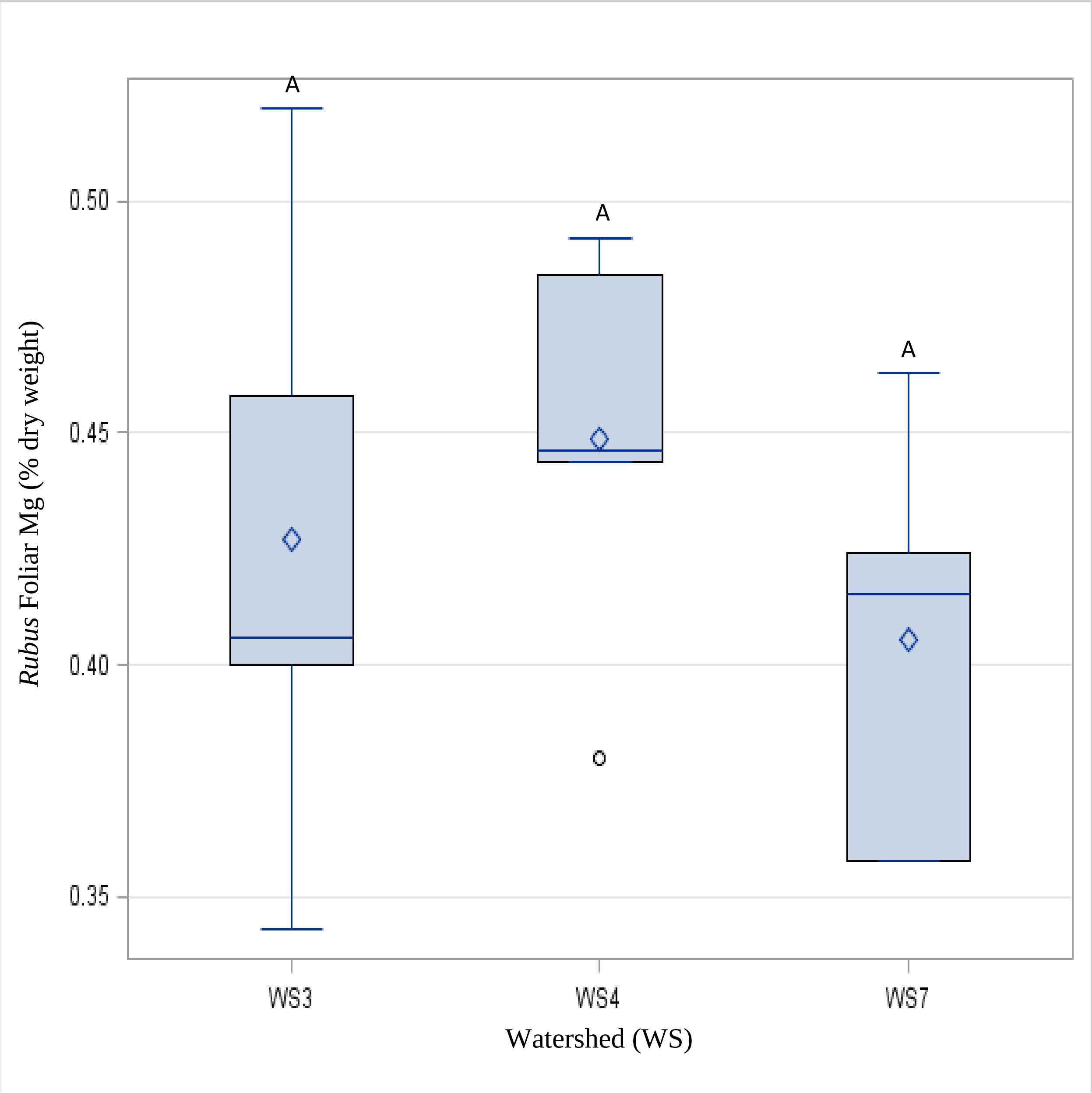

Figure 3.4: Mean % dry weight of *Rubus* foliar magnesium (Mg) during 2013 and 2014 growing seasons combined for each watershed separately. Different letters represent a significant difference ($p<0.05$) between WS3 (Treatment), WS4 (reference), and WS7 (reference).

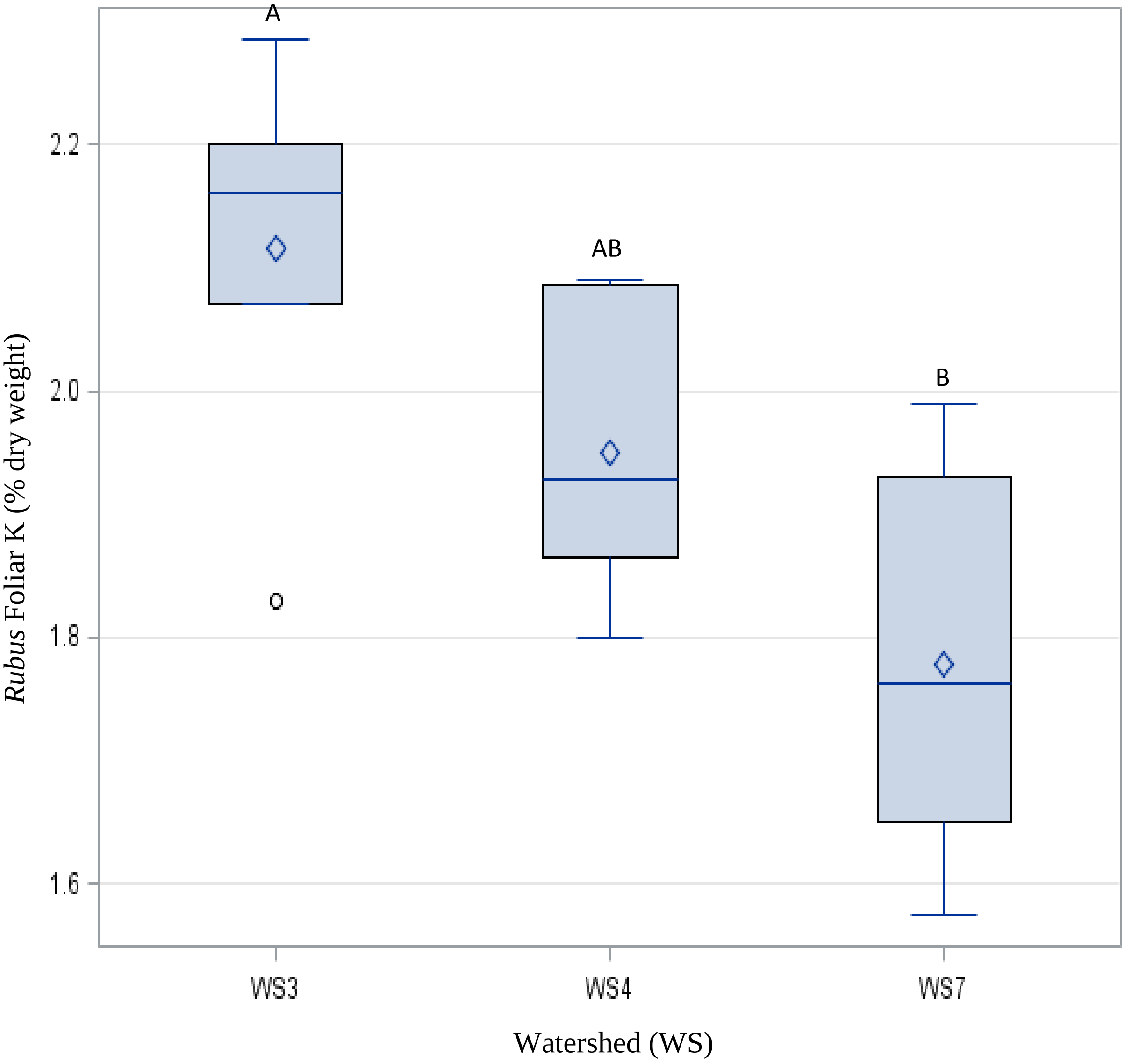

Figure 3.5: Mean % dry weight of *Rubus* foliar potassium (K) during 2013 and 2014 growing seasons combined for each watershed separately. Different letters represent a significant difference ($p<0.05$) between WS3 (Treatment), WS4 (reference), and WS7 (reference).

