AN ABSTRACT OF THE DISSERTATION OF

Jenny M. Dauer for the degree of Doctor of Philosophy in Forest Science presented on March 16, 2012.

Title: Calcium-oxalate in Sites of Contrasting Nutrient Status in the Oregon Coast Range

Abstract approved:

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Steven S. Perakis

Calcium (Ca) is an essential macronutrient that is increasingly recognized as a biogeochemical factor that influences ecosystem structure and function. Progress in understanding the sustainability of ecosystem Ca supply has been hampered by a lack of information on the various forms and pools of Ca in forest ecosystems. In particular, few studies have investigated the role of Ca-oxalate (Ca-ox), a ubiquitous and sparingly soluble biomineral formed by plants and fungi, on Ca cycling. I investigated Ca-ox pools in two young Douglas-fir forests in the Oregon Coast Range, and found that Ca-ox comprised 4 to 18% of total ecosystem Ca in high- and low-Ca sites, respectively, with roughly even distribution in vegetation, detritus and mineral soil to 1 m depth. The proportion of ecosystem Ca existing as Ca-ox varied by ecosystem compartment but was highest in needle litterfall, foliage and branches. Calcium-ox could be a large amount of Ca in mineral soil; across nine sites comprising a local soil Ca gradient, we found as much as 20% of available Ca in 0 - 10 cm depth mineral soil occurs as Ca-ox. Ca-ox was the dominant form of Ca returned from plants to soil, but disappeared as rapidly as bulk Ca from decomposing litter, suggesting an important pathway for Ca recycling. In mineral soil, Ca-ox was a larger portion of total available Ca in the low-Ca site, which had lower Ca-ox concentrations overall, suggesting that Ca-ox has limited potential to buffer against Ca depletion in forests where Ca is in shortest supply. I investigated foliar chemistry as a method for diagnosis of nutrient deficiencies in

high and low-Ca sites where Ca varied inversely with soil nitrogen (N), and which had received fertilization with urea (for nitrogen, N), lime, and calcium chloride three years prior. Foliar vector diagrams suggested N limitation at the low-N site and N sufficiency at the high-N site, but did not suggest Ca deficiency at either site after urea, lime and Ca-chloride fertilization. The high-Ca site displayed 20-60 times higher concentrations of foliar Ca-oxalate than the low-Ca site, although this was unaffected by fertilization. Soil nitrification responded to both N and lime fertilization at both sites, suggesting that fertilization with N may stimulate nitrification that could accelerate soil Ca loss. I also investigated how Ca-ox may influence cation tracers such as Ca and strontium (Sr) ratios (i.e., Ca/Sr) and Ca-isotopes ($^{44}Ca/^{40}Ca$), which are used to identify sources and pathways of Ca cycling in ecosystem studies. Laboratory synthesis of Ca-ox crystals exhibited preference for Ca over Sr, and for ^{40}Ca over ^{44}Ca. In the field, discrimination between Ca and Sr was detected in bulk plant tissues due to Ca-ox accumulation, suggesting that Ca-ox accumulation related to tree Ca supply status could influence interpretations of Ca/Sr as a tracer of Ca cycling. I also found that standard methods of soil exchangeable Ca extraction could dissolve Ca-ox crystals and potentially contribute an additional 52% to standard measurements of exchangeable-Ca pools in low-Ca sites, thus complicating long-standing interpretations of available soil Ca pools and dynamics in many studies. Results of this work show overall that Ca-ox is found in large quantities in plants, detritus, and mineral soil in forest ecosystems, and is a more dynamic component of ecosystem Ca cycling than previously recognized.

Calcium-oxalate in Sites of Contrasting Nutrient Status in the Oregon Coast Range

by
Jenny M. Dauer

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degree of

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Doctor of Philosophy dissertation of Jenny M. Dauer presented on March 16, 2012.

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Jenny M. Dauer, Author

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CONTRIBUTION OF AUTHORS

Dr. Steven Perakis was involved in the planning and design of this research as well as interpreting data and editing this dissertation. Emily Sinkhorn provided biomass data for bole-wood, branches litter, forest floor mass and soil nutrient data as part of her Master's thesis in Chapter 2. Dr. Douglas Maguire and Douglas Mainwaring provided pre-treatment data, allometric equations for foliage, branches, bole-wood and bark and tree volume, basal area and height growth in Chapter 2. Joselin Matkins provided leaf litter decomposition mass data and samples in Chapter 2 as part of her Master's thesis. Dr. Thomas Bullen provided calcium isotope and calcium/strontium ratio data in Chapter 4.

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For E. Marie Edwards and Pauline M. Cordner, my grandmothers.

CHAPTER 1 – INTRODUCTION

Calcium (Ca) is an essential macronutrient that is increasingly recognized as a biogeochemical factor that influences ecosystem structure and function (Schaberg et al., 2001; Bigelow and Canham, 2007; Lautner and Fromm, 2010). Calcium has an effect on a wide range of ecosystem processes and functions (McLaughlin and Wimmer, 1999) including buffering the effects of acid deposition (Johnson et al., 1991), stability and structural integrity of trees (Lautner and Fromm, 2010), plant cellular signaling in response to environmental stimuli (McAinsh and Schroeder, 2009) and water flow regulation (Gilliham et al., 2011). In some forests Ca from mineral weathering is likely to be insufficent for plant growth and ecosystem functioning (Bedison and Johnson, 2010) on soils that are highly weathered (Cuevas and Medina, 1988; Reich et al., 1995; Bockheim and Langley-Turnbaugh, 1997) which may be exacerbated in regions that experience acid rain deposition (Federer et al., 1989; Driscoll et al., 2001; Liu et al., 2011) or in stands undergoing sequential whole-tree harvests (Hornbeck and Kropelin, 1982; Perakis et al., 2006; Siemion et al., 2011). Progress in understanding the sustainability of ecosystem Ca supply has been hampered by a lack of information on the various forms and pools of Ca in forest ecosystems. For example, there has been considerable interest and methodological development in understanding how various chemical forms of other macronutrients such as nitrogen (N) and phosphorous (P) are distributed in plants and soils (Schlesinger, 1997), for example organic N versus inorganic ammonium and nitrate (Sparks, 1996), and inorganic versus organic P (Compton and Cole, 1998). Comparable approaches for investigating Ca forms and partitioning in plant-soil systems are poorly developed (Sparks, 1996), in fact, nearly all ecosystem and nutrition studies measure plant tissues as bulk-Ca and in forest floor and soils via exchangeable-Ca (Mead, 1984; Johnson and Lindberg, 1992; Sparks, 1996), omitting the ability to investigate single biogeochemical or physiological processes that influence Ca dynamics.

Calcium-oxalate (Ca-ox) is a biomineral form of Ca that may play an important ecosystem role, but its contribution to pools of Ca in ecosystems is poorly understood. Ca-ox is created by nearly all plants (Franceschi and Nakata, 2005) and by both mycorrhizal and saprophitic fungi (Cromack et al., 1979; Arnott, 1995; Dutton and Evans, 1996). Both plants

and fungi internally create Ca-ox crystals to sequester Ca away from the cytoplasm, where ionic Ca is kept in micromolar concentrations (Arnott, 1995; Franceschi and Nakata, 2005). Plants and fungi also release oxalate ions into soil or detritus to regulate pH and cation balance of their external soil or detrital microenvironment (Jellison et al., 1997) or to enchance mineral weathering or decomposition for nutrient uptake (Landeweert et al., 2001; Ryan et al., 2001; Arvieu et al., 2003). Oxalate ions can subsequently bind to free soil Ca ions forming highly insoluble Ca-ox crystals (K_{sp} 2.57 x 10^{-9}, Ringbom, 1963). Other cation complexes with oxalate are often less soluble than Ca-ox and are much less common in natural systems (Baran and Monje, 2008; Echigo and Kimata, 2011). Oxalate ions in soil can range in concentration from 1 to 600 μM (Allison et al., 1995; Strobel, 2001), though concentrations of crystalline Ca-ox are infrequently quantified. Observational studies report that Ca-ox is found widely and can accumulate in soils (Graustein et al., 1977; Cromack et al., 1979) and is particularly observed *in situ* associated with decomposing leaf litter (Arnott, 1995; Horner et al., 1995) and ectomycorrhizal fungal mats (Cromack et al., 1979; Entry et al., 1991). Despite progress in identifying mechanisms and controls on Ca-ox formation in plants (Franceschi and Nakata, 2005), understanding the significance of Ca-ox occurrence at the ecosystem-scale requires more detailed information on Ca-ox pools sizes in vegetation, detritus and mineral soils.

The objectives of this dissertation are to investigate: 1) amount, distribution and degradation rates of Ca-oxalate in forests along a nutrient availability gradient, 2) the use of Ca-oxalate for foliar diagnosis of response to fertilization, 3) influence of Ca-ox on Ca/Sr discrimination and Ca-isotope ($^{44}Ca/^{40}Ca$) fractionation in biological systems, 4) Ca-oxalate measurement in soil and separation from other chemical forms of Ca in soil. For the first objective to estimate the amount and distribution of Ca-ox, I sampled major ecosystem plant and soil pools and analyzed Ca-oxalate concentrations using a sequential extraction procedure to separate chemical forms of Ca in tissue (Fink, 1991). I used sequential extraction of leaf litter pools of Ca-ox to track changes in Ca-ox concentrations over time in decomposing leaf litter. Other studies have implied that Ca-ox is an important and non-labile reservoir for Ca in the soil (Bailey et al., 2003; Smith et al., 2009), although, Ca-ox decomposition rates in ecosystems is unstudied. For the second objective to determine if Ca-ox can be used as a foliar diagnosis in response to Ca and nitrogen (N) additions, I measured Ca-ox in leaf tissue of two

sites fertilized with urea, lime and calcium-chloride, expecting that Ca fertilization would increase Ca accumulation in foliar chemical Ca fractions. Foliar diagnosis is useful if trees indicate changes in foliar nutrition before or in association with changes in growth or health, allowing managers to more easily measure fertilization response compared to tree biomass or volume changes. For the third objective to determine the influence of Ca-ox on Ca/Sr discrimination and Ca-isotope fractionation in biological systems I first synthesized Ca-ox in the laboratory to estimate strength of preference for Ca over Sr and for ^{40}Ca over ^{44}Ca. I then sampled ecosystem compartments to compare discrimination of Ca/Sr and fractionation of ^{40}Ca/^{44}Ca in patterns at whole-plant level, plant organ- and tissue-level scales and whether Ca-ox accumulation in tissues influenced these patterns. While discrimination of Ca/Sr and fractionation of ^{40}Ca/^{44}Ca are known to occur in ecosystems due to biological processes, little work has been done in determining mechanisms and the importance of these mechanisms to bulk Ca/Sr and ^{40}Ca/^{44}Ca values. For the fourth objective I investigated Ca-ox measurement in soil and separation from other chemical forms of Ca in soil. It is generally assumed that insoluble Ca-ox is not detected in measurements of exchangeable Ca with neutral pH salts (Cromack et al., 1979), so assumed omission of Ca-ox from previous soil measurements of Ca results in speculation over the size and influence of this pool. I measured the solubility of Ca-ox in salt solutions that are typically used in exchangeable cation analysis to determine expected Ca-ox solubility. Then I used a spike-recovery approach in soils with additions of Ca-ox crystals, which were then analyzed for soluble Ca using typical salt extractions of exchangeable Ca.

Throughout this dissertation I contrasted sites with differing nutrient status to more fully understand differences between Ca-rich and Ca-poor sites. I used this approach based on the range of ecosystem nutrient properties found in the Oregon Coast Range region that have been studied more intensely previously (Perakis and Sinkhorn, in progress). These nine sites had an inverse relationship between Ca and N in terms of both soil supply and plant accumulation. Across the sites with a nearly 3-fold range in soil N, soil exchangeable pools of Ca, Mg, and K to 100 cm decreased by an order of magnitude and soil pH declined from 5.8 to 4.2 (Perakis and Sinkhorn, in progress). The inverse relationship between Ca and N was due to long-term N enrichment leading to acidified soils and depletion of much of the available base

cation pool prior to establishment of the current conifer stands. At the lower-Ca sites, both biomass and surface soil contained a larger fraction of available ecosystem Ca than in Ca-rich sites, suggesting tight biotic retention and recycling of Ca as its overall supply decreased in N-rich sites. Fertilized plots studied in this dissertation also represent range of Ca found in the Oregon Coast Range, for example, soil exchangeable Ca in 0-10 cm deep mineral soils are similar in range between the fertilized sites and the sites in the other chapters (low-Ca, Site 16: 194 μg Ca g^{-1}, low-Ca fertilized site, GDH: 110 μg Ca g^{-1}, high-Ca site, Site 5: 2031 μg Ca g^{-1}, high-Ca fertilized site, OSU: 2680 μg Ca g^{-1}) and have similar inverse relationships with N. The sites I chose for this dissertation are the ends of a natural gradient of soil Ca-supply that represent contrasting models of cation cycling in this region.

More detailed information about chemical forms of Ca is important to understanding perturbations to ecosystem nutrient cycling. Calcium can be deficient in areas of acid rain deposition in the Northeastern U.S. and Europe (Shortle et al., 1997; Gebauer et al., 1998) and increasingly in China (Liu et al., 2011), on older weathered soils in tropical regions (Cuevas and Medina, 1988; Reich et al., 1995) or in managed temperate zone forests that have undergone biomass removal with repeated harvest (Hornbeck and Kropelin, 1982; Perakis et al., 2006). Greater understanding of chemical forms of Ca is important to understanding soil supply and resilience to disturbance as different chemical forms of Ca may be involved in single biogeochemical or physiological processes that respond in various ways to disturbances. This dissertation represents an attempt to advance the understanding and interest in studying ecosystem Ca with more attention to chemical forms of Ca and their varying influence on long-term soil Ca supply, tree Ca deficiency, and tools for studying Ca dynamics.

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CHAPTER 2 – CALCIUM-OXALATE POOLS IN FORESTS OF CONTRASTING NUTRIENT STATUS

Abstract

Calcium-oxalate (Ca-ox) is an insoluble biomineral that forms in plants and fungi and occurs in soils across many types of ecosystems. However, to date no studies have quantified whole-ecosystem pools of Ca-ox collectively considering plants, detritus, and mineral soil. We studied two Douglas-fir forests of contrasting Ca status in the Oregon Coast Range and compared sizes of Ca-ox pools across these sites. We also evaluated the contribution of Ca-ox to total Ca in plants and detritus, and to the sum of exchangeable Ca plus Ca-ox in mineral soil to 100 cm depth. We found that Ca-ox comprised 4% and 18% of total ecosystem Ca in the high-Ca and low-Ca site, respectively. The largest pools of Ca-ox were living biomass (42 to 40% of total Ca-ox), detritus (37 to 31% of total Ca-ox) and mineral soil (21 to 29% of total Ca-ox). The forest floor contained 17 to 13% of the total Ca-ox at both sites, with estimated Ca-ox mean residence times of 9 to 18 years, which was shorter than bulk Ca, 15 to 25 years, at the high- and low-Ca site respectively. Ca-ox was more concentrated in fresh litterfall than in living biomass, particularly at the high-Ca site, where it was the dominant form of Ca returned from plants to soil. Ca-ox disappeared rapidly from decomposing litter (0.28 yr^{-1} or faster) at both sites, yet did not appear to be associated with changes in litter pH, suggesting biodegradation of Ca-ox in decomposing litter. In mineral soil, Ca-ox was a larger portion of total available Ca (exchangeable Ca plus Ca-oxalate Ca) in the low-Ca site (9%) than the high-Ca site (1%), and this general pattern was supported across seven additional sites that constitute a wide local forest Ca gradient. However, the mineral soil Ca-ox concentration was lower overall in low-Ca site (16 μg Ca g^{-1} soil, 0-10 cm depth) than the high-Ca site (37 μg Ca g^{-1} soil, 0-10 cm depth), and therefore has limited potential to buffer against Ca depletion in forests where Ca is in shortest supply. Overall, our results suggest that differences in soil Ca among sites can directly influence Ca and Ca-ox accumulation in plant and detrital pools, and that Ca-ox does not resist decomposition to accumulate over time in the forest floor as previously assumed, but instead is an important pathway for Ca recycling.

Introduction

Calcium (Ca) is an essential macronutrient that is increasingly recognized as a biogeochemical factor that influences ecosystem structure and function (Schaberg et al., 2001; Bigelow and Canham, 2007; Lautner and Fromm, 2010). In some forests Ca from mineral weathering is likely to be insufficent for plant growth and ecosystem functioning (Bedison and Johnson, 2010) particularly on soils that are highly weathered (Cuevas and Medina, 1988; Reich et al., 1995; Bockheim and Langley-Turnbaugh, 1997), and which may be exacerbated in regions that experience acid rain deposition (Federer et al., 1989; Driscoll et al., 2001; Liu et al., 2011), in stands undergoing sequential whole-tree harvests (Hornbeck and Kropelin, 1982; Perakis et al., 2006; Siemion et al., 2011). Progress in understanding the sustainability of ecosystem Ca supply has been hampered by a lack of information on the various forms and pools of Ca in forest ecosystems. For example, while there has been considerable interest and methodological development in understanding how various chemical forms of other macronutrients such as nitrogen and phosphorous are distributed in plants and soils (Schlesinger, 1997), comparable approaches for investigating Ca forms and partitioning in plant-soil systems are poorly developed (Sparks, 1996). In fact, nearly all ecosystem and nutrition studies measure plant tissues as bulk-Ca and in forest floor and soils via exchangeable-Ca (Mead, 1984; Johnson and Lindberg, 1992; Sparks, 1996), omitting the ability to investigate single biogeochemical or physiological process that influence Ca dynamics.

Calcium-oxalate (Ca-ox) is a biomineral form of Ca that may play an important ecosystem role, but its contribution to pools of Ca in ecosystems is poorly understood. Ca-ox is created by nearly all plants (Franceschi and Nakata, 2005) and by both mycorrhizal and saprophitic fungi (Cromack et al., 1979; Arnott, 1995; Dutton and Evans, 1996). Both plants and fungi internally create Ca-ox crystals to sequester Ca away from the cytoplasm, where ionic Ca is kept in micromolar concentrations (Arnott, 1995; Franceschi and Nakata, 2005). Plants and fungi also release oxalate ions into soil or detritus to regulate pH and cation balance of their external soil or detrital microenvironment (Jellison et al., 1997) or to enchance mineral weathering or decomposition for nutrient uptake (Landeweert et al., 2001; Ryan et al., 2001; Arvieu et al., 2003). Oxalate ions can subsequently bind to free soil Ca ions forming highly insoluble Ca-ox crystals (K_{sp} 2.57 x 10^{-9}; Ringbom, 1963). Other cation complexes with

oxalate are often less soluble than Ca-ox and are much less common in natural systems (Baran and Monje, 2008; Echigo and Kimata, 2011). Oxalate ions in soil can range in concentration from 1 to 600 μM (Allison et al., 1995; Strobel, 2001), though concentrations of crystalline Ca-ox are infrequently quantified. Observational studies report that Ca-ox is found widely and can accumulate in soils (Graustein et al., 1977; Cromack et al., 1979) and is particularly observed *in situ* associated with decomposing leaf litter (Arnott, 1982; Horner et al., 1995) and ectomycorrhizal fungal mats (Cromack et al., 1979; Entry et al., 1991). Despite progress in identifying mechanisms and controls on Ca-ox formation in plants (Franceschi and Nakata, 2005), understanding the significance of Ca-ox occurrence at the ecosystem-scale requires more detailed information on Ca-ox pools sizes in vegetation, detritus and mineral soils.

Ca-ox creation in plant tissue and soil, and the subsequent longevity of insoluble Ca-ox crystals, may influence both short- and long-term Ca supply and recycling in ecosystems. In plants, once Ca^{2+} is deposited in Ca-ox crystals it becomes unavailable for other physiological processes unless the Ca supply is completely truncated (Franceschi, 1989; Volk et al., 2002). Because general Ca mobility in plant tissue is low, the sequestration of available Ca^{2+} in Ca-ox crystals may become a limiting process for other plant functions involving Ca. As leaves age, Ca-ox accumulates (Borer et al., 2004; Littke and Zabowski, 2007; Smith et al., 2009), which when dropped as leaf litter deposits Ca-ox to the forest floor. If highly insoluble Ca-ox from leaf litter, roots and fungi accumulates in soil and then resists degradation, it could potentially reduce soil Ca availability overall. The rate of Ca-ox degradation in soil is unknown; some authors suggest that Ca-ox is a "slow-release" form of Ca that is retained in soils and an important long-term source in regions where ecosystem Ca inputs are relatively low (Bailey et al., 2003; Smith et al., 2009), while others indicate that Ca-ox may turnover quickly in leaf litter microcosms (O'Connell et al., 1983). Information on rates of Ca-ox delivery from plants to soils via leaf litter, on the longevity of Ca-ox in decaying detritus, and on how these fluxes may vary with sites Ca status, are significant unknowns in resolving the importance of Ca-ox to ecosystem Ca dynamics overall.

Douglas-fir forests of the Oregon Coast Range exhibit wide variation in plant and soil Ca status, with evidence of long-term Ca depletion to low levels that limit tree growth at low-

Ca sites (Perakis et al., 2006). These sites provide an opportunity to examine the importance of Ca-ox in ecosystem Ca partitioning and dynamics among forests that differ widely in overall Ca status. The objectives of this study were: 1) to quantify Ca-ox in major ecosystem compartments of living biomass, detritus and mineral soil at high and low Ca sites, 2) to examine which factors may shape variation in soil Ca-ox concentrations, and 3) to determine the potential role of foliar Ca-ox in shaping subsequent Ca dynamics during leaf litter decomposition.

Methods

Study sites

We quantified Ca-ox in major ecosystem compartments of living biomass, detritus and mineral soil at two sites of sites of contrasting Ca status in the north-central Oregon Coast Range. The high-Ca site (site 5) is located at N44°38' W123°48', 322 m elevation with a SW aspect and a 55% slope. The low-Ca site (site 16) is located at N45°10' W123°55', 115 m elevation with a SW aspect and a 15% slope. Soils at both sites are classified as Andic Dystrudepts, and are derived from ocean floor sedimentary parent material that was uplifted during the tertiary (Orr et al., 1992). Both sites experience maritime, temperate climates with cool, wet winters and warm, dry summers. The sites receive most precipitation as rain from October-April, and average annual precipitation from September 2004 to June 2006 was 173 and 196 cm yr^{-1} for the high and low-Ca site respectively (Daly et al., 1994; Perakis and Sinkhorn, 2011). January mean minimum and July mean maximum temperatures range from –2 to +2°C and from 20 to 28°C, respectively (Maguire et al., 2002). The vegetation at these two sites is dominated by planted Douglas-fir (*Pseudotsuga menziesii*), with occasional volunteer western hemlock (*Tsuga heterophylla*), red alder (*Alnus rubra*), Sitka spruce (*Picea sitchensis)*, vine maple (*Acer circinatum* Pursh), and bigleaf maple (*Acer macrophyllum*). Douglas-fir trees were planted in 1977 at the high-Ca site and 1980 at the low-Ca site (Table 1). Additional information on these sites is available in Perakis et al. (2006) and Perakis and Sinkhorn (2011).

Tree tissue sampling and biomass estimation

To measure concentrations of Ca-ox in living biomass compartments of the ecosystem, we collected samples of Douglas-fir tree tissue during 2007 to 2010 within a 0.5 ha plot at the high and low-Ca sites, including: leaves by age cohorts, branches, bark, bole-wood and roots. Sun-exposed foliage was composited by site from three trees at the end of the growing season (Sept and Oct 2007). Live foliage was separated into age cohorts (5 age cohorts at the high-Ca site and 4 age cohorts at the low-Ca site). Branch samples collected in 2007 from each of three trees at both sites were removed and cut into representative samples (Perakis and Sinkhorn, 2011). In May 2010 we cored three trees from each site twice with an increment borer to a depth of 20 cm, discarding any heart-wood, and separating the remainder into bark and bole-wood tissues. Four additional bark samples were taken with a 2 cm diameter corer. Samples were composited into one bark and one bole-wood sample per site. In June 2010 we collected root biomass by taking a composite of three 6.7 cm diameter soil cores to 10 cm depth, followed by washing the sample with tap water through three nested sieves of 2mm, 1 mm and 0.425 mm sizes. Representative samples of <2 mm and 2-10 mm fine roots were picked from the sieves and composited by site. Leaves, bark and bole-wood samples were flash frozen in an -80°C cooler and subsequently freeze-dried (Virtis 35L Genesis Super XL, SP Industries, Warminster, PA, USA). Branches and roots were dried at 65°C for 48 hrs. All samples were ground to fine powder prior to chemical analysis.

We determined pool sizes of each biomass compartment for subsequent determination of Ca pool sizes. We used estimates of foliage and branch biomass from a previous nutrient cycling study (Perakis and Sinkhorn, 2011) that were based on allometric equations developed at these sites (Maguire et al., 2002; Weiskittel et al., 2010). Bark and bole-wood biomass were estimated using an allometric equation (Maguire and Hann, 1990) updated with individual tree parameters measured on the sites in 2008. The density of < 2 mm and 2-10 mm diameter fine roots was determined from eight (Site 5) and six (Site 16) 0-10 cm deep soil cores in June 2006 using 6.7 cm diameter bulb corers and the same sieving technique as above. To calculate root biomass to 80 cm depth, we assumed root density measured in our 0-10 cm cores also applied to 10-20 cm depths, and used estimates of < 2 mm and 2 – 10 mm root density for the 20-80 cm depth determined from 16 cores at a nearby coastal Douglas-fir site (Lee et al., 2007).

Detrital sampling and biomass estimation

We measured Ca-ox in detrital pools as fine and coarse woody debris and forest floor. In five 16 m^2 subplots at each site, we measured the amount of fine (1 to 10 cm diameter) and coarse (> 10 cm diameter) woody detritus during May 2010 (Harmon and Sexton, 1996). All coarse downed wood in each subplot was measured for width, diameter, and decay class (Fasth et al., 2010) and a representative sample was collected. Fine downed wood was collected from a 1 m^2 subplot in each of the five main woody debris subplots and separated into decay classes for each site. Both fine and coarse wood were dried at 65°C for 48 hrs prior to weighing, grinding, and chemical analysis. Ten litter traps (1.4 m^2 each) were placed in random locations within each plot in June 2010, during the summer dry season. We collected litter prior to the onset of fall rains in September 2010, separated needles from twigs and other debris, and composited needles into a single sample by site. Samples were dried at 65°C for 48 hrs prior to weighing, grinding, and chemical analysis.

Samples of the forest floor (30 x 30 cm) were collected in September 2010 from each of five 16 m^2 subplots per site, and composited by site. Forest floor samples were dried at room temperature and then ground to a fine powder. To determine the amount of mineral soil in the forest floor samples, dry ash content was determined by weighing before and after combustion at 500°C. To control for Ca in the mineral soil mixed with the forest floor samples, we collected mineral soil directly below the forest floor from 0-3 cm (30 x 30 cm), sieved to 2 mm to remove rocks and debris and to homogenize the soil, then dried, ground and analyzed the samples in the same manner as the forest floor. After chemical analysis the forest floor Ca-ox concentrations were corrected for the weight of dry ash and HCl-extractable Ca in the 0-3 cm mineral fraction collected directly beneath the forest floor.

Coarse downed wood biomass was estimated by calculating each sample's volume with Huber's formula (Husch et al., 1972). Wood density was determined by using the mean moisture content measured on the fine downed wood and published data on Douglas-fir specific gravity (Forest Products Laboratory, 2010), then multiplying by a softwoods decay class reduction factor (Waddell, 2002). The biomass of all coarse downed wood samples in the

16 m^2 plots was summed and reported as biomass (Mg) per ha for each site. The mean total dried fine downed wood weighed within 1 m^2 plot at each site was scaled to report data as biomass (Mg) per ha. We used forest floor biomass measurements determined in 2003 and 2005 and Douglas-fir whole litter flux estimates determined 2004-2006 (Perakis and Sinkhorn, 2011) adjusted by the proportion of foliage in litterfall during the summer estimated on these sites (Weiskittel, 2003).

Sequential extraction of Ca

For all biomass, woody debris, and forest floor samples we used a sequential extraction technique to separate Ca into soluble Ca^{+2} ions (Ca_{aq}), structurally bound Ca in Ca-pectin and Ca-lignin (Ca_{st}) and insoluble Ca in Ca-ox (Ca_{ox}) (Fink, 1991; Borer et al., 2004). Ground tissue (5 g subsamples) of leaf, branch, bark, bole-wood, roots, and litter were sequentially extracted for Ca using three 10 mL solutions: water (Ca_{aq}), 2 *N* acetic acid (Ca_{st}), and 2 *N* hydrochloric acid (Ca_{ox}). Each extraction was centrifuged for 8 min and 11,000 rpm, the supernatant reserved for analysis, and the remaining solid rinsed with 40 mL Nanopure water and dried at 60 C prior to extraction of the next sequential fraction. This sequential extraction method, developed for biomass, was also applied to determine Ca fractions in forest floor and 0-3 cm depth mineral soil (see above). For total Ca concentrations (Ca_t), 2 mg of dried tissue or forest floor were weighed into a glass scintillation vial and burned for 12 hrs at 500°C. Samples were digested with 12 *N* HCl, and diluted to 2 *N* HCl with nanopure water. Calcium concentrations were measured using atomic absorption spectrophotometry on an AAnalyst 200 Spectrometer, Perkin Elmer (Waltham, MA, USA). The sum of the three extractions (Ca_{sum}) was compared to Ca_t to determine the completeness of the Ca extraction.

Ca-ox in mineral soil

We estimated mineral soil Ca-ox concentrations in 0-100 cm depth at the high and low-Ca status sites for quantifying total ecosystem Ca distribution. Additionally, to more widely understand the importance of Ca-ox in soils, we analyzed seven additional sites in the

Coast Range of Oregon along a 35-fold gradient of mineral soil exchangeable Ca, as described elsewhere (Perakis and Sinkhorn, 2011, Perakis and Sinkhorn, in progress). We estimated Ca_{ox} concentrations (amount of Ca within Ca-ox crystals) in surface to deep mineral soil by measuring acid extractable oxalate anion concentrations and converting these to Ca_{ox} and to moles of Ca-ox using stoichiometric relationships. Mineral soil samples were collected in summer 2005 in depth increments of 0-10 cm, 10-20 cm, 20-30 cm and 70-100 cm. Four locations within each site were randomly selected for soil sampling. A pit excavation method was used for the first 10 cm and a 4 cm diameter corer was used for the lower soil depths. Mineral soils were sieved to 2 mm to remove rocks and debris and to homogenize the soil. Subsamples were composited by depth within sites and stored at 4°C for up to 2 months before extraction and analysis. We also used Ca_{exch} data from these sites determined previously by 40 mL of 1 N NH_4OAc extraction of 2 g of soil (Perakis and Sinkhorn, in progress). Soil bulk density, soil volume, layer depth, and nutrient concentrations were used to determine total soil nutrient content (Perakis and Sinkhorn, 2011).

There is no standard method for Ca-ox determination in mineral soil, and we selected a conservative oxalate extraction that was based on relatively dilute HCl and short extraction times in comparison to other studies (discussed below), corrected for spike-recovery of Ca-ox addition to each sample. This approach assumes that the oxalate measured by dilute acid extraction was bound primarily to Ca in Ca-ox crystals, as previously reported in similar soils (Cromack et al., 1979). Our selection of extraction procedure followed from preliminary tests where stronger HCl and longer extraction times failed to yield additional oxalate in test soils collected from our study sites. Soil samples of 5 g were extracted with 0.5 M HCl for 5 hours (Lilieholm et al., 1992). Duplicate samples were spiked with 10 mg of Ca-ox and extracted as above. Spike recovery ranged from 14% to 66% on individual samples across all 9 sites, and was used to correct for non-extracted Ca-ox. Oxalate ions were measured by Dionex Ion Chromatography system with a carbonate-bicarbonate eluent with the ASRS-4mm suppressor and AS4-A analytical column (4x250mm) with AG4-A guard column (4x250mm). The concentration of the Ca_{ox} soil pool was calculated using the stoichiometric relationship between oxalate (88 g mol^{-1}) and Ca (40 g mol^{-1}) in Ca-ox anhydrous (88/40=2.2). For example: 2.2 * oxalate (μg oxalate g^{-1}) = Ca_{ox} (μg Ca g^{-1}). Soil oxalate ion concentrations were

not measured for 30 to 50 and 50 to 70 cm depth, but were estimated by interpolations using power model relationships (all sites $P<0.05$) between median depth and Ca_{ox} measurements for each of the nine sites (for example: Site 5, high-Ca site, $y=556.8x^{-1.05}$, $R^2=0.86$, $P<0.001$; Site 16, low-Ca site, $y=52.2x^{-0.54}$, $R^2=0.38$, $P=0.03$). The power model was a better fit than other tested models at all sites.