Up to 37% of K from throughfall was contributed to herbaceous foliar tissue and foliar litter productions in a deciduous forest from the herbaceous layer and can account for ~30% of total litter fall (Muller, 2014). *Rubus* foliar tissue contained significantly higher ($P \leq 0.05$) levels of K on WS3 compared to WS7 (Fig 3.4) and total cover of the herbaceous layer was higher in 2013 and 2014 on WS3 compared to WS4 because of significantly higher *Rubus* cover (Fig 2.1). This suggests that *Rubus* foliar material contributes to high concentrations of K in the soil on WS3. High concentrations of K in foliar material have also increased herbaceous species growth during experimental settings (Grewal and Singh, 2008), contributing to higher *Rubus* cover on WS3. In experimental settings, increases in foliar K led to more efficient water retention in herbaceous species (Grewal and Singh, 1980).

Levels of K in *Rubus* foliar tissue influence water retention, where K is responsible for stomata opening and closing. In turn, stomatal opening influences transpiration rates through *Rubus* tissue to aid in more water absorption for the higher *Rubus* cover on WS3. Luxury consumption of K by *Rubus* most likely inhibits K from leaching out of the soil when it is stored in foliar material, possibly due to an increased water stress through competition from higher *Rubus* cover on WS3. Water stress from competition is also supported by the generalized linear regression of mean annual precipitation versus mean annual *Rubus* cover (Fig 2.4), where increased *Rubus* foliar K on WS3 could be responsible for precipitation versus *Rubus* cover increasing at a higher rate on WS3 compared to WS4.

Micronutrients

Effects of excess N on foliar nutrients can vary greatly among specific nutrients, especially when comparing macro- versus micronutrients. Excess N deposition can acidify soils and promote increased mobility of metal ions in soil solution (Bobbink et al., 2010). There are no significant differences ($p \leq 0.05$) among watersheds for Cu or Zn (Fig 3.5 and Fig 3.6, respectively). Mineral soils at FEF were acidic prior to starting the N treatment to WS3 and high mobility of these micronutrients prior to the N treatment on WS3 may be responsible for the similarity of some *Rubus* foliar nutrient levels across watersheds, suggesting that excess N deposition cannot further alter these micronutrient levels. There was also failure to detect any differences ($p \leq 0.05$) among watersheds for B concentrations in *Rubus* foliar tissue (Fig 3.8).

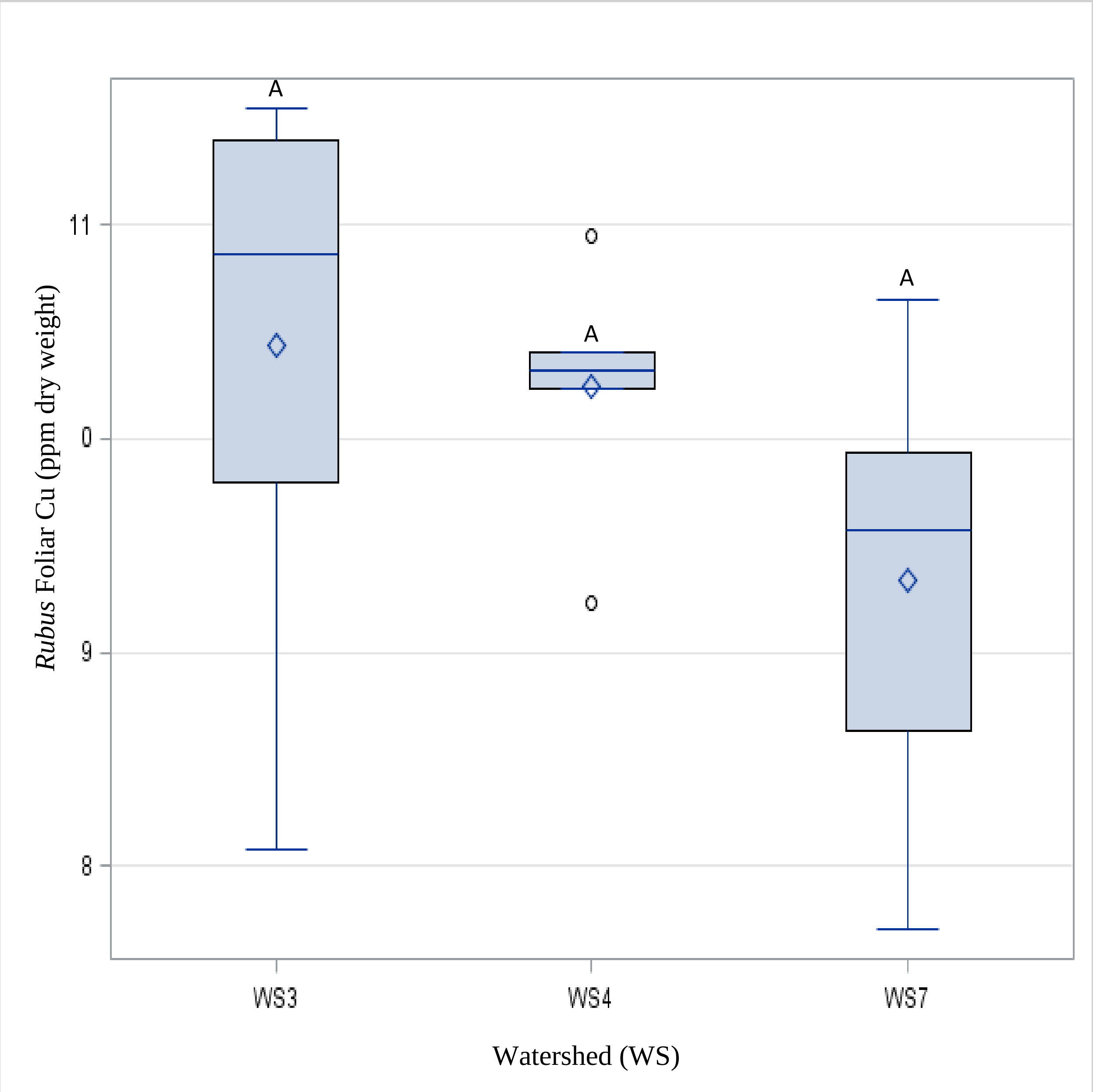

Figure 3.6: Mean parts per million dry weight of *Rubus* foliar copper (Cu) during 2013 and 2014 growing seasons combined for each watershed separately. Different letters represent a significant difference ($p<0.05$) between WS3 (Treatment), WS4 (reference), and WS7 (reference).

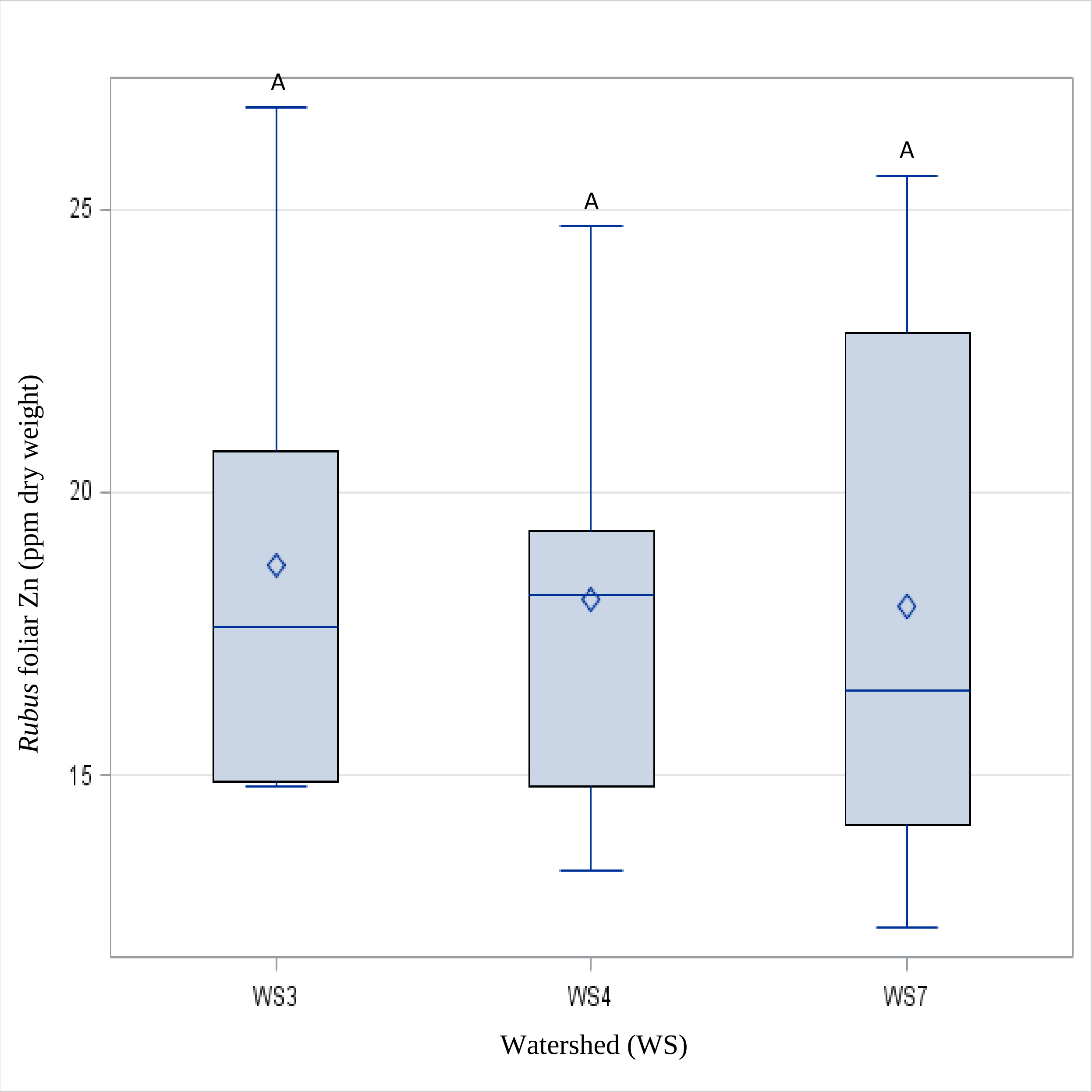

Figure 3.7: Mean parts per million dry weight of *Rubus* foliar zinc (Zn) during 2013 and 2014 growing seasons combined for each watershed separately. Different letters represent a significant difference ($p<0.05$) between WS3 (Treatment), WS4 (reference), and WS7 (reference).

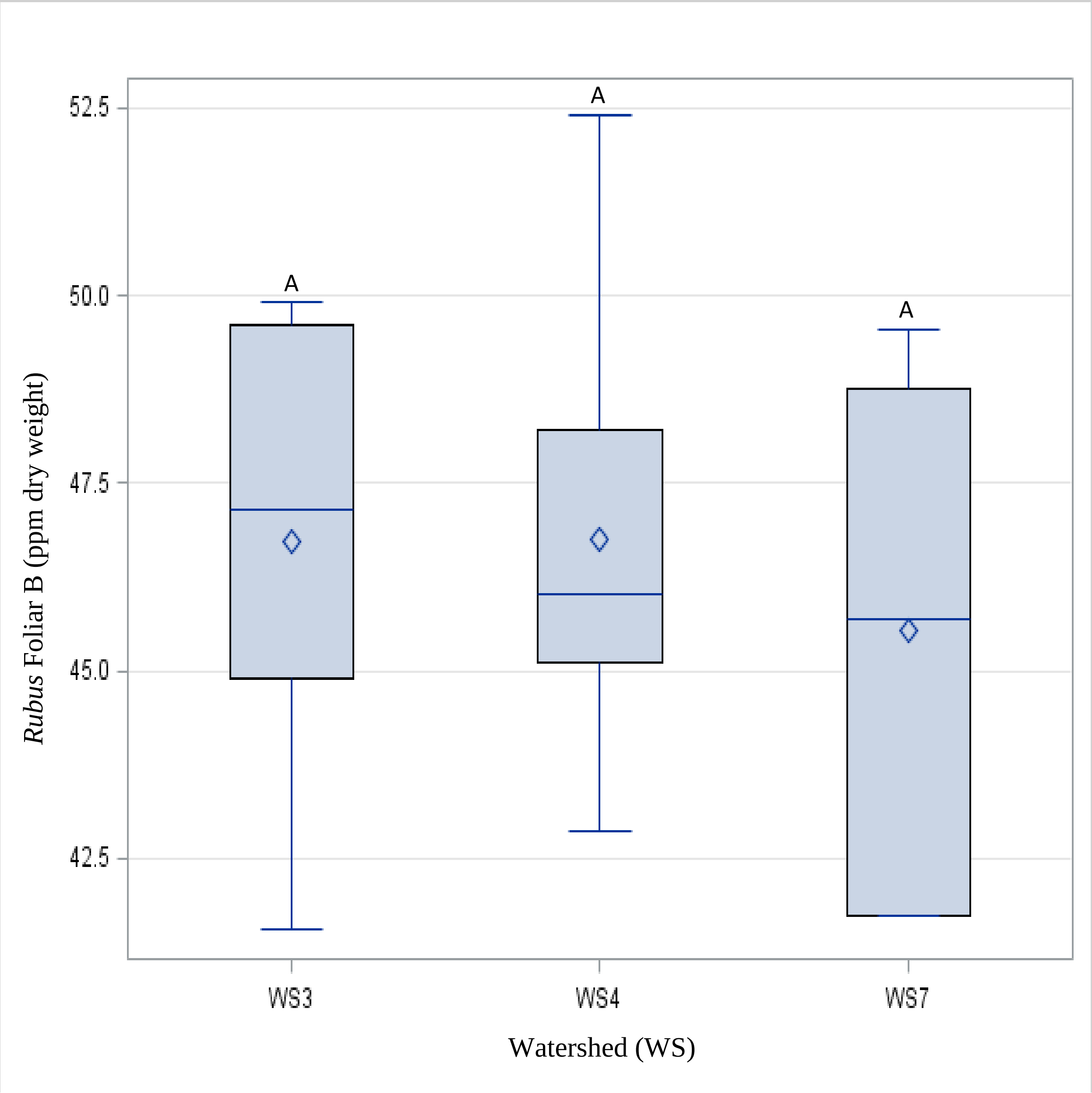

Figure 3.8: Mean parts per million dry weight of *Rubus* foliar boron (B) during 2013 and 2014 growing seasons combined for each watershed separately. Different letters represent a significant difference ($p<0.05$) between WS3 (Treatment), WS4 (reference), and WS7 (reference).