Ca-ox in decomposing leaf litter

To examine whether Ca-ox in the forest floor is selectively concentrated or lost from bulk Ca during litter decomposition, we used a two-year leaf litter decomposition study with Douglas-fir needles with different Ca status, one high-Ca (14.2 mg Ca g^{-1}) and one low-Ca (7.2 mg Ca g^{-1}) (Matkins, 2009). The two source litters were decomposed at four Douglas-fir dominated study sites in the Oregon Coast Range for two years (as described by Matkins 2009); the four sites were used as experimental replication. Litterbags (20 x 20 cm) were constructed using a 1 mm nylon mesh upper layer and a water permeable, non-mesh nylon bottom layer to contain Douglas fir needles. Approximately 5 grams of air-dried litter were weighed then placed in a bag. Litterbags were deployed in November 2003. Source needles and the two-year collection in November 2005 were used for this study. During collection, freshly fallen materials and moss growth were removed from individual litterbags, oven dried at 65° C then reweighed to determined mass loss. Subsamples of dried whole needles from initial and 2 years decomposed samples were placed in a beaker with nanopure water in a ratio of 1:10, left for 10 minutes, then measured with a pH probe (Fisher Scientific Accumet AR20 meter, with an AccuFast pH combination electrode). Additional subsamples of initial and decomposed litters were ground to a fine powder using a roller grinder. Ca_{ox} concentrations were determined using the sequential extraction technique described above. Images of unground and finely sliced open initial and decomposed needles were imaged on a Philips CM-12 scanning transmission electron microscope (STEM). To confirm the presence of Ca in crystals, chemical composition analysis was performed using X-Ray Energy Dispersive Spectrophotometry.

Data analysis

To quantify pools of Ca-ox at high and low status Ca sites, average plant and detrital Ca_{ox} concentrations (mg Ca g^{-1} dry tissue) were multiplied by biomass estimates (Mg ha^{-1}) to determine Ca_{ox} pools (kg Ca ha^{-1}). Similar calculations were used to quantify Ca_t (total Ca, kg Ca ha^{-1}) in plant and detrital pools at both sites, except average Ca_t concentration (mg Ca g^{-1} dry tissue) were multiplied by biomass estimates (Mg ha^{-1}). The Ca_{ox}-fraction (percent of Ca_t existing as Ca_{ox}) for each plant part and detritus type was calculated as 100 * Ca_{ox} (kg Ca ha^{-1})/Ca_t (kg Ca ha^{-1}). Soil pools of Ca_{exch} (Perakis and Sinkhorn, in progress) and Ca_{ox} were estimated by multiplying concentrations with soil bulk density for each sampling depth for the two sites. We also report Ca_s as the sum of mineral soil Ca_{ox} and Ca_{exch} (Cromack et al., 1979). The Ca_{ox}-fraction in soil was calculated as 100 * Ca_{ox} (kg Ca ha^{-1})/Ca_s (kg Ca ha^{-1}). The Ca_{ox}-fraction contained in total biomass, detritus, and mineral soil compartments of the high-Ca and low-Ca sites was calculated as the summed weighted average of Ca_{ox}-fractions contained in each individual component part (for example for live biomass; 100* $\Sigma_{\text{foliage, branches, bole-wood, bark, <2 mm roots, 2-10 mm roots}}$ Ca_{ox} (kg Ca ha^{-1})/ $\Sigma_{\text{foliage, branches, bole-wood, bark, < 2mm roots, 2-10 mm roots}}$ Ca_t (kg Ca ha^{-1})). Due to the time-intensive nature of sequential extractions for Ca, replicate samples from each site were not measured, so no statistical analysis was performed.

Leaf litter (1) mass loss percentage and (2) Ca loss percentage were estimated with the following equations:

(1) Mass loss (%) = 100 * $(M_0-M_2) / M_0$

(2) Ca loss (%) = 100 * $(Ca_0 - (Ca_2 * M_2/M_0)) / Ca_0$

where M=litterbag dry weight mass, Ca= Ca concentration, subscripts denote time of sampling in years. A nutrient release rate was determined for Caox using a single exponential model: ln(Ca2/Ca0) = k * t where Ca = Ca concentration, subscripts denote time of sampling in years, k = release rate per year, t = time in years (Olson, 1963; Cuevas and Medina, 1988) and averaged within litter source types. Although we lacked detailed time-resolved data during decomposition, we choose a single-exponential model as it corresponds well with general understanding of the biology of litter decomposition (Wieder and Lang, 1982) and in particular provides the best fit for decomposition of the Douglas-fir needles used in this study (Matkins, 2009). This approach assumes consistent exponential decomposition behavior over time, and that decomposition rates approach zero as time progresses, as has been observed for

Ca (Rustad, 1994). The exponential model allowed us to calculate the time required to lose 95% of the Ca_{ox} (3/*k*) (Gosz et al., 1973). Additionally, the mean residence time (MRT, in years) of Ca_t and Ca_{ox} in leaf litter calculated as initial leaf litter pool mg Ca g^{-1} / leaf litter loss mg Ca g^{-1} yr^{-1}.

For forest floor, mean residence time, in years, of Ca_t and Ca_{ox} was also estimated by calculating forest floor pool kg Ca ha^{-1} / leaf litterfall kg Ca ha^{-1} yr^{-1} assuming steady state of forest floor Ca_{ox}. We did not measure fungal production of Ca_{ox}, so the calculation of forest floor Ca_{ox} MRT does not include all possible inputs of Ca_{ox}, which results in an over-estimate of forest floor MRT. The amount of Ca_{ox} in the forest floor originating in leaf litterfall as compared to root or fungal sources was calculated as leaf litterfall kg Ca_{ox} ha^{-1} yr^{-1} / decomposition rate Ca_{ox} yr^{-1} under the assumption that the forest floor is in steady state (Olson, 1963). We used leaf litter decomposition rates for high- and low-Ca source needles determined on four different sites with the assumption that they apply to the high- and low-Ca sites (respectively).

We estimated soil pools of Ca_{exch} and Ca_{ox} across the Ca gradient of seven additional sites using the same methods described for the two intensively studied sites described above. Collectively, across all nine sites, we used Bayesian information criterion (BIC) to evaluate site, depth, soil pH, and soil exchangeable Ca as potential factors explaining variation in soil Ca-oxalate concentrations from 0-100 cm deep (excluding interpolated values in 30-50 and 50-70 cm deep samples). All data were natural log transformed, except for site and depth, which were included in the model as categorical variables. Models of soil Ca_{ox} pools were fit to the data using PROC MIXED in SAS v9.2 (SAS Institute Inc., Cary, NC).

In the leaf litter decomposition experiment, effects of source litter, decomposition stage, and sequentially extracted Ca pool on Ca concentrations were analyzed using a three-way analysis of variance (ANOVA). Differences in percent loss of either Ca or mass by source litter or sequential extraction were determined with a two-way ANOVA. High versus low-Ca source litter and initial versus decomposed leaves were treated as categorical variables. The four sites were treated as replicates, so site comparisons were not analyzed.

Models were fit to the data using PROC GLM in SAS v9.2 (SAS Institute Inc., Cary, NC), and data were unbalanced due to a missing data point for Ca_t, so LSMEANS was used to estimate means and the differences between means. Statistical significance for all models was at the level of $\alpha = 0.05$.

Results

Total ecosystem Ca was 12-times larger at the high-Ca site (6314 kg Ca ha^{-1}) than in the low-Ca site (534 kg Ca ha^{-1}, Figure 2.1). Mineral soil was the largest Ca pool in the high-Ca site (91% of total ecosystem Ca) and approximately half of ecosystem Ca at the low-Ca site (58%). The mineral soil pool of Ca_s (i.e., sum of Ca_{exch} and Ca_{ox}) was 19-times larger in the high- than low-Ca site. Summed woody debris Ca_t pools and summed living biomass Ca_t pools were 3-times larger at the high-Ca site than at the low-Ca site. The high-Ca site contained more Ca than the low-Ca site in individual component pools, including forest floor and foliage across all age classes and also in leaf litterfall flux (high-Ca site: 7 kg Ca ha^{-1} yr^{-1}, low-Ca site: 2 kg Ca ha^{-1} yr^{-1}, Figure 2.1). The MRT of Ca_t in forest floor was 15 yrs and 25 yrs at the high- and low-Ca sites, respectively. The high-Ca site appeared to have higher concentrations of Ca than the low-Ca site in all compartment pools except bark and coarse woody debris (Table 2.2). At both sites the live tissues most concentrated in Ca were older foliage and branches, and the dead tissues most concentrated in Ca were leaf litter, forest floor and less-decayed fine woody debris (Table 2.2).

Total ecosystem Ca_{ox} was 3-times larger at the high-Ca site (268 kg Ca ha^{-1}) than the low-Ca site (95 kg Ca ha^{-1}, Figure 2.2). The high-Ca site consistently had larger pools of Ca_{ox} across all compartments, which varied from approximately 2-times larger (branches, fine woody debris, mineral soil), 4-times larger (roots, coarse woody debris, forest floor), 5-times larger (bole-wood, bark) to 9-times larger (foliage). At each site about a third of the ecosystem Ca_{ox} was found each in live biomass, detritus and mineral soil. Live biomass was the largest pool of Ca_{ox} comprising 42% and 40% of total ecosystem Ca_{ox} of the high- and low-Ca sites, respectively. Of live biomass component pools, branches comprised 20% and 29% (Figure 2.2) of the total ecosystem Ca_{ox}. The remaining live biomass pools other than branches (foliage, bole-wood, bark and roots) together comprised 22% and 12% of the total Ca_{ox} at each

site (Figure 2.2) but each alone made up less than 10% of the total Ca_{ox}. Combined detrital pools were the second largest pool of Ca_{ox} comprising 37% and 31% of the total ecosystem Ca_{ox} of the high-Ca and low-Ca site, respectively. Of detrital component pools, woody detritus contained 20 and 18% of total ecosystem Ca_{ox}, and forest floor contained 17 and 13% of total ecosystem Ca_{ox} (Figure 2.2). Within woody debris component pools, coarse (>10 cm) downed-wood contained more Ca_{ox} than all combined decay classes of fine downed-wood (Figure 2.2). The third largest pool of Ca_{ox} was the mineral soil, comprising 21% and 29% of the high-Ca and low-Ca site respectively. Leaf litterfall rates of Ca_{ox} were 5.0 kg Ca ha^{-1} yr^{-1} at the high Ca site, and 0.7 kg Ca ha^{-1} yr^{-1} at the low-Ca site (data not displayed). The MRT of Ca-ox in forest floor was 9 yrs and 18 yrs at the high- and low-Ca sites (respectively). The amount of Ca_{ox} in forest floor originating from leaf litterfall versus root and fungal sources was 25 kg Ca ha^{-1} (55% of forest floor Ca_{ox}) at the high-Ca site and 17 kg Ca ha^{-1} (135% of forest floor Ca_{ox}) at the low-Ca site, which was estimated using leaf litterfall rates of Ca_{ox} (kg Ca ha^{-1} yr^{-1}) and leaf litter Ca_{ox} decomposition rates (yr, reported below) in a high and low-Ca source litter in four sites (for example: leaf litter addition rate 5 kg Ca_{ox} ha^{-1} yr^{-1} * MRT of Ca_{ox} in leaf litter 5 yrs = 25 kg Ca_{ox} ha^{-1}).

Calcium concentrations in tissues are shown in Table 2.2. The highest concentration of Ca_{ox} in living biomass at the high-Ca site occurred in 4 to 5 year-old leaves, whereas branches contained the highest Ca_{ox} concentrations in living biomass at the low-Ca site. All high-Ca site pools were more concentrated in Ca_{ox} than low-Ca site pools with the exception of coarse woody debris and 70-100 cm depth mineral soil. Leaf litter Ca_{ox} concentrations exceeded the oldest live leaf cohort at both sites. Fine wood Ca_{ox} generally decreased in concentration with increasing decay class within each site.

We estimated the potential importance of Ca_{ox} to ecosystem Ca pools as the proportion of Ca_{ox} in Ca_t in biomass and detritus, as the proportion of Ca_{ox} in Ca_s in mineral soil, and as the proportion of Ca_{ox} in the ecosystem overall (Figure 2.3, Table 2.2). In total ecosystem pools, the Ca_{ox} fraction was higher at the low-Ca site, than the high-Ca site, primarily because mineral soil was the dominant ecosystem pool of Ca, and contained a greater fraction of Ca_{ox} at the low-Ca site than the high-Ca site. Nevertheless, at both sites the

living biomass and detrital pools had a higher Ca_{ox} fraction than the mineral soil (Figure 2.3). Ca_{ox} accounted for approximately 1/3 of live biomass Ca at both sites (Table 2.2). Within live biomass pools at the high-Ca site, Ca_{ox} comprised a similar proportion of branch and foliar Ca pools whereas at the low-Ca site, branches had a higher Ca_{ox} fraction than foliage (Figure 2.3). The proportion of leaf litter Ca flux as Ca_{ox} was twice as large at the high-Ca site compared to the low-Ca site (Figure 2.3). Ca-ox accounted for 41% and 25% of Ca_t in combined detritus and forest floor pools at the high-Ca and low-Ca sites, respectively. Within detritus pools the least decayed fine wood (<2mm and 2-10 mm, decay class 2) contained the highest Ca_{ox} fraction at both the high-Ca site and at the low-Ca site.

To compare how Ca_{ox} varies with Ca_t or Ca_s across sites, we calculated the multiplicative factors for which Ca_t (biomass kg Ca ha^{-1}), Ca_s (mineral soil kg Ca ha^{-1}), and Ca_{ox} (kg Ca ha^{-1}) were greater at the high-Ca than low-Ca sites. We found only 3-times more total ecosystem Ca_{ox} in the high- than low-Ca site despite a 12-fold difference in total ecosystem $Ca_t + Ca_s$. These site differences are attributable primarily to mineral soil, as Ca_{ox} was only 2-times greater in the high-Ca site than low-Ca site despite a 19-fold differences in total mineral soil Ca_s (Ca_{exch} and Ca_{ox} combined) between sites. In contrast, foliar differences were amplified between sites, with 9-times greater foliar Ca_{ox} in the high-Ca than low-Ca site despite only a 3-fold difference in foliar Ca_t.

At the nine sites where mineral soil Ca_{ox} concentrations were measured, Ca_{ox} concentrations were highest in the 0-10 cm depth, mean = 44 (S.E. = 6) μg Ca g^{-1} and declined rapidly while converging with depth (Figure 2.4). The difference in mineral soil Ca_{ox} among sites was smaller than differences in Ca_{exch}. The upper 10 cm of mineral soil varied 7-fold in Ca_{ox} concentrations across all nine sites (16 to 114 μg Ca g^{-1}) but Ca_{exch} concentrations varied 14-fold (153 to 2194 μg Ca g^{-1}). The total mineral soil Ca_{ox} pool to 100 cm depth ranged only 4-fold (27 to 102 kg Ca ha^{-1}) among sites, compared to Ca_{exch} which ranged 35-fold (171 to 6022 kg Ca ha^{-1}). The low-Ca site (Site 16) that was examined intensively for other ecosystem Ca pools contained among the smallest mineral soil pools of both Ca_s (308 kg Ca ha^{-1}) and Ca_{ox} (27 kg Ca ha^{-1}) of the nine sites examined. The intensively studied high-Ca site (Site 5) contained among the largest mineral soil pools of Ca_s (5745 kg Ca ha^{-1}), but was similar to the

mean Ca_{ox} across all nine sites (55 at the high-Ca site versus a mean 68 kg Ca ha^{-1}). The fraction of Ca_{ox} in total available mineral soil Ca (Ca_s) ranged from 0.2 to 23.9% depending on site and depth, with a mean of 5.8% (Figure 2.5). Higher Ca sites generally had a lower fraction of Ca_s in Ca_{ox}. At most sites, the fraction of Ca_s as Ca_{ox} remained relatively consistent with soil depth, and averaged 1.0% at the high-Ca site (Site 5) and 8.8% at the low-Ca site (Site 16, Figure 2.5).

Variation in mineral soil Ca_{ox} concentrations across all nine sites was best explained ($R^2 = 0.75$) by a combination of soil depth ($P<0.001$) and pH ($P<0.001$), as determined by the lowest model selection criteria (BIC). Soil exchangeable Ca correlated positively with soil pH ($R^2=0.70$), leaving only soil pH and depth included in the final model:

$$\log_e(Ca_{ox}) = \beta_0 + \beta_1 \log_e(\text{soil pH}) + \beta_2 \text{soil depth} + \varepsilon$$

where Ca_{ox}= Ca_{ox} (μg Ca g^{-1}); soil pH= soil pH; soil depth = class variable for each median depth (5, 15, 25, 85 cm), β is the variation associated with each variable, and ε= random variation with the final model. Including site as an explanatory variable was less effective than soil depth in explaining soil Ca_{ox} concentrations. The relationship of Ca_{ox} with soil pH was stronger in 0-10 cm (R^2= 0.43, P<0.001) and 10-20 cm depths ($R^2 = 0.45$, P<0.01) than in the two lower soil depths ($R^2 \leq 0.13$). Ca_{exch} was not included in the larger model due to correlation with soil pH, although a model using Ca_{exch} instead of soil pH explained a similar amount of variation in mineral soil Ca_{ox} concentrations after accounting for soil depth across all sites ($R^2 = 0.72$).