Biochemical Function

Significantly correlated foliar nutrients suggest similar biochemical functions within that plant. Garten (1976) identified three common correlations within 54 terrestrial and aquatic species; P:N, Ca:Mg, and Mg:K. Garten (1978) further confirmed these same relationships within 110 plant species and identified plant nutrients based on three functions: 1) nucleic acid-protein (P, N, Cu, S, and Fe), 2) structural/photosynthetic (Mg, Ca, and Mn), and 3) enzymatic (Mn, K, and Mg). Ca:Mg was significantly correlated ($r=0.77$, $P\leq0.05$) on WS3 (Table 3.1), but not significantly correlated for WS4 or WS7 (Table 3.2 and 3.3, respectively). There were no significant correlations ($P\leq0.1$) on WS3, WS4, and WS7 for P:N or Mg:K (Table 3.1, 3.2, and 3.3, respectively), suggesting that *Rubus* absorbs nutrients at a different capacity through luxury consumption of specific nutrients (eg. N and K) that can alter the correlated nutrients based on function. The amount of significantly correlated ($P\leq0.05$) nutrients on WS3 was higher than WS4 and WS7 suggesting that excess N deposition may alter the nutrient cycling on WS3.

Potential toxicity

Aluminum comprises ~7% of earth's crust, making it the most abundant metal (Delhaize and Ryan 1995). Aluminum is toxic to plants at very low concentrations and inhibits root growth (Delhaize and Ryan 1995; Miyasaka and Hawes 2001). Toxic effects of Al are not specifically reliant upon levels of Al in mineral soil, but rather controlled by Ca levels in roots (Boudot et al. 1994). Calcium deficiencies are typically associated with levels of Al, therefore, Ca:Al ratios in foliar tissue are used to identify potential toxicity (Godbold et al., 1988; Rengel 1992; Delhaize and Ryan, 1995). There was failure to detect differences ($P\leq0.05$) among watersheds for Al (Fig 3.9), but Ca:Al ratio is significantly lower ($P\leq0.05$) on WS3 compared to WS4 and WS7 (Fig 3.10) due to significantly ($P\leq0.05$) lower Ca on WS3 compared to WS4 and WS7 (Fig 3.3). Mobility of Al is enhanced when soils are acidic, where Al can then become the major limiting factor of plant growth (Kochian et al., 2005). The pH of WS3, WS4 and WS7 are 4.3, 4.5 and 4.1, respectively (Gilliam et al., 1994), and this similarity of pH is potentially responsible for no differences among watersheds for Al. Results suggests potential for Al toxicity, wherein *Rubus* may have high tolerance for Al toxicity, given the significantly ($P\leq0.05$) higher cover on WS3 compared to WS4 and WS7 (Fig 2.4).

Table 3.1: Spearman rank correlation matrix of means for nutrients in *Rubus* foliar tissue from treatment WS3 during the growing seasons of 2013 and 2014. * indicates significant correlation (P<0.05) and ** indicates significant correlation (P<0.01).

WS3 Spearman Correlation Coefficients, N = 7

	N	**Ca**	**K**	**Mg**	**P**	**Al**	**B**	**Cu**	**Fe**	**Mn**	**Zn**
N	1										
Ca	-0.39286	1									
K	-0.75679 *	0.07207	1								
Mg	-0.32434	0.77481 *	0.35455	1							
P	0.32143	-0.85714 *	-0.18019	-0.75679 *	1						
Al	0.54056	-0.90094 **	-0.38182	-0.8 *	0.95499 **	1					
B	-0.39641	-0.23424	0.10909	-0.23636	0.07207	0.1	1				
Cu	0.92857 **	-0.35714	-0.73877	-0.30632	0.42857	0.59462	-0.46849	1			
Fe	0.10714	-0.21429	0.41443	-0.09009	-0.03571	-0.05406	-0.50452	0	1		
Mn	0.5	-0.17857	-0.52254	-0.41443	0.53571	0.50452	-0.63066	0.67857	0.03571	1	
Zn	-0.03571	0.75	-0.23424	0.45047	-0.42857	-0.46849	-0.55858	0.17857	-0.17857	0.42857	1

Table 3.2: Spearman rank correlation matrix of means for nutrients in *Rubus* foliar tissue from reference WS4 during the growing seasons of 2013 and 2014. * indicates significant correlation (P<0.05) and ** indicates significant correlation (P<0.01).

WS4 Spearman Correlation Coefficients, N = 6

	N	**Ca**	**K**	**Mg**	**P**	**Al**	**B**	**Cu**	**Fe**	**Mn**	**Zn**
N	1										
Ca	0.02857	1									
K	-0.14286	0.14286	1								
Mg	0.54286	0.37143	0.54286	1							
P	-0.25714	0.82857 *	0.31429	0.42857	1						
Al	-0.71429	-0.14286	0.02857	-0.54286	0.2	1					
B	0.54286	0.6	-0.25714	0.08571	0.08571	-0.42857	1				
Cu	-0.48571	0.08571	0.25714	-0.54286	-0.08571	0.42857	0.2	1			
Fe	-0.6	0.14286	-0.02857	-0.25714	0.54286	0.88571 *	-0.37143	0.08571	1		
Mn	-0.42857	0.48571	-0.37143	-0.6	0.2	0.2	0.48571	0.6	0.14286	1	
Zn	0.82857 *	0.37143	-0.48571	0.31429	0.02857	-0.54286	0.77143	-0.42857	-0.31429	0.02857	1

Table 3.3: Spearman rank correlation matrix of means for nutrients in *Rubus* foliar tissue from reference WS7 during the growing seasons of 2013 and 2014. * indicates significant correlation (P<0.05) and ** indicates significant correlation (P<0.01).

WS7 Spearman Correlation Coefficients, N = 6

	N	**Ca**	**K**	**Mg**	**P**	**Al**	**B**	**Cu**	**Fe**	**Mn**	**Zn**
N	1										
Ca	0.6	1									
K	-0.42857	-0.37143	1								
Mg	0.11595	0.40584	0.49281	1							
P	0.65714	0.94286 **	-0.25714	0.60876	1						
Al	0.02857	-0.14286	-0.31429	-0.57977	-0.37143	1					
B	0.63775	0.52179	-0.69573	-0.08824	0.43483	0.57977	1				
Cu	0.37143	0.77143	0.08571	0.7537	0.88571 *	-0.71429	-0.02899	1			
Fe	0.25714	0.54286	-0.31429	0.23191	0.42857	0.54286	0.81168 *	0.08571	1		
Mn	0.08571	-0.54286	-0.2	-0.63775	-0.48571	-0.08571	-0.28989	-0.42857	-0.77143	1	
Zn	-0.08571	0.65714	-0.2	0.40584	0.54286	0.08571	0.40584	0.42857	0.77143	-0.88571 *	1

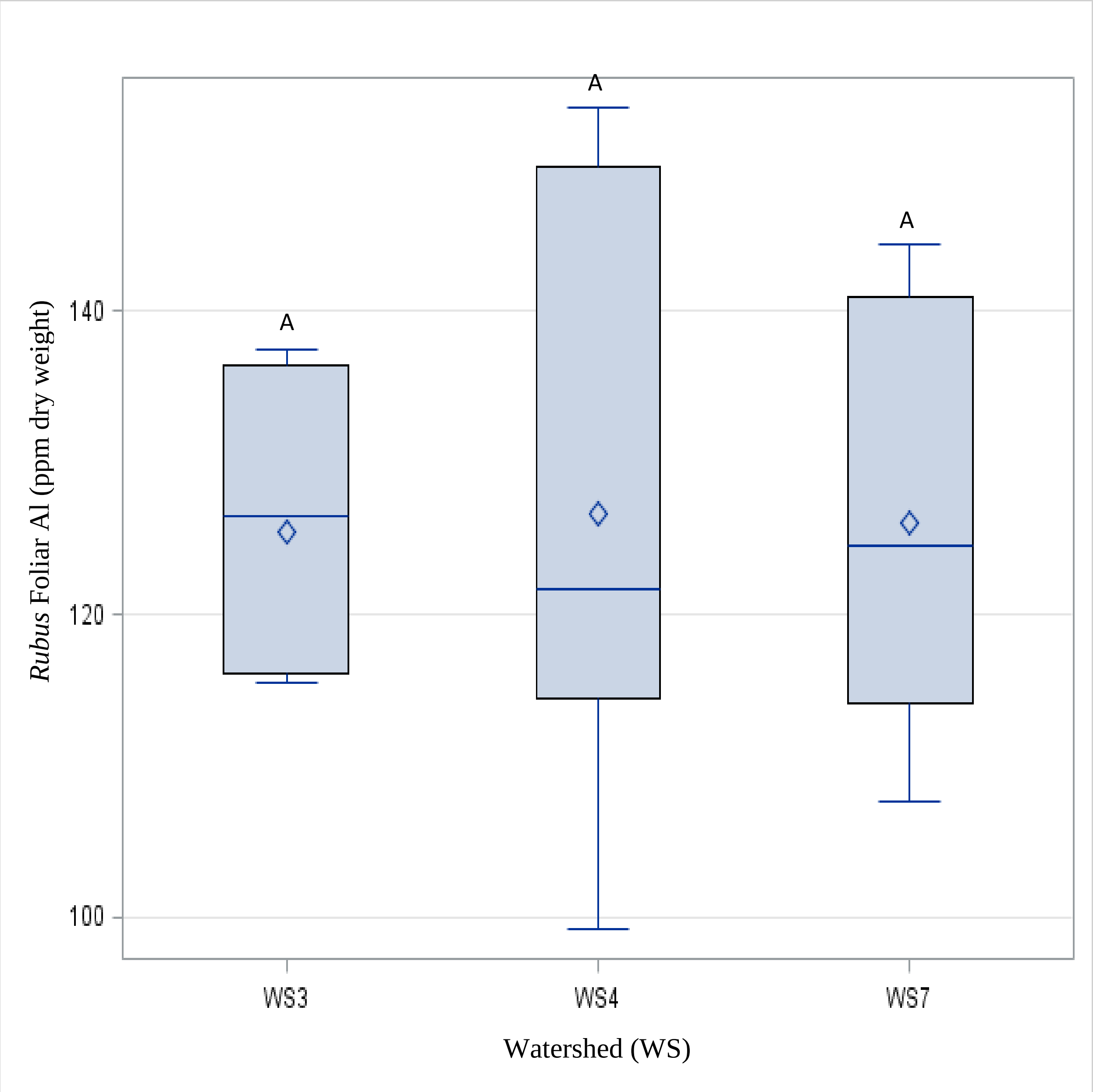

Figure 3.9: Mean parts per million (ppm) dry weight of *Rubus* foliar aluminum (Al) during 2013 and 2014 growing seasons combined for each watershed separately. Different letters represent a significant difference ($p<0.05$) between WS3 (Treatment), WS4 (reference), and WS7 (reference).