STEM image analysis revealed the presence of Ca-ox crystals in newly shed leaf litter of high-Ca concentration (Figure 2.6A) but not in decomposed litter (Figure 2.6B). Ca-ox crystals were not found in the low-Ca source undecomposed litter, and no Ca-ox crystal images were found in decomposed litter from any of the sites. X-Ray Energy Dispersive Spectrophotometry was used to confirm the presence of Ca in the crystals in the image (Appendix).

Comparisons of Ca concentrations in sequential fractions between high and low-Ca source litters before and after decomposition revealed significant differences in Ca_{aq}, Ca_{st}, Ca_{ox}, Ca_{sum} and Ca_t concentrations after two years decomposition that varied between high and low-Ca source litter (source*time*pool interactive effects, F-value=158.08, DF=78, $P<0.001$, three-way ANOVA, Table 2.3). In the high-Ca source litter, Ca_t, Ca_{sum} and Ca_{aq} concentrations remained constant during decomposition while Ca_{st} increased ($P<0.001$) and Ca_{ox} decreased ($P<0.001$, Table 2.3). In low-Ca source litter, Ca_t, Ca_{aq} and Ca_{ox} concentrations remained constant during decomposition, but Ca_{sum} ($P=0.011$) and Ca_{st} ($P=0.026$) significantly increased (Table 2.3). The high-Ca litter had a mean initial pH of 5.32 (0.03 S.E.), which was not significantly different from decomposed litter pH of 5.24 (0.08 S.E.). The low-Ca litter had a mean initial pH of 5.48 (0.09 S.E.), which decreased in decomposed litter to a pH of 4.77 (0.10 S.E.; $P= 0.002$).

After two years of leaf litter decomposition, percent Ca loss differed significantly among Ca pools in a way that varied by Ca source litter (F=7.46, DF=5, pool*source interaction, two-way ANOVA, P<0.001, Table 2.3). Ca_{ox} had a higher percent loss than Ca_{st} in the low-Ca source litter and a higher percent loss in both Ca_{st} and Ca_{aq} in the high-Ca source litter (Table 2.3). Ca_{st} increased significantly (negative% loss) over two years in both source litters, but Ca_{st} percent loss did not differ from Ca_t or mass loss in low-Ca source litter only. The percent of Ca_t loss (43% in high-Ca source litter, 26% in low-Ca source litter) did not differ from mass loss (42% in high-Ca source litter, 38% in low-Ca source litter, Table 2.3). The rate of Ca_{ox} disappearance was 0.28 yr^{-1} and 0.04 yr^{-1} in high- and low-Ca source litter respectively; assuming the difference between initial and final source litters is representative of long-term behavior. From this Ca removal rate, we estimate a 95% disappearance of Ca_{ox} ($3/k$) after 15 years and 72 years for the high- and low-Ca source litters, respectively. The loss of Ca_{ox} in 2 years was equivalent to 2 mg Ca_{ox} g^{-1} yr^{-1} at the high-Ca site, and 0.15 mg Ca_{ox} g^{-1} yr^{-1} at the low Ca site (for example: [10 mg Ca_{ox} g^{-1} initial minus 6 mg Ca_{ox} g-1 final] / 2 years = 2 mg Ca_{ox} g^{-1} yr^{-1}). Using this loss rate, and the amount of Ca_{ox} in initial litter (Table 2.3), the MRT of Ca_{ox} was estimated as 5 and 24 years at the high- and low-Ca source litters, respectively (for example: 10 mg Ca_{ox} g^{-1} / 2 mg Ca_{ox} g^{-1} yr^{-1} = 5 yrs).

Discussion

Ca-ox quantities in two ecosystems

To our knowledge, this is the first study to estimate the quantity of Ca-ox on an ecosystem scale, providing novel information about Ca-oxalate concentration and accumulation, and the potential influence of Ca-ox on ecosystem processes. We found that Ca-ox was present in all biomass, detrital, and mineral soil pools measured at both sites, and that the contribution of Ca-ox to total Ca varied with underlying site Ca supply. However, the difference in Ca-ox pools between sites was lower than expected given the large difference in total Ca; the high-Ca site had 12-times more Ca, but only 3-times more Ca-ox than the low-Ca site, suggesting that Ca availability is not the only factor that influences Ca-ox accumulation.

We found that Ca_{ox} had a roughly equitable distribution among vegetation, detrital and mineral soil pools, although living biomass accounted for the largest fraction of ecosystem Ca_{ox} at both the high-Ca (42% Ca_{ox} fraction) and low-Ca site (40% Ca_{ox} fraction, Figure 2.2). Within living biomass compartments, branches were the largest pool of Ca-ox, and comprised 35 to 48% of living biomass Ca-ox and 20 to 29% of total ecosystem Ca-ox (Figure 2.2). This was surprising considering high concentrations of Ca-ox in foliage (Table 2.2), though foliage comprised only 3 to 8% of the total site Ca-ox at both sites (Figure 2.2). Other living biomass pools of bole-wood, bark and roots were small proportions of the total Ca-ox at both sites (< 6% Ca_{ox} fraction each, Figure 2.2). The high amount of Ca-ox in non-bole compartments like branches has interesting implications, as removal of Ca in biomass during harvest is a concern in Ca-depleted areas (Federer et al., 1989). In the Pacific Northwest, branches, foliage and roots are typically left on-site after harvest (Cramer, 1974), making them an important source of Ca return to harvested sites, although much of this return is likely to be localized near burn piles (Little and Klock, 1985; Feller, 1988). Crystalline Ca-ox has different decomposition dynamics than soluble or structural Ca, as discussed below, so the Ca return rate from these sources may be influenced by the Ca-ox concentration, though this effect is currently unknown because existing measurements of Ca in harvest residue only report total Ca (Fahey et al., 1991; Olsson et al., 1996).

We expected woody debris to be a fairly large pool of Ca-ox, because Ca-ox is created by wood decomposers (Dutton and Evans, 1996; Jellison et al., 1997; Schilling and Jellison, 2007) and there is potential for high woody debris accumulation in Pacific Northwest forests (Smithwick et al., 2002). At both study sites, detritus including both woody debris and forest floor accounted for roughly one-third of ecosystem Ca-ox. Indeed, we observed a large pool of Ca-ox despite relatively low quantities of coarse woody debris, (high-Ca site: 61 Mg ha^{-1}, low-Ca site: 17 Mg ha^{-1}) compared to nearby managed sites of Douglas-fir of variable ages (dead wood biomass ~73 Mg ha^{-1}, Kennedy and Spies, 2007), which indicates that this pool of Ca-ox may be even more significant in sites with larger stocks of coarse woody debris. Ca-ox concentrations in coarse woody debris that were approximately equal to fine woody debris are interesting considering that Ca-ox concentrations in live bole-wood were lower than any other live biomass compartment at both sites (Table 2.2). Low Ca in bole-wood tissues indicates that bole-wood does not accumulate Ca-ox as a repository for excess Ca, in contrast to foliage and branches which likely use Ca-ox to sequester Ca away from the cytoplasm (Franceschi and Nakata, 2005). Interestingly, decomposing coarse woody tissue had higher concentrations of Ca-ox than bole-wood tissue, even after accounting for 45% mass loss of wood (decay class 4 reduction factor, Waddell, 2002), confirming the idea that decomposers contribute to Ca-ox accumulation in coarse woody debris. In contrast to bole-wood and coarse woody debris, leaf litter Ca-ox concentration decreased during decomposition (discussed below), suggesting different microbial communities may be associated with these two pools; microbes that use Ca-ox in leaf litter versus wood-rot decay fungi that accumulate Ca-ox in woody debris. Fine woody debris (1 to 10 cm diameter) had lower Ca-ox concentrations than live branches, suggesting that decomposition processes in these pools may be more similar to litter than bole-wood, with no accumulation of additional Ca-ox during decomposition.

Our finding of large quantities of Ca-ox in the forest floor builds on prior reports of visible Ca-ox crystal accumulation in forest floor (Cromack et al., 1979; Horner et al., 1995). Ca-ox in the forest floor can originate from plant litter and from root or fungal oxalate production and concomitant Ca-ox precipitation, that together shape Ca-ox accumulation. Leaf litterfall accounts for a sizeable input of Ca-ox; the majority of the Ca in leaf litter flux is in the form of Ca-ox at the high-Ca site (70% of Ca_t), compared to about a third of the total Ca in

leaf litter flux at the low-Ca site (34% of Ca_t). Yet, using leaf litter decomposition rates (discussed below) and Ca-ox of the overall forest floor pool we estimated that Ca-ox originating from leaf litterfall is 55% and 135% of the forest floor Ca-ox pool at the high- and low-Ca sites, respectively. This estimation assumes that the forest floor is in steady state, and that leaf litter decomposition rates for high- and low-Ca source needles apply to the high- and low-Ca sites. This suggests that the majority of Ca-ox in the forest floor at the low-Ca site originated as leaf litter. Although we have no way to resolve the discrepancy of an estimate that exceeds 100% of the forest floor, the calculation suggests that either our forest floor pool calculations are underestimates, or the MRT of leaf Ca_{ox} in the low-Ca needles or leaf litter fall addition rate are over-estimates. At the high-Ca site, half of the Ca-ox in the forest floor is either from non-leaf litter (which we did not measure for Ca_t or Ca_{ox}) or from root and fungal sources. It is known that ectomycorrhizal fungal mats can concentrate Ca-ox up to 200-times greater than non-mat soils in coastal Oregon forests (Cromack et al., 1979), suggesting that Ca-ox accumulation in the forest floor can be strongly influenced by belowground biotic processes. Additionally, we found that the 4-fold greater forest floor Ca-ox pool at the high-Ca site compared to the low-Ca site (Figure 2.2) was entirely due to the 4-fold difference in Ca-ox concentration between sites (Table 2.2) rather than differences in forest floor mass, in contrast to woody debris, which had similar concentrations of Ca-ox at both sites. Although our data suggest that Ca-ox is the main contributor to forest floor Ca-ox accumulation, some high-Ca sites accumulate more Ca-ox than can be accounted for by leaf litter additions. These accumulations are likely due to fungal or root oxalate production and may be due to other site properties, such as rates of soil Ca and/or oxalate supply, soil pH or microbial communities.

Decomposer communities that reside in different detritus types may control differences in Ca-ox accumulation in detritus compartments. Species of fungi studied in culture mediums have different rates of oxalic acid production that can vary with available Ca and pH (Arvieu et al., 2003; Schilling and Jellison, 2007; Guggiari et al., 2011) as well as carbon and nitrogen sources (Dutton and Evans, 1996). This suggests that substrates with different properties (wood versus litter) may have different potential for oxalate production and concomitant Ca-ox precipitation depending on the decomposer community, which makes prediction of Ca-ox concentrations in detritus more complex than in living tree tissues.

Analysis of decomposer communities was not performed by this study, but may be useful, as it could help explain differences in Ca-ox concentrations in detrital ecosystem compartments.

Mineral soil (0-100 cm depth) comprised 21% of total ecosystem Ca-ox at the high-Ca site, and 29% of total ecosystem Ca-ox at the low-Ca site (Figure 2.2). While these pools are sizeable, they were not much greater than the forest floor, suggesting that Ca-ox accumulates to a greater extent in association with organic matter than in mineral soil alone. The relatively small mineral soil Ca-ox pool was surprising given that mineral exchangeable-Ca comprises a large percent of total ecosystem Ca, especially at the high site (91%), though less so at the low Ca site (58%, data not displayed). In the upper mineral soil (0-10 cm depth), the concentration of Ca_{exch} was 54-times (high-Ca site) and 12-times (low-Ca site) higher than Ca_{ox} (Table 2.2). Ca-ox has been implied to be a "storage" pool of Ca that resists degradation over long-time periods due to its insolubility (Bailey et al., 1996; Smith et al., 2009), yet our data suggest that Ca-ox is unlikely a significant repository for extra Ca in the mineral soil of acid forest soils, especially on Ca-rich sites. However, Ca-ox may still exert leverage on Ca dynamics if it makes up a sizeable proportion of total available Ca, or if it cycles at a different rate than other ecosystem Ca pools (discussed below).

Proportional contribution of Ca-ox to total Ca pools

While total Ca-ox pools are informative in understanding the location of Ca-ox and patterns of Ca-ox accumulation in ecosystems, the proportion of the total Ca that resides as Ca-ox (Table 2.2) provides information on potential leverage that Ca-ox exerts on ecosystem (or compartment) Ca dynamics. At the ecosystem scale, Ca-ox contributed 4 to 5 times as much (18% versus 4% Ca_{ox} fraction of Ca_t) to total Ca in the low- than high-Ca site (Table 2.2). Therefore, assuming similar Ca-ox dynamics among ecosystems pools between high versus low-Ca sites, a greater proportion of total ecosystem Ca may be cycled through Ca-ox pools in low-Ca sites, which raises the possibility that Ca-ox is more important in sustaining Ca supplies where Ca is relatively scarce. In particular, Ca-ox contributed more to mineral soil Ca at the low-Ca (9% Ca_{ox} fraction of Ca_s) than high-Ca (1% Ca_{ox} fraction of Ca_s) site (Table 2.2). The greater proportion of Ca_{ox} relative to Ca_s in soils at lower Ca sites was also observed more broadly across the nine-site Ca gradient, including the contrasting high-Ca (site 5) and

low-Ca (site 16) sites (Figure 2.5). Previous work has shown that a larger proportion of total available ecosystem Ca is sequestered in living biomass in low- than high-Ca Douglas-fir forests, which may reduce the susceptibility of Ca to leaching loss in low-Ca sites (Perakis et al., 2006; Perakis and Sinkhorn, in progress). Our results suggest that this idea may be extended to soil biological regulation of ecosystem Ca, where a greater proportion of Ca occurs as Ca-ox in soils of low-Ca than high-Ca sites. In mineral soils where exchangeable-Ca is relatively scarce, Ca-ox decomposition dynamics are important for either restricting or amplifying leaching loss and providing a pool of Ca for tree growth. These results suggest that sites with low-Ca likely have a greater proportion of their mineral soil Ca in Ca-ox, making them more reliant on this biomineral as a potential to buffer Ca depletion below the forest floor. However, a low absolute quantity of Ca-ox in low-Ca sites limits the ability of this pool to buffer ecosystem Ca depletion overall.

The forest floor was not only a sizeable pool of Ca-ox at both sites, but Ca-ox was also a substantial portion of total Ca (Ca_t) in the forest floor, comprising 41% at the high-Ca site and 23% at the low-Ca site. This suggests that Ca-ox is an important source for biocycling of Ca. The primary source of Ca uptake for trees is recycled Ca from organic matter as indicated by cation tracer studies (Blum et al., 2008; Fahey and Blum, 2011), rather than exchangeable-Ca in mineral soil, and our data support the idea of biotic controls on Ca return to soil and mineralization. This also highlights the importance of understanding the rate of Ca-ox decomposition in decomposing materials. Both the current study and previous work (O'Connell et al., 1983) highlight that Ca-ox is not permanently insoluble in decaying organic matter (discussed below), although the dynamics of Ca-ox turnover in mineral soils are not well known.

In living biomass the proportional contribution of Ca-ox to total Ca was somewhat equitable between sites, with slightly more Ca-ox contribution to total Ca in high-Ca sites. Ca_{ox} accounted for 34% fraction of Ca_t (up to 56% in individual pools) in the high-Ca sites, compared to 29% Ca_{ox} fraction of Ca_t in the low-Ca site (up to 42% in individual pools, Table 2.2). These patterns suggest that Ca-ox may be equally or slightly more important in regulating plant Ca dynamics at Ca-rich sites. However the influence of Ca-ox concentration

in plant tissues on aboveground productivity or resiliency is unknown. It is hypothesized that Ca-ox crystal formation in plants regulates bulk Ca levels (Franceschi and Nakata, 2005), as a defense to herbivory (Hudgins et al., 2003; Franceschi et al., 2005; Korth et al., 2006) and detoxifies aluminum or other heavy metals (Ma, 2000; Ma et al., 2001; Mazen, 2004). Because the high-Ca site tree tissues accumulated a slightly higher proportion of Ca-ox than the low-Ca site, with much higher absolute concentrations of Ca-ox as well, the high-Ca site may have increased mechanisms for herbivory defense or detoxification of heavy metals, especially in foliage where there are in Ca-ox proportions between sites (Table 2.2).