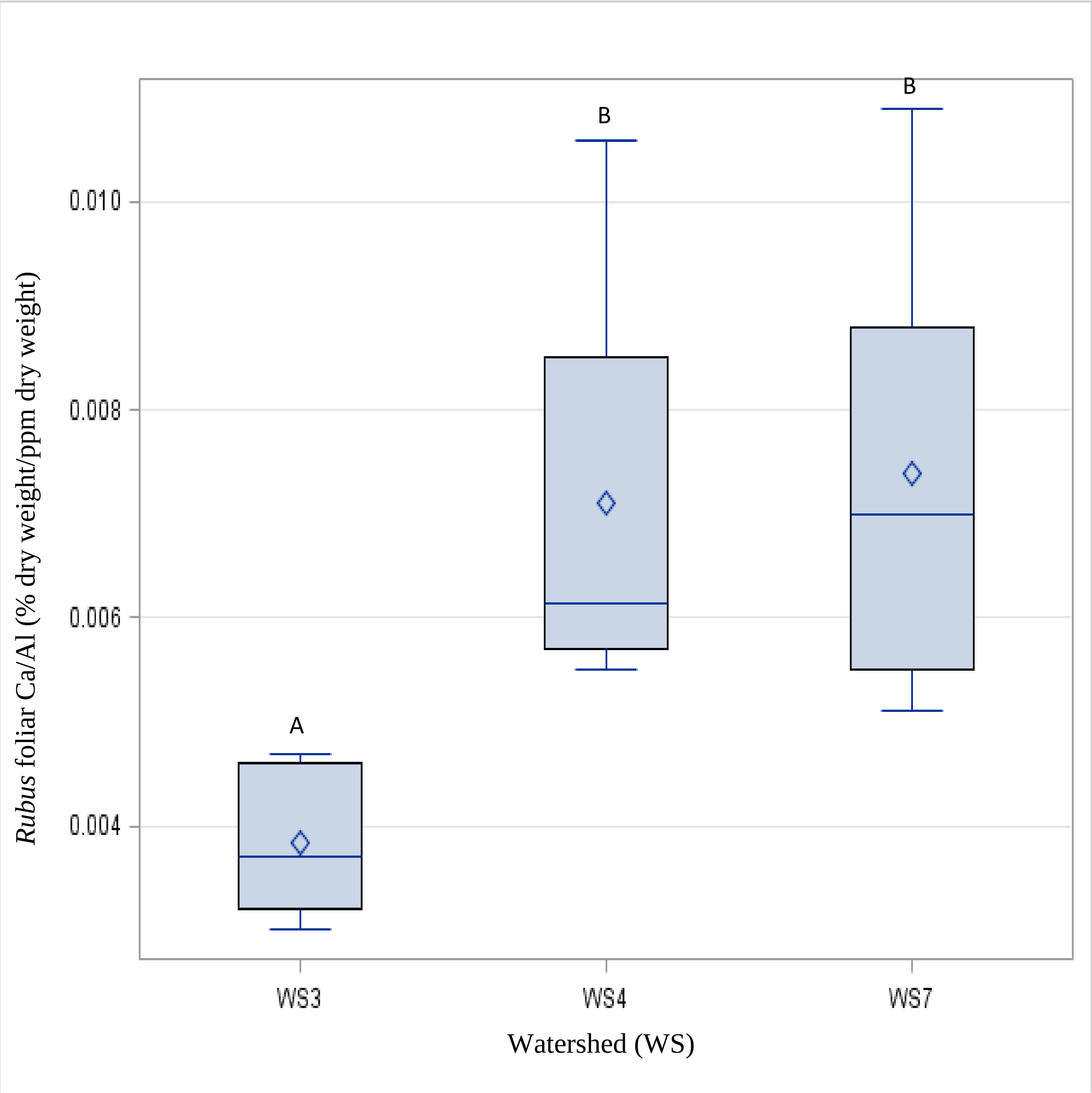

Figure 3.10: Mean % dry weight Ca:ppm dry weight Al in *Rubus* foliar tissue for 2013 and 2014 growing seasons combined. Different letters represent a significant difference ($p<0.05$) between WS3 (Treatment), WS4 (reference), and WS7 (reference).

Acidic soils increase exchangeable Mn in soil solution, particularly in the form of Mn^{2+} at pH<5.5 (Millaleo et al., 2010). Soils of WS3, WS4 and WS7 have a pH of 4.3, 4.5 and 4.1, respectively (Gilliam et al., 1994), which should show increased mobility of Mn. Kula et al. (2012) found *Rubus idaeus* L. (raspberry) and *Rubus fructicosus* L. (blackberry) to have the highest concentrations of Mn in foliar tissue along with other herbaceous species (e.g. *Sorbus aucuparia* L., Rowan). Consistent with the high concentration of Mn in the results of Kula et al. (2012), Mn concentration were significantly (P≤0.05) in *Rubus* foliar tissue on WS3 compared to reference WS4 and WS7 (Fig 3.11). Even though soils of all three watersheds are acidic, significantly higher (p≤0.05) Mn concentration in *Rubus* foliar tissue implies higher mobility of Mn in the mineral soil of WS3 due to excess N deposition. Significantly higher (p≤0.05) Mn concentrations in foliar tissue is due to more available Mn in soil solution from excess N deposition on WS3 along with luxury consumption of Mn by *Rubus*.

Micronutrients are utilized at low concentrations within plants, where higher concentrations can promote toxicity. *Rubus* shows high tolerance for micronutrients, more specifically metal ions (e.g. Mn^{++} and Fe^{+++}) (Donoso and Nyland, 2006). Alam et al. (2005) examined the effects of K on Mn toxicity in barley and found that K represses Mn toxicity. Higher K in *Rubus* foliar tissue (Fig 3.4) could be a result of Mn toxicity stress or could be responsible for high tolerance of Mn. Toxic symptoms of Mn could be affecting some species in the herb layer on WS3 and not having a significant impact on *Rubus* due to luxury consumption of K in *Rubus* foliar tissue. Increased K is associated with alleviating Fe toxicity in herbaceous species, decreasing foliar concentrations of Fe by two-fold in foliar tissue (Cakmak, 2005). There was no significant difference in *Rubus* foliar Fe concentrations among watersheds at FEF (Fig 3.12), but concentrations of Fe are similar (WS3>WS4>WS7) to K concentrations in *Rubus* foliar tissue (Fig 3.4). This suggests that Fe levels in foliar material are not affected by K concentrations because Fe may not be toxic to *Rubus*, but levels of Mn may be toxic and promoting luxury uptake of K to alleviate toxic stress.

Micronutrients in high amounts typically present herbaceous plants with toxic symptoms, where *Rubus* is able to potentially tolerate conditions that are not suitable for other herbaceous species. *Rubus* cover was significantly (P≤0.0003) related to soil Mn concentrations at FEF in 2011, where higher *Rubus* cover was associated with higher Mn concentrations in mineral soil (Chris Walter, unpublished data). High tolerance of *Rubus* for toxic levels of metal ions and micronutrients promote a competitive advantage for space, light, essential nutrients, and moisture. Luxury consumption of nutrients like K can provide tolerance for *Rubus* against effects of toxicity from excess N deposition, but other herbaceous species may be impacted by toxicity from Mn.

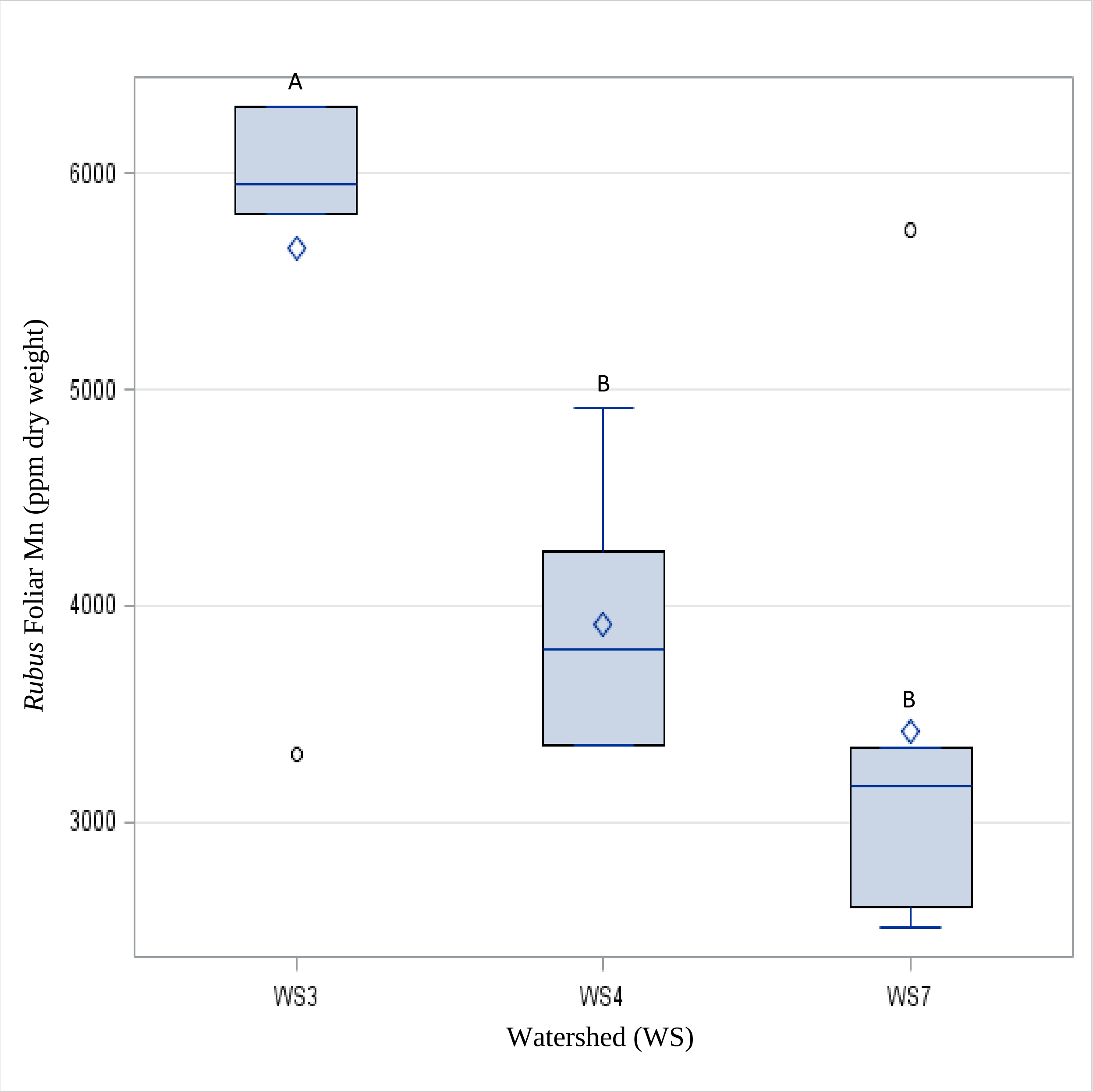

Figure 3.11: Mean parts per million dry weight of *Rubus* foliar manganese (Mn) during 2013 and 2014 growing seasons combined for each watershed separately. Different letters represent a significant difference ($p<0.05$) between WS3 (Treatment), WS4 (reference), and WS7 (reference).

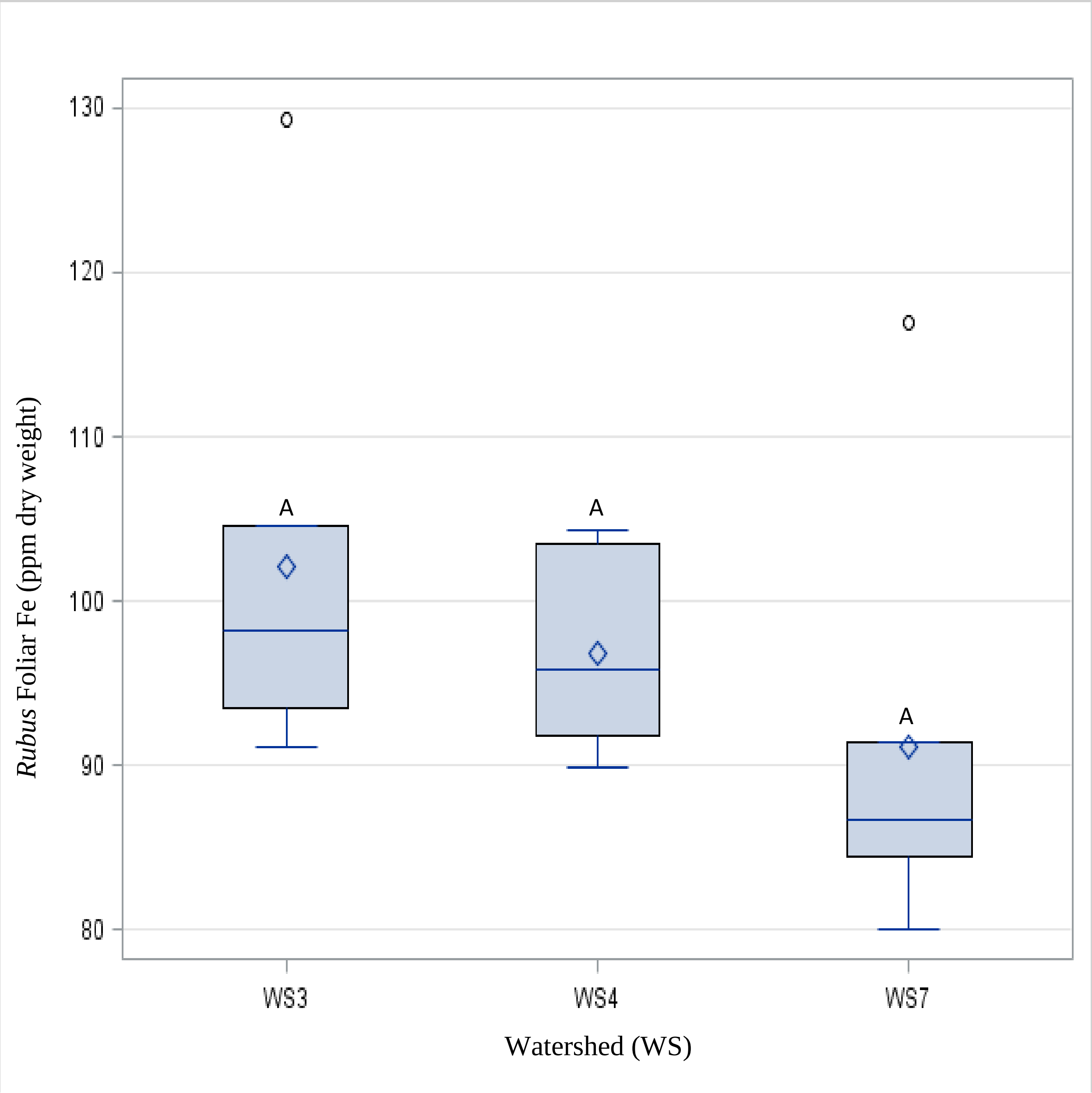

Figure 3.12: Mean parts per million dry weight of *Rubus* foliar iron (Fe) during 2013 and 2014 growing seasons combined for each watershed separately. Different letters represent a significant difference ($p<0.05$) between WS3 (Treatment), WS4 (reference), and WS7 (reference).

CHAPTER 4

CONCLUSION

Nitrogen deposition is increasing globally due to anthropogenic increases from high-energy combustion of fossil fuels and use of N-based fertilizers for agriculture, but despite efforts to regulate anthropogenic increases (Clean Air Act of 1990), rates are not expected to decrease in the near future (Bobbink et al. 2010). Excess N deposition has the potential to saturate the capacity for N retention in the eastern hardwood region, possibly altering herb layer dynamics and nutrient status, acidify soils, and lead to forest decline (Magill et al., 1997, 2004). This study assessed the effects of excess N deposition on the herbaceous layer of a mixed hardwood forest by comparing a watershed (WS3) that has been receiving N additions since 1989 to two adjacent watersheds (WS4 and WS7) that have not received any treatment. The objectives of this study were to: 1) quantify *Rubus* response to N treatment on WS3 from 1991 to 2014, 2) relate *Rubus* cover to species richness of the herbaceous layer during this period, and 3) determine effects of N additions on foliar nutrients of *Rubus,* providing an indication of nutrient status.

Herb layer dynamics

Herbaceous layers of hardwood forest ecosystems are particularly sensitive to fluctuations in N deposition, wherein excess N deposition can alter herb layer composition by decreasing biodiversity. Results of this study contrast earlier findings by Gilliam et al. (2006), who found no significant response after six years of N treatment to WS3 at FEF up to 1994. Excess N deposition has significantly altered herb layer composition on WS3 compared to WS4 through impacting herb layer dynamics after 1994. Total cover on WS3 significantly increased from 1991-present mainly due to the significant increase of *Rubus* cover. Results for increased *Rubus* cover from excess N deposition are consistent with many other studies that found higher *Rubus* presence associated with increasing N availability (Jobidon 1993; Truax et al. 1994;

Claussen and Lenz 1999; Hedl 2004; Strengbom and Nordin 2008; Hedwall et al. 2011; Heubner et al. 2013; McDonnell et al. 2014). As *Rubus* cover relative to total cover significantly increased from 1991-present, species richness of the herb layer decreased on WS3. Decreased biodiversity on WS3 from excess N deposition is consistent with predictions for effects of long-term excess N deposition (Gundersen et al. 1998; Aber et al. 1998; Magill et al. 2000; Aber et al. 2003; Bobbink et al. 2010). The decrease in species richness accompanied by an increase of *Rubus* cover supports the hypothesis for the herb layer study.