Ancillary data from our field sites raise the possibility that site differences in Ca-ox accumulation in plant tissues are most likely due to differences in Ca supply, rather than oxalic acid sequestration in tissues. The specific metabolic pathway for oxalic acid production in plants is subject to debate (Franceschi and Nakata, 2005; Yu et al., 2010) but evidence supports the idea that oxalate and Ca accumulation are independent processes, and that Ca-ox accumulation is independent of photorespiration (Zindler-Frank, 1974; Zindler-Frank and Horner, 1985; Yu et al., 2010). Oxalate production has been suggested to be related to nitrogen source type as a way to maintain cellular pH and ionic balance during nitrate metabolism, as observed in both plants (Franceschi and Horner, 1980; Ahmed and Johnson, 2000) and fungi (Lapeyrie et al., 1987; Gharieb and Gadd, 1999) exposed to different nitrate: ammonium ratios. However, at our sites net nitrification rates are nearly 3-times higher at the low- than high- Ca site (Perakis and Sinkhorn, 2011), which is the opposite of what would be expected if oxalate production due to this mechanism were driving Ca-ox accumulation. Generally, increasing Ca supply level increases the amount of Ca-ox crystals formed in plants (Zindler-Frank et al., 2001; Volk et al., 2002; Mazen, 2004), and it is assumed to be unlikely that crystal formation is the end product of an attempt to sequester excess oxalic acid (Franceschi and Nakata, 2005). Additionally, despite the relationship between Ca-ox creation and carbon metabolism, the impact of Ca-ox concentrations on whole-plant carbon dynamics may be minimal at our sites, as Ca-ox was estimated at both sites to be only 0.4% of C in foliage and branches, and less for bole-wood and roots. Overall, these findings support the idea that Ca supply is an important driver for differential aboveground Ca-ox production at these sites.

Overall, the proportional contribution of Ca-ox to total Ca differed by major ecosystem pools in similar patterns at both sites regardless of Ca status, with roughly comparable proportions of Ca-ox in living biomass, detritus and mineral soil at both low- and high-Ca sites (Table 2.2). These patterns by pool-type suggest that total ecosystem Ca-ox may be predictable based on quantification of traditional ecosystem Ca pools sizes alone. However, analyses of additional sites are needed to determine whether the trends in Ca-ox contribution to individual component pools varies systematically across multiple ecosystem types and sites with multiple levels of Ca supply.

Ca-ox turnover in decomposing leaf litter

Studies of Ca in decomposing materials have primarily investigated bulk Ca content, and often indicate that Ca_t is lost at the same rate as mass in decomposing leaf tissue (Attiwill, 1968; Gosz et al., 1973; Staaf and Berg, 1982). However, the rate of Ca release or immobilization in leaf litter can also vary by species (Stohlgren, 1988; Bockheim et al., 1991; van Wesemael, 1993; Rustad, 1994; Dijkstra, 2003), which may be related to the variation in species Ca concentration in leaf and root litter (Hobbie et al., 2006; Dauer et al., 2007). In this study, Ca_t loss from litterbags was similar to mass loss after 2 years, with no significant differences between mass loss and Ca_t loss within either high or low-Ca source litters (Table 2.3). Therefore, the large difference between the two litter sources in Ca_t loss (43% in high-Ca source litter versus 26% in low-Ca source litter) was due entirely to differences in initial chemical Ca pools and variations in the overall rate of litter degradation, and not to differences in bulk Ca loss relative to mass loss.

We found that the dynamics of different chemical forms of Ca within leaf litter differed greatly during decomposition. In particular, Ca_{ox} decomposed rapidly losing 66% in the high-Ca and 42% in the low-Ca source litters in two years, indicating the potential for quick Ca-ox degradation (Table 2.3). In comparison, for both litter sources, Ca_t and Ca_{aq} concentrations remained constant between initial and decomposed litter. However, the Ca_{st} pool increased nearly 4-fold in the high-Ca and nearly 2-fold in the low-Ca litter over two years (Table 2.3). The Ca_{st} pool, extracted with 2 *N* acetic acid, targets structurally bound Ca

in pectin and lignin of fresh tissue but also likely includes ionic Ca that is electrostatically bound to cation exchange sites, the latter increasing with time in decomposing conifer litter (Staaf and Berg, 1982). Such exchange sites may be the proximal fate for Ca that is released from Ca-pectin, Ca-lignin or Ca-ox in decomposing litter. The quick turnover of Ca-ox in decomposing tissues is additionally supported by the rate of Ca_{ox} release, which we estimated to be 0.28 yr^{-1} for high-Ca litter, and 0.04 yr^{-1} for low-Ca litter. We also estimated that 66% (high-Ca source litter) and 42% (low-Ca source litter) of the Ca_{ox} was lost in the first two years of decomposition (Table 2.3). Our data are comparable to Eucalyptus litter decomposition that lost 70% of Ca-ox in the first wet season during which time NH_4Cl-extractable exchangeable Ca increased (O'Connell et al., 1983). Decaying Ca-ox may not be released immediately from decomposing litter systems, but instead may bind to organic matter exchange sites, and be available for either plant uptake or leaching loss. This indicates one way that Ca-ox can influence dynamics of Ca soil availability.

The rapid release of Ca_{ox} from decomposing litter was not expected, considering that Ca-ox is a highly insoluble crystal (Ringbom, 1963). It has been speculated that Ca-ox pools remain intact until soil pH decreases (Bailey et al., 2003), however we found that litter pH did not decrease significantly during decomposition of the high-Ca source litter (initial pH = 5.32 versus final pH = 5.24) coincident with both overall Ca_{ox} loss and decreases in Ca_{ox} concentrations in remaining litter. A significant decrease in pH did occur in the low-Ca source litter (initial pH = 5.48 versus final pH = 4.77) coincident with overall Ca_{ox} loss, but the final concentration of Ca_{ox} in litter did not decrease relative to initial litter. Given that the high-Ca source litter had higher initial Ca-ox concentrations and therefore more Ca-ox degradation than the low-Ca litter, our data do not support the idea that pH changes were the main factor driving Ca-ox degradation, raising the possibility that microbial degradation may have been more important. Free oxalate anions in the soil are quickly utilized by microbes primarily as an energy source (van Hees et al., 2002; Brant et al., 2006) even though it is not a particularly high-energy yielding C compound; oxalate has a free energy yield of 45.6 J mol^{-1} K^{-1} compared to 209.2 J mol^{-1} K^{-1} of glucose (Stumm et al., 1996). Free oxalate ions can also be used as an electron or carbon source in microbial metabolism (Sahin, 2003; Verrecchia et al., 2006). During incubation of C-isotope labeled oxalate ions, the majority of oxalate is respired

in less than a few days in surface forest soils (van Hees et al., 2002, 2005; Brant et al., 2006). The rate of crystalline Ca-ox use by microbes has not been investigated, though microbial degradation of crystalline Ca-ox has been observed in petri cultures (Jayasuriya, 1955; Morris and Allen, 1994; Braissant et al., 2004) and has been implied in observations of *Streptomyces* in association with Ca-ox from fungal mats in the soil (Knutson et al., 1980). Additionally, petri-culture evidence suggests that some species of white rot and ectomycorrhizal fungi are also capable of dissolving Ca-ox crystals, especially when Ca supply is low (Tuason and Arocena, 2009; Guggiari et al., 2011). Overall, emerging lines of evidence supported by our observation of rapid Ca-ox disappearance from litter merit further examination of Ca-ox properties and dynamics *in situ*, to determine its importance as a Ca source in organic and mineral soil systems.

We found general agreement between the mean residence time estimates from the leaf litter decomposition study, and the mean residence times in forest floor at the high- and low-Ca sites. The forest floor estimate indicated faster turnover of Ca_{ox} compared to Ca_t at both sites (high Ca site: 9 years for Ca_{ox}, 15 years for Ca_t; low Ca site: 18 years for Ca_{ox}, 25 years for Ca_t). Likewise, leaf litter decomposition estimates of mean residence time for Ca-ox were similarly rapid (high-Ca site: 5 years, low-Ca site: 24 years). This parallel in Ca-ox decomposition rate estimates between independent measures of leaf litter versus whole forest floor suggests that the high-Ca site generally had more rapid turnover of Ca-ox than the low-Ca site. This result was somewhat surprising given the lower soil pH at the low-Ca site (4.61 versus 5.50 at the high-Ca site). Our findings from the leaf litter decomposition experiment suggest that Ca-ox degradation in leaf litter may be driven primarily by biotic controls rather than pH. And interestingly, in low-Ca sites despite lower Ca status, microorganisms do not decompose Ca-ox more rapidly and the supply of Ca to vegetation via the Ca-ox pathway is not necessarily amplified.

Ca-ox concentration variation in soils

Our finding that Ca-ox generally increased with exchangeable-Ca across nine sites suggests that calcium supply likely shapes Ca-ox formation in mineral soil. However, variation in Ca-ox in mineral soil between all nine sites (2-fold) was less than expected given

wide variation in exchangeable-Ca (19-fold), similar to patterns observed in all ecosystem pools of our two intensively studied sites. This lack of 1:1 relationship suggests that Ca-supply is not the only control on Ca-ox accumulation. We found across our nine sites that most of the variation in mineral soil Ca-ox concentrations was predictable based on soil depth combined with either soil pH (R^2=0.75) or soil exchangeable Ca (R^2=0.72). Because there is a strong interrelationship between soil-exchangeable Ca and soil pH, a controlled experiment is necessary to determine what specific chemical factor(s) most strongly control Ca-ox presence in soils. At all sites Ca-ox concentrations were highest in 0-20 cm surface soils (Figure 2.4), which supports the idea that Ca-ox primarily accumulates biologically either via plant or fungal exudation of oxalate and concomitant Ca-ox precipitation or via direct inputs of Ca-ox created within plant and fungal tissue. Mineral soil Ca-ox concentrations generally increased with increasing soil pH (from pH = 4.2 to 5.8) although, the relationship was strongest in 0-20 cm depths and broke down in lower soil depths. Site was not an important factor in the model; therefore concentrations of Ca-ox may vary consistently within local soil-types with soil chemistry factors that could allow for straightforward predictions of Ca-ox accumulation.

We compiled available literature data from seven studies reporting surface soil Ca-ox concentrations (Hintikka, 1970; Cromack et al., 1979; Fox and Comerford, 1990; Lilieholm et al., 1992; Tani et al., 1993; Morris and Allen, 1994; Certini et al., 2000) along with our own data (Table 2.4). All of the studies examined used acid extractants to dissolve Ca-ox, with subsequent determination of free oxalate concentrations and estimation of Ca_{ox} using stoichiometric relationships. Acid extractable oxalate is assumed to originate as Ca-ox, because while Al-oxalate and Fe-oxalates can occur in natural systems, they are less abundant and more soluble than Ca-ox (Baran and Monje, 2008). Literature values of Ca_{ox} concentrations in surface soils (0 to either 10 or 15 cm depth) ranged from 0.7 to 3318 μg Ca g^{-1} soil, but varied depending on association with fungal mats, extraction method used and type of soil. For example, the highest concentrations were found in ectomycorrhizal fungal mats (Cromack et al., 1979), suggesting that mineral soils may be extremely patchy in Ca-ox accumulation. High Ca-ox concentrations were also associated with organic horizons (Hintikka, 1970). Ca-ox concentration may be expected to have a wide range if related to oxalate ion concentration, which displays a similarly wide range in soil concentrations (at least

100-fold, Allison et al., 1995; Strobel, 2001). The mean Ca_{ox} concentration in 0-10 cm mineral soil across our nine study sites (44 μg Ca g^{-1}) was on the low end of the range of Ca_{ox} concentrations reported for mineral soil in other studies (Table 2.4), although, different extraction procedures may result in different estimates of Ca-ox concentrations. For example, we used dilute acid to avoid dissolving mineral Ca when extracting oxalate from Ca-ox across our nine sites, which likely resulted in low estimates of Ca-ox concentrations, even though our spike-recovery data was intended to correct for partial Ca-ox dissolution during extraction. However, when a more concentrated acid was used to estimate Ca-ox in 0-3 cm depth of the same soils, the Ca-ox concentrations were 11- to 18-times higher (discussed more below).

Most of the studies that measured acid-extractable oxalate ions do not report levels of Ca in the extractant, or even soil exchangeable-Ca, reflecting a main interest in examining organic acids as root exudates or microbial substrates (Fox and Comerford, 1990; Tani et al., 1993; Certini et al., 2000). The few studies we found that report Ca levels along with oxalate concentration indicate that Ca_{ox} is less than a quarter of total available Ca, ranging from 1.4 to 24% of Ca_s (Table 2.4). However, this amount (27 to 55 kg Ca ha^{-1}) could be significant in terms of plant needs or export from many regions. For example, at the high and low-Ca sites in our study, the annual uptake of Ca was estimated to be 5.7 and 3.6 kg Ca ha^{-1} respectively (Perakis and Sinkhorn, in progress), stream export was estimated to be 1.5 to 2.6 kg Ca ha yr^{-1} in a northeastern hardwood forest (Bailey et al., 1996), and supply of Ca from mineral soil was estimated to be 33 to 47 kg Ca ha^{-1} y^{-1} at Hubbard Brook Experimental Forest (Hamburg et al., 2003). Bailey et al. (2004) used the amount of Ca-ox measured in Italian soils by Certini (2000, Table 2.4) to compare to the soil exchangeable pools at Hubbard Brook Experimental Forest, and found that the Ca pool in Ca-ox of these Italian soils was twice as large as the exchangeable Ca pool at Hubbard Brook. Overall, this evidence supports the idea that Ca-ox comprises an ecologically significant pool of available Ca, especially in low-Ca ecosystems that rely on Ca from recycled plant sources, and on the rapid turnover of Ca-ox in the forest floor.

Although all of studies we found that quantified Ca-ox in soils were temperate forests (Table 2.4), other types of ecosystems worldwide have been observed to accumulate Ca-ox,

though concentrations were not quantified. For example, Ca-ox excreted by fungi accumulates and participates in the genesis of calcrete (hardened deposits of Ca-carbonate) in xeric soils of semi-arid ecosystems (Verrecchia and Dumont, 1996; Verrecchia et al., 2006). In tropical systems where accumulations of Ca-ox are unexpected due to the acidic conditions of soils, since the iroko tree (*Milicia excelsa*) creates and accumulates Ca-ox in association with decaying plant tissues (Cailleau et al., 2011). The variation in concentrations of Ca-ox in mineral soils, and observations of Ca-ox across multiple ecosystem types, indicate the likelihood that Ca-ox may be fairly ubiquitous with the potential to widely influence Ca dynamics in terrestrial ecosystems.

A comparison of soil Ca-ox measurement methods

There is no accepted method for measuring Ca-ox in soil, so we compared two possible methods of measuring Ca-ox in soils, which resulted in different estimations of Ca-ox concentrations. The method used for calculations in this study (Table 2.2) was likely a low estimate of Ca-ox due to the dilute acid (0.5 *N* HCl) and the relatively short extraction time of 5 hours. The second technique used was a sequential extraction, which was likely a high estimate of Ca-ox due to using a concentrated acid (2 *N* HCl) for a long time period (24 hrs) that may have dissolved mineral-Ca or organic-Ca in the sample. However, this methodological comparison is also confounded in that the high estimate method was performed on a shallower soil depth (0-3 cm) that likely has higher Ca-ox concentrations than the deeper soils (0-10 cm) evaluated using the low estimate method. However, this comparison may be useful to inform soil Ca-ox methods development, which is particularly challenging (Dauer, Chapter 5).

Using the dilute acid/short extraction time (low-estimate) method of Ca-ox in soils, we found that mineral soil (0-10 cm depth) Ca_{ox} concentrations were 37 and 16 μg Ca g^{-1} at the high and low Ca sites, respectively. This was approximately equivalent to Ca_{ox} concentrations of 14 μg Ca g^{-1} measured at similar sites (Cromack et al., 1979), but 10 to 18-fold less than the (high estimate) method of Ca_{ox} that we measured in 0-3 cm soils. And, using the low-estimate concentrations, the Ca-ox in mineral soil was estimated to be 21 to 29% of the total ecosystem Ca-ox. However, if we assume the difference between the low-estimate

and high-estimate for surface soils holds for the entire soil profile, the resulting soil pool of Ca-ox would be the largest pool of total ecosystem Ca-ox measured; 99% and 96% of high and low Ca site, respectively. Soil stores of Ca-ox are likely extremely heterogeneous in both time and space, and remain unclear in terms of their contribution to total ecosystem Ca pending refinement of methods to measure Ca-ox unambiguously in a range of soil types.