Nutrient status

Herbaceous layers of hardwood forest ecosystems are particularly sensitive to fluctuations of nutrient dynamics. Thus, foliar tissue analysis of *Rubus* in the herb layer can provide insight as to how excess N deposition is affecting nutrient status in the eastern hardwood region. Results of this study are consistent with previous finding at FEF by Gilliam et al. (1996), wherein excess N deposition has significantly increased N concentrations in herbaceous foliar material. Decrease of Ca concentrations in foliar material indicates that excess N deposition has saturated the capacity for N retentions at FEF, partially supporting the previous hypothesis; excess N treatment will increase foliar N of *Rubus* while decreasing foliar cation nutrients. Increased K concentrations from a shift of the N cycle to high NO_3^- mobility also indicate that soils are N saturated, with NO_3^- being the main source of available N for plant uptake.

This study also provides evidence for two potential toxic effects that can arise from excess N deposition. Even though there was no significant difference across watersheds for Al concentrations, excess N deposition significantly decreased Ca concentrations on WS3. Thus, the Ca:Al ratio was significantly lower on WS3, providing indication for potential Al toxicity that can result in forest decline (Godbold et al. 1988). Micronutrients are utilized at very low concentrations, with potential for toxicity arising when plants are exposed to levels that are higher than the biotic demand. Manganese was significantly higher on WS3, suggesting that *Rubus* may have high tolerance for Mn toxicity that can be potentially harmful to other herbaceous species.

Broader implication

The N treatment to WS3 of 35 kg N ha^{-1} yr^{-1} is double the ambient N deposition that was determined prior to the initiation of this treatment in 1989. Gilliam et al. (2006) concluded that this N addition to WS3 increased available N relative to the amount generated from net N mineralization by 25%, representing a relatively small addition of a no longer growth-limiting nutrient. This makes the N treatment (2x total ambient N deposition) to WS3 at FEF more biologically relevant compared to other studies on excess N deposition, with the lowest N treatments prior to this study being ~5x and ~7x total ambient N deposition (Fernandez et al. 2010 and Magill et al. 2004, respectively). The effects of excess N deposition to WS3 is also appearing to be representative of the gradual increase of *Rubus* cover on WS4 from ambient N deposition over this time period. These findings could be either unique to this region of the central Appalachians or could be applied as an indication of excess N deposition to the entire eastern hardwood region. Regardless, Gilliam (2006) found that there are far less N manipulation studies in hardwood forest ecosystems compared to other ecosystems (e.g. grass prairie); therefore, future studies are needed to justify if the summarized data from this study can be used as a baseline comparison for just the central Appalachians or the entire eastern hardwood region.

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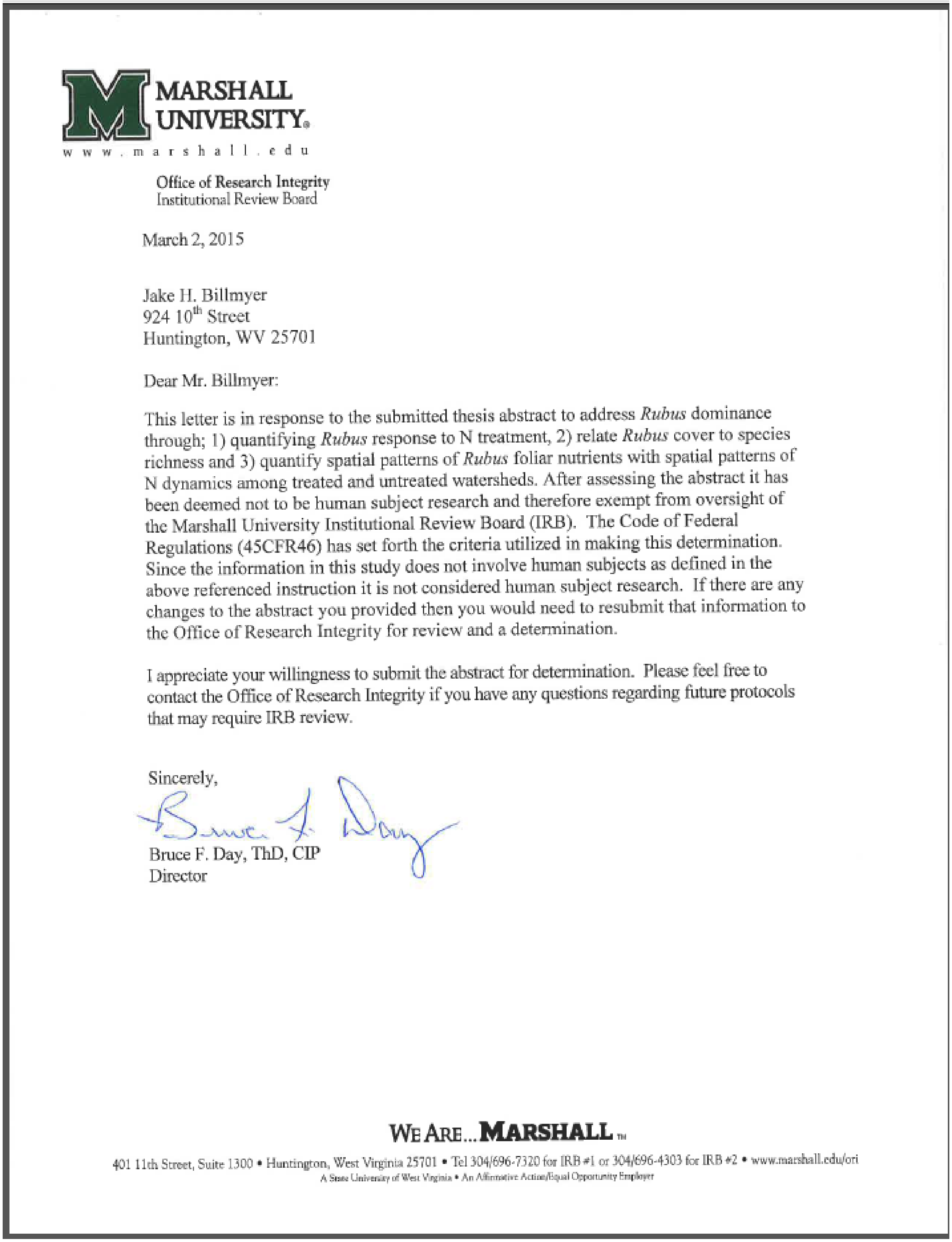
APPENDIX



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March 2, 2015

Jake H. Billmyer
924 10th Street
Huntington, WV 25701

Dear Mr. Billmyer:

This letter is in response to the submitted thesis abstract to address *Rubus* dominance through; 1) quantifying *Rubus* response to N treatment, 2) relate *Rubus* cover to species richness and 3) quantify spatial patterns of *Rubus* foliar nutrients with spatial patterns of N dynamics among treated and untreated watersheds. After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the information in this study does not involve human subjects as defined in the above referenced instruction it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day

Bruce F. Day, ThD, CIP
Director

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