A useful protocol for measuring Ca-ox in soils will measure both Ca and oxalate ions, and will necessarily keep oxalate anions in solution, as well as dissolve Ca-ox but not other Ca sources. Extracting oxalate anions once freed from Ca is difficult, as the movement and availability of oxalate anions may be influenced by soil solid phase reactions (sorption and desorption), rapid degradation by soil microorganisms or complexation and precipitation reactions (Ryan et al., 2001). Additionally, the strength of acid and the time for extraction could affect the extraction of both Ca and oxalate ions. Pairing measurements of both Ca and oxalate ions in a moderate concentration acid extraction, using a spike-recovery technique, and matching Ca and oxalate ions stoichiometrically may be a way to resolve some of these issues.

Conclusions

Ca-ox is ubiquitous in temperate forest ecosystems, existing in live and decomposing biomass pools, as well as in organic and mineral soil horizons, and because it makes up a large portion of ecosystem Ca, it may have a profound effect on the availability of Ca. About one-third of ecosystem Ca was found in plants, detritus and mineral soil at each site, and leaf litterfall, foliage and branches had the largest proportion of total Ca in Ca-ox. The Ca status of a site can influence both the absolute amount of Ca-ox present in each ecosystem pool, as well as the proportion of Ca that occurs as Ca-ox. The forest floor may be influenced via multiple inputs from senesced tree tissue or oxalate ion exudation from roots and fungi followed by complexation with free Ca^{2+}, and via multiple losses from Ca-ox losses due to microbial degradation or acidic dissolution. We found that despite high initial quantities of Ca-ox in high-Ca leaf litter, and a large pool of Ca-ox in the forest floor, Ca-ox does not appear to

accumulate over time as an insoluble crystal. Instead Ca-ox loss paralleled or exceeded bulk Ca loss during decomposition, indicating that Ca-ox release from decomposing leaf litter in the forest floor is an important pathway for Ca return to plants, especially at the high-Ca site. The low-Ca site leaf litter and forest floor residence times were longer than the high-Ca site suggesting that release of Ca from Ca-ox is not necessarily accelerated in sites with low Ca supply, regardless of apparent biotic control over Ca-ox decomposition. Processes involving Ca-ox are poorly understood, but may be important in shaping long-term Ca availability of ecosystems under stress from acidic atmospheric deposition, repeated biomass harvest or, as in our study sites, naturally high N sites that result in soil acidification and Ca loss.

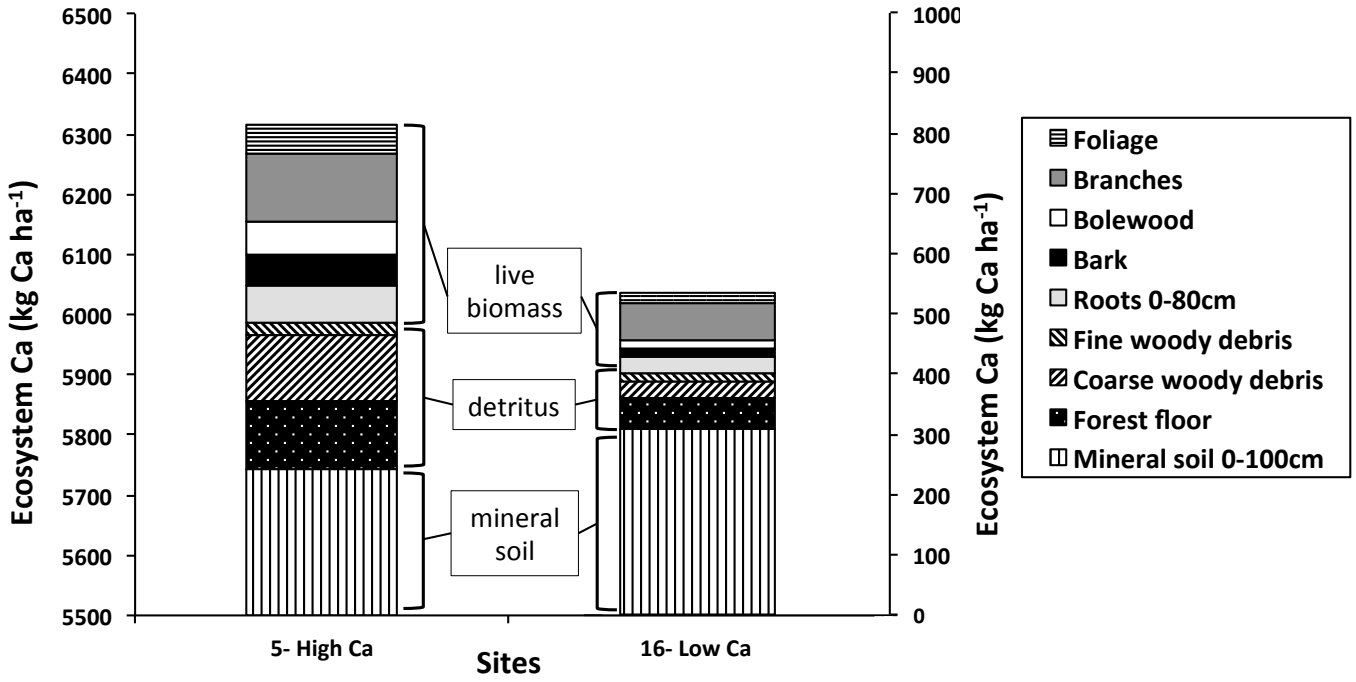

Figure 2.1. Ca content (kg Ca ha^{-1}) in different ecosystem pools at a high Ca and low Ca site. Note different scales for the high-Ca (left) and low-Ca sites (right).

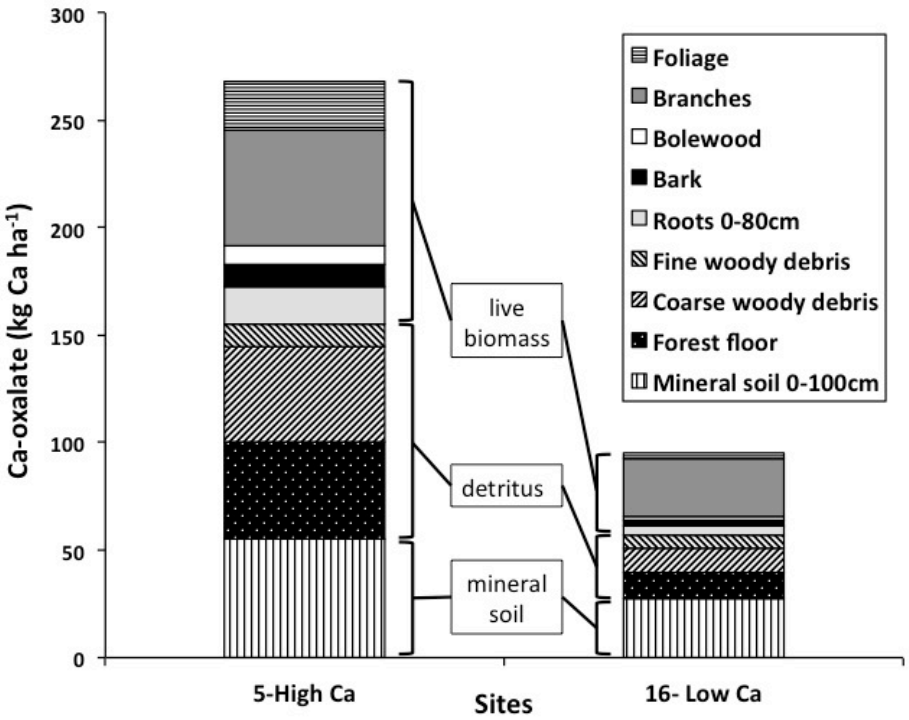

Figure 2.2. Ca_{ox} content (kg Ca ha^{-1}) in different ecosystem pools at a high Ca and low Ca site.

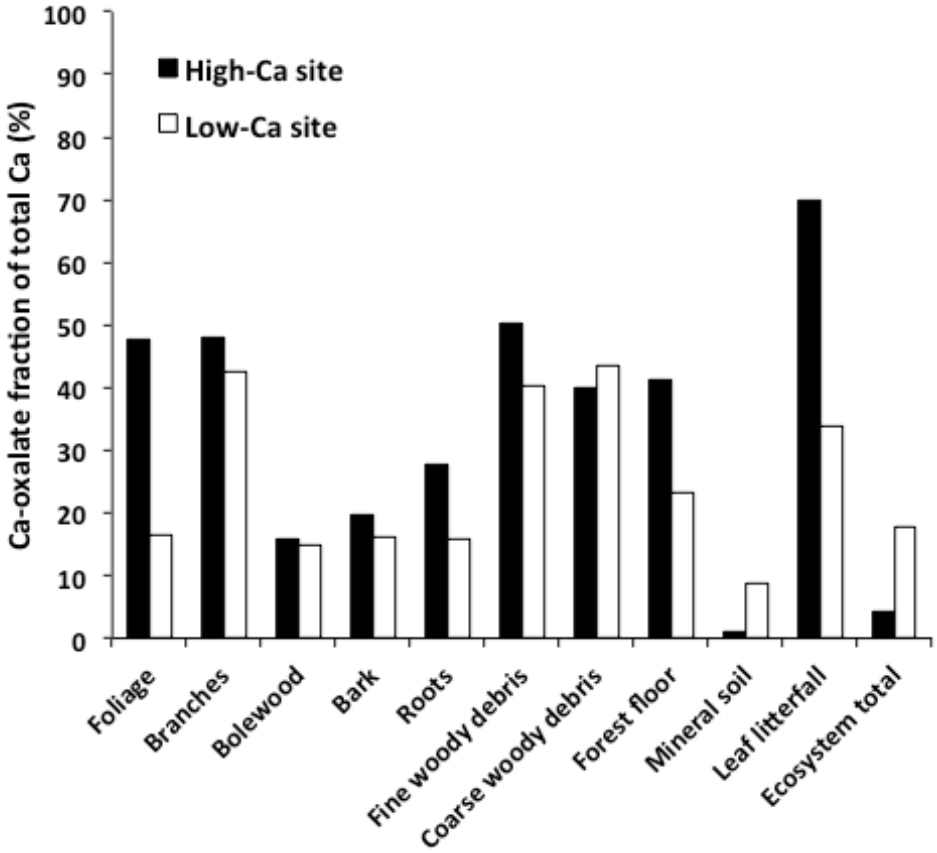

Figure 2.3. Ca_{ox} proportion of total Ca (%) in different ecosystem pools at a high Ca and low Ca site.

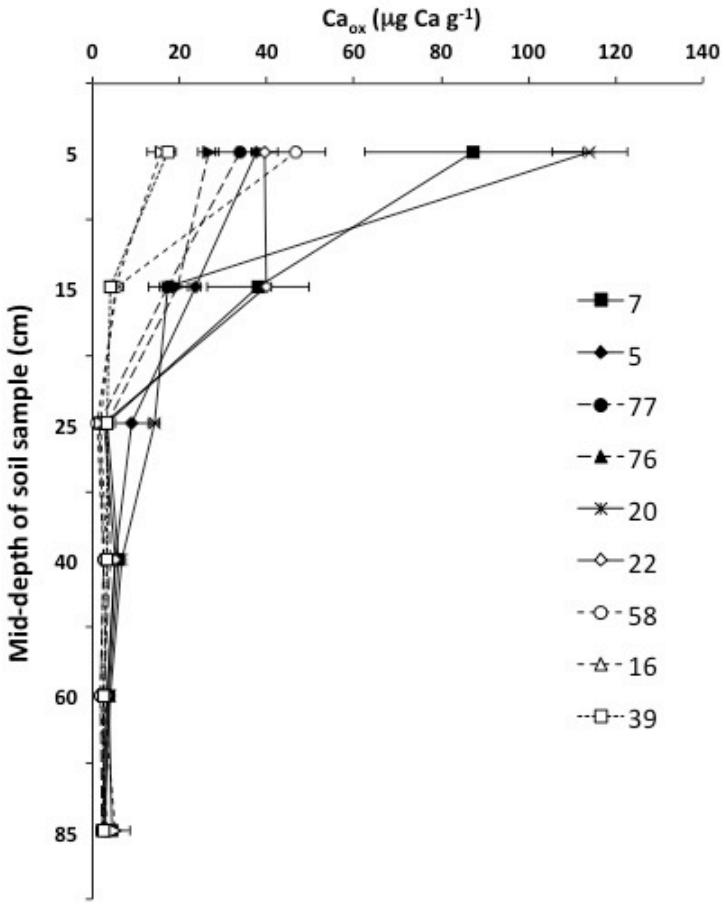

Figure 2.4. Calcium oxalate concentration (µg Ca g^{-1} soil) of soil depths in nine sites across the Coast Range of Oregon. Sites are arranged in order of largest exchangeable Ca pool from 0-100 cm depth of mineral soil; Site 7, 12,843 kg ha^{-1}; Site 5, 11,295 kg ha^{-1}; Site 77, 9286 kg ha^{-1}; Site 76, 4238 kg ha^{-1}; Site 20, 3697 kg ha^{-1}; Site 22, 3593 kg ha^{-1}; Site 22, 3593 kg ha^{-1}; Site 58, 1123 kg ha^{-1}; Site 16 624 kg ha^{-1}; Site 39, 440 kg ha^{-1} with filled in symbols representing the four most Ca-rich sites. Bars represent standard error (n=3, for all depths except 40 and 60 which were determined by interpolation).

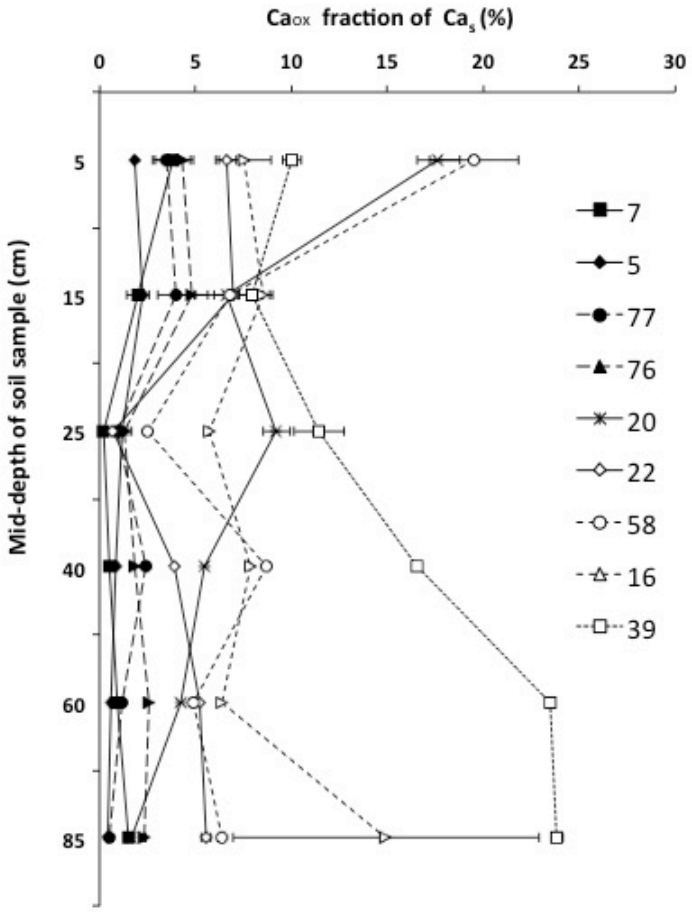

Figure 2.5. Fraction of Ca_s (Ca_{exch} + Ca_{ox}) that exists as Ca_{ox} by percentage of soil depths in nine sites across the Coast Range of Oregon. Sites are arranged in order of largest exchangeable Ca pool from 0-100 cm depth of mineral soil, see Figure 2.2. Bars represent standard error (n=3) for all mid-depths except 40 and 60, which were determined by interpolation.

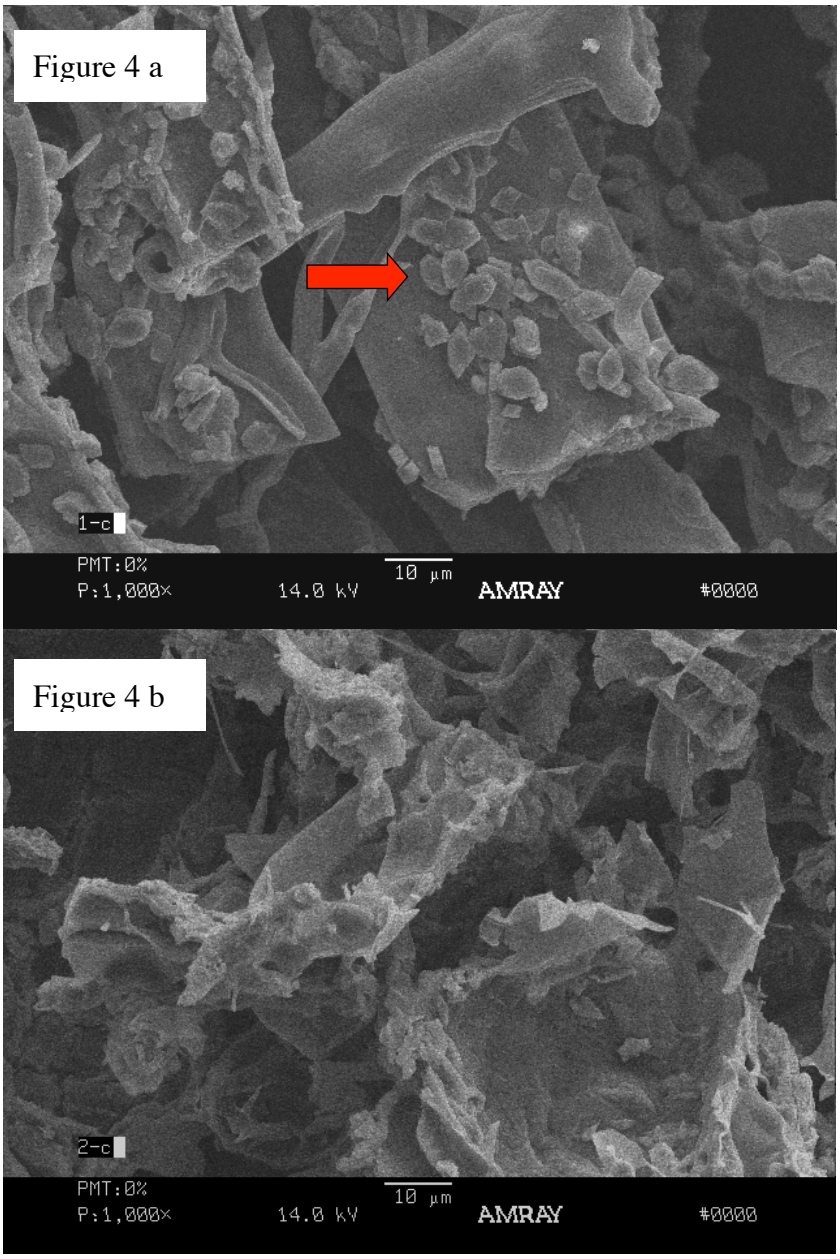

Figure 2.6. High-Ca source litter a) before decomposition and b) after 2 years decomposition, imaged with STEM and magnified 1000 times. Ca-oxalate crystals are present in image (a) as indicated by arrow, and Ca content of the crystals in (a) were determined by X-Ray Energy Dispersive Spectrophotometry.

Table 2.1. Characteristics of two sites in the Coast Range of Oregon including soil exchangeable Ca pools to 100 cm depth, soil pH and texture in surface soils, Ca in plant biomass, tree age in 2007 and total aboveground biomass. ANPP calculated as the net annual aboveground in stems, branches, foliage mass plus litterfall (Perakis and Sinkhorn 2011).

Site	Soil exchangeable Ca 0-100 cm depth	Soil pH(H_2O) 0-10 cm depth	Soil texture (%) 0-10 cm depth			Ca in aboveground plant biomass	Tree age	Total aboveground biomass	ANPP
	kg ha^{-1}		sand	silt	clay	kg ha^{-1}	years	Mg ha^{-1}	Mg ha^{-1} yr^{-ha}
high-Ca (5)	5680	5.50	53	23	25	172	30	163	21.5
low-Ca (16)	280	4.61	30	31	39	98	27	122	14.3

Table 2.2. The concentration of total Ca (Ca_t), the concentration of Ca-oxalate (Ca_{ox}), and the percent of Ca_t that exists as Ca_{ox}, at a high (site 5) and low (site 16) Ca-status site in the Coast Range of Oregon. Ca_t and Ca_{ox} concentration are reported for each plant part, detritus type (mg Ca g^{-1}) and soil depth (μg Ca g^{-1}). The percentage of Ca_t existing as Ca_{ox} for each plant part, detritus type (100*Ca_{ox}(kg ha^{-1})/Ca_t (kg ha^{-1}) and soil depth (100*Ca_{ox} (kg ha^{-1})/(Ca_{exch} + Ca_{ox} (kg ha^{-1}) are reported. The percentage of Ca_t existing as Ca_{ox} weighted by mass for live biomass, detritus and soil pools (example for live biomass; 100* $\Sigma_{\text{foliage, branches, bole-wood, bark, <2mm roots, >2mm roots}}$ Ca_{ox} (kg ha^{-1})/ $\Sigma_{\text{foliage, branches, bole-wood, bark, <2mm roots, >2mm roots}}$ Ca_t (kg ha^{-1})) is reported in bold type). Foliage 1-5 = leaf age cohorts from youngest (1) to oldest (5). Roots are from 0 to 80- cm depth. FWD= fine woody debris (<10 cm diameter). CWD= coarse woody debris (>10 cm diameter). Mean live biomass, mean detritus, mean mineral soil and mean ecosystem total were weighted by mass. Table cells with n.a. denotes not applicable.

Ecosystem Compartment	Ca concentration		Ca_{ox} concentration		Ca_{ox} fraction	
	High-Ca Site (5)	Low-Ca Site (16)	High-Ca Site (5)	Low-Ca Site (16)	High-Ca Site (5)	Low-Ca Site (16)
	Tissue Ca_t mg Ca g^{-1}		**Tissue Ca_{ox} mg Ca g^{-1}**		**Tissues Ca_{ox} % of Ca_t**	
foliage 1 yr	3.2	1.5	1.4	0.1	43	10
foliage 2 yr	7.1	2.3	3.0	0.4	42	17
foliage 3 yr	8.5	2.9	4.1	0.9	48	30
foliage 4 yr	11.5	2.7	6.4	0.8	56	30
foliage 5 yr	12.9	n.a.	6.6	n.a.	52	n.a.
branches	6.2	4.9	3.0	2.1	48	42
bole-wood	0.3	0.2	0.0	0.0	16	15
bark	1.5	1.6	0.3	0.3	20	16
roots <2mm	2.2	1.4	0.5	0.2	20	16
roots >2mm	4.3	1.6	1.5	0.3	35	16
mean live biomass					**34**	**29**
annual leaf litter	10.3	3.0	7.3	1.0	70	34
FWD decay 2	4.6	3.3	2.5	1.5	55	45
FWD decay 3	2.9	2.7	1.1	0.9	37	35
FWD decay 4	2.8	1.9	0.8	0.7	28	34
CWD	1.8	1.8	0.6	0.9	40	44
forest floor	9.1	4.3	3.7	1.0	41	23
mean detritus					**41**	**25**
	Soil Ca_{exch} µg Ca g^{-1}		**Soil Ca_{ox} µg Ca g^{-1}**		**Soil Ca_{ox}% of Ca_s**	
soil 0 - 10 cm	2031	194	37.4	15.9	1.8	8.2
soil 10 - 20 cm	1047	55	23.7	5.1	2.3	9.3
soil 20 - 30 cm	767	29	9.0	1.8	1.2	6.0
soil 30 - 50 cm	641	38	5.3	3.3	0.8	8.6
soil 50 - 70 cm	570	38	3.5	2.6	0.6	6.9
soil 70-100 cm	521	27	2.1	5.4	0.4	19.8
mean mineral soil					**1.0**	**8.9**
mean ecosystem total					**4**	**18**

Table 2.3. Ca concentrations (mg g^{-1}) of Ca_t, Ca_{sum} and Ca fractions and mass (g) of initial and two-years decomposition of high-Ca and low-Ca Douglas-fir source litter at four sites in western Oregon. Values represent arithmetic means with standard errors in parentheses. Bold numbers represent significant differences between Ca (mg g^{-1}) initial and two-years decomposition (three-way ANOVA, $P<0.001$). Percent loss of Ca and mass after two-years decomposition reported in italics. Net accumulation of Ca in Ca_{st} occurred, resulting in negative "loss" values. Letters in lower-case represent within-source litter differences in percent loss (two-way ANOVA, $P<0.001$). Ca_{aq}, Ca_{st} and Ca_{ox} Ca concentrations were determined by sequential extraction on the same dried tissue, Ca_{sum} is the sum of those fractions, and Ca_t was determined independently by digestion of the whole tissue.

	High Ca source litter			Low Ca source litter		
Ca fraction	Initial mg Ca g^{-1} (g)	Two years mg Ca g^{-1} (g)	*Loss (%)*	Initial mg Ca g^{-1} (g)	Two years mg Ca g^{-1} (g)	*Loss (%)*
Ca_t	14.0 (0.1)	13.6 (0.4)	*43 (4) ac*	7.4 (0.2)	8.8 (0.5)	*26 (5) ab*
Ca_{sum}	12.5 (0.1)	13.0 (0.5)	*40 (4) ac*	**6.7 (0.1)**	**8.8 (0.2)**	*18 (5) ab*
Ca_{aq}	0.9 (0.0)	1.5 (0.1)	*4 (10) a*	0.7 (0.1)	1.1 (0.3)	*6 (20) ab*
Ca_{st}	**1.6 (0.0)**	**5.9 (1.1)**	*-102 (27)b*	**2.4 (0.0)**	**4.4 (0.4)**	*-13 (10) a*
Ca_{ox}	**10.0 (0.1)**	**6.0 (1.2)**	*66 (5) c*	3.6 (0.1)	3.3 (0.2)	*42 (4) b*
Mass	4.5 (0.0)	2.6 (0.1)	*42 (3) ac*	4.5 (0.0)	2.8 (0.1)	*38 (2) ab*

Table 2.4. A comparison of surface soil measurements of either oxalate ions or Ca_{ox} ions of forest soils. Bold values are measured values versus calculated values in italics, based on the assumption that all the acid-extractable measured oxalate or Ca ions were once bound as Ca-oxalate crystals. The oxalate or Ca ions were calculated stoichiometrically using the ratio of Ca cations to oxalate anions in Ca-oxalate (2.2). Moles of Ca-oxalate were calculated based on the formula weight of Ca-oxalate anhydrous (128.1 g mol^{-1}). Details of the extraction procedure, soil depth and soil pH are reported.

Soil measurement via acid extraction in forest soils:	**method**	**Soil depth (cm)**	**Oxalate mg oxalate g^{-1}**	**Ca_{ox} mg Ca g^{-1}**	**Ca-oxalate mmol g^{-1}**	**Ca_{ox}% of total available Ca (Ca_s)**	**Soil pH**
This study	0.5 *N* HCl for 5 hrs	0-10					
Site 5 (High Ca)			**82**	*37*	*0.93*	*1.8*	5.5
Site 16 (Low Ca)			**35**	*16*	*0.40*	*7.5*	4.6
Site 7			**191**	*87*	*1.10*	*7.9*	5.8
Site 20			**251**	*114*	*2.85*	*3.8*	5.2
Site 22			**88**	*40*	*1.00*	*6.7*	5.6
Site 39			**37**	*17*	*0.43*	*10.0*	4.1
Site 58			**103**	*47*	*1.18*	*19.5*	4.8
Site 76			**59**	*27*	*0.68*	*4.3*	5.0
Site 77			**75**	*34*	*0.85*	*3.5*	5.3
Mean of 9 sites			**97**	*44*	*1.1*	*7.9*	5.1
This study	2 *N* HCl for 24 hrs	0-3					
High Ca site (5)	after H_2O and HOAc		*1452*	**660**	*17*	n.d.	5.6
Low Ca site (16)	extraction		*374*	**170**	*4.3*	n.d.	4.6
Hintikka, 1970 (range of values)	1.5 N HCl for 12 hours	Humus					
alder stand			**4910**	*2237*	*55.9*		
spruce stand			**880**	*401*	*10.0*		
Cromack et al 1979	0.65 *N* HCl in methanol	0-10					
mat			**7300**	*3318*	*83*	*24*	4.9
non-mat			**30**	*14*	*0.34*	*1.9*	6.1

Table 2.4 Continued

Fox & Comerford 1990	adj with HCl to pH	0-15					
Psamment	of 4.3, 12 hrs		**1.5**	*0.7*	*0.02*	n.r.	4.0-4.5
Utisol			**5.3**	*2.4*	*0.06*	n.r.	
Spodosol			**9.2**	*4.2*	*0.11*	n.r.	
Spodosol			**5.1**	*2.3*	*0.06*	n.r.	
Spodosol			**4.1**	*1.9*	*0.05*	n.r.	
Liliehom et al 1992	1 N HCl	0-10					
mesic Typic	6 h		**0**	*0*	*0*	n.r	7.9
mesic Xerollic	16 h		**110**	*50*	*1.3*	n.r	7.7
mesic Calcic	16 h		**52**	*24*	*0.6*	n.r	7.5
overburden	6 h		**278**	*127*	*3.2*	n.r.	6.9
Tani et al 1993	0.1 M ammonium						
greywacke Cambisol	phosphate buffer,	0-10	**3.6**	*1.6*	*0.04*	n.r.	4.34
volcanic Cambisol	pH 2, 30 min.	0-10	**7.2**	*3.3*	*0.08*	n.r.	4.38
Andisol		1-15	**7.9**	*3.6*	*0.09*	n.r	4.61
Andisol		0-11	**8.4**	*3.8*	*0.10*	n.r.	5.06
Morris and Allen 1994	1 N HCl						
Pachic Haploxerol		0-10	**17.6**	*8.0*	*0.20*	0.69	5.7 – 6.3
Certini et al 2000	1.2 M HCl for 12						
volcanic entisols	hrs and repeated	2-12	**220**	*100*	*2.5*	n.r.	4.5
sandstone inceptisol		0-10	**214**	*97*	*2.4*	n.r.	4.9

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CHAPTER 3 – FOREST RESPONSE TO NITROGEN AND CALCIUM FERTILIZATION IN SITES OF CONTRASTING NUTRIENT STATUS

Abstract

The diagnosis and management of nutrient deficiency is a major challenge in regions where key soil nutrients vary widely over small geographic areas. In Douglas-fir forests of the Oregon Coast Range, levels of soil nitrogen (N) often vary inversely with soil calcium (Ca), which has prompted the evaluation of both N and Ca fertilization as approaches to alleviate nutrient deficiency. However, it remains unclear whether foliar chemistry can be used to diagnose nutrient deficiencies across this region, whether fertilization ameliorates apparent deficiencies, and how different fertilizers affect soil N cycling processes. To address these questions, we investigated live foliage and senesced foliage nutrients and soil N cycling at two sites with contrasting nutrient status (i.e., low-N and high-Ca versus high-N and low-Ca) after 3-years of fertilization with urea (227 kg N ha^{-1}), lime (1020 kg Ca ha^{-1}) and $CaCl_2$ (105 kg Ca ha^{-1}). Response to fertilization examined with foliar vector diagrams suggested N limitation at the low-N site and N sufficiency at the high-N site, but did not suggest Ca deficiency at either site. Calcium fertilization did not influence foliar concentrations of physiologically active Ca at either site, but the high-Ca site displayed 20-60 times higher concentrations of physiologically inactive Ca-oxalate than the low-Ca site. Soil nitrogen mineralization did not respond strongly to fertilization overall, with the exception of increased forest floor N mineralization after urea fertilization at the low-N site. In contrast, nitrification responded to N fertilization at both sites, and was also stimulated by liming at the high-N site. Nutrient management in these forests must be carefully considered to manage interactions between N and Ca that shape long-term site fertility. Fertilization with N may not result in sustained increases in soil N availability, but may stimulate nitrification that could accelerate soil Ca loss. In N-rich sites, fertilization with $CaCl_2$ rather than lime shows promise as a way to provide supplemental Ca without further stimulating nitrification.

Introduction

Nitrogen (N) and calcium (Ca) availability are important to plant productivity in forest ecosystems, and coupled interactions between these two elements are important for soil pH, nutrient availability, and ecosystem nutrient loss (McNulty et al., 1996; Aber et al., 1998; Asner et al., 2001). Much evidence for N and Ca nutrient couplings comes from temperate forests receiving elevated N deposition where increased nitrate mobility has resulted in increased loss of soil base cations (Likens et al., 1996; Currie et al., 1999; Bedison and Johnson, 2010). As a result of base cation losses many sites with high N fertility are deficient in Ca (Aber et al., 1989; Perakis et al., 2006; Lucas et al., 2011). An increase in available inorganic N supply can occur after fertilization with either Ca (Yavitt and Newton, 1990; Smallidge et al., 1993; Nohrstedt, 2002) or N (Nave et al., 2009). Ca fertilization influences on long-term ecosystem function and productivity may be similar to N fertilization, which could either be positive, ameliorating tree growth limitation of either N or Ca, or negative, increasing nitrate availability and resulting in coupled soil Ca losses. Mechanisms for these mutual influences are not well understood, and may vary depending on underlying site Ca and N status. Additionally, lags in the influence of fertilization on tree growth (Mainwaring et al., in review) can lead to a potential mismatch where fertilizer management efforts to increase short-term productivity may lead to long-term declines in soil nutrient availability. However, changes in foliage nutrient concentrations or soil nutrient dynamics may be more immediate, easier to observe, and enable predictions of longer-term tree and soil response.

Fertilization to increase forest productivity typically focuses on N, which is commonly limiting in many ecosystems (Tamm, 1991; Vitousek et al., 1997; LeBauer and Treseder, 2008). Nitrogen additions frequently increase soil N supply by amplifying inorganic N production via increased N mineralization rates (Nilsen, 2001; Saarsalmi and Malkonen, 2001; Nave et al., 2009). This is especially true in soil ecosystems where microbial activity is thought to be N limited (Aber et al., 1993, 1998). However, in some cases N fertilization has resulted in brief increases in soil N cycling, followed by subsequent declines (Aber et al., 1993; Magill et al., 1996; McNulty et al., 1996), while other sites have seen no effect of N addition (Emmett et al.,

1995; Chappell et al., 1999). It may be that a lack N cycling response to N fertilization is due to inherently high-N availability (Zogg et al., 1996; Prietzel et al., 2004), to excessive levels of N addition that decrease soil organic matter quality (Fox, 2004) or to nitrogen saturation (Aber et al., 1998). Alternatively, a lack of response to N fertilization may be due to stability in site factors like soil texture, soil moisture, and site history that also govern N cycling and responses to N fertilization (Reich et al., 1997; Chappell et al., 1999; Prescott et al., 2000). Whether or not N-fertilization stimulates N cycling may also be important for long-term availability of soil Ca. For example, in N-limited sites, N-addition may accelerate Ca cycling via increased soil mobilization, tree uptake, and litterfall return (McLaughlin and Wimmer, 1999) without negative long-term effects on productivity. However, in N-rich sites, added N fertilizer may intensify deficiency in soil Ca supply via soil leaching loss or uptake and sequestration in accumulating biomass (Akselsson et al., 2007; Johnson et al., 2008; Lucas et al., 2011). If N fertilization stimulates inorganic N production, then there is evidence that: 1) N cycling is N limited and 2) N-fertilization can alter N cycling and concomitant changes in Ca cycling.

Additions of Ca fertilizers to forest ecosystems are less common than N additions (Bengtson, 1979; Chappell et al., 1991; Fox et al., 2007) despite evidence that soil Ca supply influences tree growth (Bigelow and Canham, 2007; Dauer et al., 2007; Mainwaring et al., in review) and long-term productivity in some forest ecosystems (Gradowski and Thomas, 2008; Park et al., 2008; Vadeboncoeur, 2010). For Ca fertilizers to be used wisely in forest management, long-term influences of Ca additions on soil N processes need to be better understood. For example, Ca additions can increase available inorganic N supply rates (Yavitt and Newton, 1990; Smallidge et al., 1993; Nohrstedt, 2002). These increases in N cycling may either benefit low-N systems by ameliorating N limitation, or aggravate problems of nitrate loss in high-N systems (Kreutzer, 1995; Gebauer et al., 1998). However, amplified N cycling responses to Ca fertilization are not consistently positive (Smolander et al., 1995; Neale et al., 1997; Groffman et al., 2006) which could be due to multiple factors including pre-treatment soil pH, time since fertilization, or site N-status. Mechanisms by which Ca addition influence N mineralization are unclear especially since Ca additions typically use lime fertilizer, which, in

increasing soil pH (Smallidge et al., 1993), makes it difficult to evaluate independent influences of Ca versus pH.

Plant nutritional deficiencies and potential growth responses to fertilizer are typically analyzed using foliar nutrient levels. Most commonly, total Ca or N concentration on a dry weight basis is compared to critical values of foliar nutrient concentrations to identify deficiencies (Van den Driessche, 1974; Ballard et al., 1986; Walker et al., 1991). An alternative approach uses vector diagrams to simultaneously compare changes in nutrient concentration to changes in leaf weight, thus accounting for potential confounding effects of fertilization on increased nutrient uptake versus nutrient dilution due to increased tree growth (Timmer and Stone, 1978; Haase and Rose, 1995; Braekke and Salih, 2002). Quantification of specific nutrient fractions within foliage is another approach taken for N, carbon, and phosphorus (Kedrowski, 1983; Chapin et al., 1986; Mulligan, 1988; Warren and Adams, 2000) that allows a deeper characterization of nutrient status, although this approach is less well-developed for chemical fractions of Ca (Fink, 1991). Physiologically active pools of Ca include soluble Ca and structural Ca while pools of crystalline Ca-oxalate are inactive in foliar tissues (DeHayes et al., 1997; Schaberg et al., 2001; Borer et al., 2004). Changes in the levels of each pool after fertilization can give a clearer understanding of sufficient Ca soil supply levels in an ecosystem. An increase in foliar Ca after fertilization can be explained primarily by increases in Ca-oxalate, (Littke and Zabowski, 2007; Smith et al., 2009) suggesting that the inactive Ca pool is most sensitive to Ca supply. However, little is known about mature trees and the active versus inactive Ca concentrations that would satisfy physiological demand, nor how Ca concentrations may vary with soil nutrient supply. Comparative fertilization studies provide an opportunity to determine the elasticity of Ca-oxalate concentrations in relation to Ca supply in mature trees, and to link Ca-oxalate concentrations to soil status and tree physiological characteristics.

Both foliar and soil responses are important for understanding how fertilization influences forest nutrition. In this study we compare foliar and soil responses to urea, lime, and calcium chloride ($CaCl_2$) fertilization to differentiate among soil inorganic N supply, Ca supply,

and changes in soil pH as factors shaping forest response to fertilization. We distinguish between responses to Ca versus soil pH by use of two Ca fertilizers that either increase soil Ca and pH (lime) or increase soil Ca without changes in pH ($CaCl_2$). Our choice of both N and Ca treatments reflects both past and emerging issues in nutrient management in Pacific Northwest forests (Chappell et al., 1991; Mainwaring et al., in review). Nitrogen fertilization with urea has been widespread in forests of the Pacific Northwest, even in naturally N-rich forests, because although Douglas-fir growth on a large proportion of forest sites is demonstrably N limited (Peterson and Hazard, 1990), our ability to discriminate between responding and non-responding sites is weak. However, recent evidence on relatively N-rich sites suggests a developing Ca deficiency (Perakis et al., 2006). Throughout this work we are especially interested in how native site fertility influences plant and soil responses to fertilization, and consider this by comparing responses to fertilization across two sites of contrasting nutrient status (i.e., a site with low-N, high-Ca versus a site with high-N, low-Ca). Specific objectives of this study were to determine: 1) whether contrasting N and Ca native soil fertility and fertilizer response in the Oregon Coast Range is reflected in foliar diagnoses of nutrient deficiency, using three approaches: Ca and N concentrations compared to critical values in literature, Ca and N graphical vector analysis, and assays of biologically active Ca (soluble, structural) versus inactive Ca (oxalate bound) in foliage, and 2) if fertilization by urea, lime, and $CaCl_2$ stimulates soil N mineralization and nitrification. It is hypothesized that sites with differences in native fertility will differ in foliar and soil responses to nutrient addition due to inherent differences in nutrient sufficiency and deficiency.

Methods

Sites and treatment

The two sites used in this study have contrasting soil nutrients status, specifically, high in Ca and low in N (OSU), and low in Ca and high in N (GDH) (Table 3.1). These sites are part of a larger and longer-term study of fertilization of 16 sites in the Coast Range of Oregon ((Mainwaring et al., in review). The low-N, high-Ca site (OSU) is located in Polk County, Oregon (W44.724, N-123.314) with mean annual precipitation of 156 cm and mean annual

temperature of 9.6 C degrees. Soils are fine-loamy, mixed, active, mesic Ultic and Aquultic Haploxeralfs on loam colluvium and residuum derived from sandstone and siltstone. The high-N, low-Ca site (GDH) is located in Tillamook County, Oregon (W45.325, N-123.818) with mean annual precipitation of 242 cm and mean annual air temperature of 9.5 C degrees (Daly et al., 1994). Soils are fine, isotic, isomesic Aquandic and Andic Dystrudepts on colluvium and residuum derived from sedimentary rock. Both stands were Douglas-fir (*Pseudotsuga menziesii*) plantations approximately 20-years-old that received no previous thinning within the previous ten years, no previous fertilization treatment and have closed canopies with few understory plants.

Fertilizer treatments were applied during the winter of 2006-2007 on fixed-area plots (0.01 ha; radius= 5.67 m) centered on an undamaged, dominant or co-dominant measurement tree. Suitable measurement trees were selected on a 20 m grid, skipping grid points if no suitable subject tree was available. Treatments were randomly assigned to ten trees per treatment per site (this study = 70 trees), and fertilizers were applied as solid pellets at the following rates: 225 kg N ha^{-1} urea, 1020 kg Ca ha^{-1} lime, 105 kg Ca ha^{-1} $CaCl_2$, and control (no fertilizer). Pre-treatment foliar data, site and growth characteristics (Table 3.1) were determined in 2006 (Mainwaring et al., in review).

Foliage nutrients and needle weight

In October of 2009, live foliage was collected at both sites from the largest 4-yr-old secondary lateral on the southernmost branch of the 5th whorl on all subject trees (10 trees per treatment per site). The fall season was chosen to represent annual foliar nutrient concentration as this season displayed the highest Ca nutrient concentrations (Mainwaring and Maguire, 2008). The live foliage was separated into annual cohorts based on bud scars on the stem of the branch. OSU branches generally had four live foliage cohorts, whereas GDH generally had two live cohorts. Senesced needles were collected by climbing the tree and shaking it while collecting needles on a tarp (OSU) (10 trees per treatment), or by one collection of litter baskets that were placed close (<1 m) to the tree from October to December (GDH) (3-5 trees per treatment). In order to compare the two methods, at OSU needles were also collected from baskets placed out

during October to December to collect senesced needles from two trees per treatment and analyzed for N. Concentration of N between shaken needles and senesced needles in litter baskets varied by less than 11% indicating that the two methods are comparable. From each age class of live needles and senesced needles (leaf litter) samples, approximately 50 needles were separated for measuring specific leaf area, SLA = leaf area per unit dry weight (cm^2/g) and for measuring weight on a needle count basis. These 50 needles were scanned on a desktop scanner at 100 dpi as black and white images. Image J 1.43 (Research Services Branch, National Institutes of Mental Health, Bethesda, Maryland, USA) software was used to count the needle numbers and measure total needle area. These same needles were dried at 65°C for 48 hrs and weighed. The remaining foliage from the sample secondary branch was dried at 65°C for 48 hrs and ground for chemical analysis.

Subsamples of live needles (2 and 4 age classes at GDH and OSU, respectively) and senesced needles from 5 treated trees per site were processed by drying at 65°C for 48 h and grinding to fine powder on a custom roller grinder (Kansas State University, Department of Physics). Carbon and N were analyzed on 3-5 mg subsamples using a Costech ECS-4010 elemental combustion analyzer (Costech Analytical, Valencia, CA, USA). Another subsample of 0.5 g each was digested with 5 ml of concentrated nitric acid and 3 ml of 30% hydrogen peroxide using a block digestor (DEENA, Thomas Cain Inc, Omaha, NE, USA) then analyzed for Ca, along with other selected cations (P, K, Mg, S, B, Zn, Cu, Fe, Mn, Mo, Al and Na), using inductively coupled plasma optical emission spectrometry (SPECTRO ARCO, Analytical Instruments, Kleve, Germany) at Louisiana State University Ag Center's Soil Testing and Plant Analysis Lab (STPAL, LSU, Baton Rouge, LA, USA).

A sequential extraction technique was used to separate tissue Ca into three fractions: soluble Ca^{+2} ions, structural Ca (bound as Ca-pectin and Ca-lignin) and insoluble Ca-oxalate forms (Fink, 1991). Although first-year needles are typically used for foliar diagnosis (Bowen, 1985) and first year needles were used for vector analysis (below), only the two-year-old age class were analyzed for Ca-ox foliar chemistry because Ca-oxalate concentration increases as

needles age, and use of these needles maximized the expected treatment response. Additionally, two-year old needles were the oldest age-class available at GDH, so only two-year old needles were analyzed to avoid confounding effects of needle age, precluding analysis across older age classes. A subsample of 0.5 g for each site was sequentially extracted for Ca using three 10 mL solutions: water (soluble Ca), 2 *N* acetic acid (structural Ca), and 2 *N* hydrochloric acid (Ca-oxalate). Separation of the needle tissue from the solutions was performed by centrifuging IEC Multi 200 Centrifuge, Thermo Electron Corporation (Boston, MA, USA) for 8 min at 11,000 rpm. Between extractions samples were rinsed with 40 mL Nanopure water and dried at 65°C. Ca concentrations were measured using atomic absorption spectrophotometry on an AAnalyst 200 Spectrometer, Perkin Elmer (Waltham, MA, USA).

Soil nitrogen cycling rates

Soil N cycling rates were determined in forest floor and 0-10 cm mineral soil at both sites. Soil nitrogen cycling was measured for only one month in late-spring / early-summer (June 2009), when rates are seasonally highest (Perakis, unpublished data) and forest management treatment differences are strongest (Thiel and Perakis, 2009). Buried-bag incubations (Eno, 1960) were used to measure cycling rates. Forest floor samples (20 cm × 20 cm) were collected from each plot (i.e., 10 samples per treatment per site), discarding freshly-fallen needles and cones, moss, and twigs >1 cm in diameter. For forest floor, samples were collected down to the mineral soil at a random location, with one half of each sample placed in a Ziploc bag for immediate extraction and the other half sealed in a gas-permeable polyethylene bag (15 cm × 5 cm, 1.5 mil gauge). The latter bag was then incubated on top of the forest floor near the location of collection for 30 days. Below each forest floor sample, mineral soil (0 – 10 cm depth) was collected using a 5.1 cm diameter soil corer, sealed in a gas-permeable polyethylene bag and incubated uncovered in its original hole for 30 days. Another mineral soil sample was collected from a nearby random location within each plot to determine initial extractable inorganic N. Both initial and incubated samples were stored at 4 °C and processed in the laboratory within 72 h of collection. A sample of field-moist forest floor equivalent to 2.5 g dry weight was extracted with 35 mL of 0.5 M K_2SO_4 for 1 h on a shaker table. For mineral soil, all samples were sieved to 2 mm, and a 7-g

field-moist subsample was extracted with 35 mL of 0.5 M K_2SO_4 on a shaker table for 1 h. For both forest floor and mineral samples, the solution was allowed to settle for 40 min before the supernatant was passed through a pre-leached filter (Whatman #20) and frozen until analysis. Masses were corrected for water content using gravimetric percent moisture, obtained by drying 10 g subsamples of either forest floor or mineral soil at 105 °C for 48 h.

Concentrations of extractable inorganic N in forest floor and mineral soil extracts were analyzed colorimetrically using a Lachat QuikChem 8000 flow-injection autoanalyzer (QuikChem Methods 12-107-06-2-E, 12-107-04-1-F, and 12-107-04-1-H, Lachat Instruments, Milwaukee, WI, USA). Standing pools of extractable inorganic N (mg kg^{-1}) in forest floor and mineral soil were calculated by adding the total initial NO_3^- (nitrate) and NH_4^+ (ammonium) concentrations. Net N mineralization (mg kg^{-1}) was calculated by subtracting total inorganic N concentrations in initial extracts from those in incubated extracts, and net nitrification (mg kg^{-1}) was calculated by subtracting initial from incubated NO_3^-. Initial percent NO_3^- was calculated based on the proportion of the total inorganic N that was in the NO_3^- form in the initial samples.

Soil C, N, pH and cations

Soil pH was determined on organic and mineral soil using a 20:1 ratio Nanopure water: soil with a Fisher Scientific Accumet AR20 meter, with an AccuFast pH combination electrode). Forest floor C and N were determined by drying subsamples at 65°C for 48 h, grinding them to fine powder on a roller mill, and analyzing 3 mg via elemental combustion (as above). Soil exchangeable Ca was extracted with 1M ammonium acetate on 0-10 cm mineral soil collected in 2010, and composited by site and by treatment (Mainwaring et al., in review).

Data analysis

For the first objective, to diagnose N or Ca deficiency and responses of foliar nutrients to fertilization between sites, two approaches were taken. First, needle nutrient and weight data were analyzed statistically for the effect of fertilization and site (fixed effects) with each individual tree

as the replicated observational unit. For needle nutrients across both sites, a two-way analysis of variance (ANOVA) was performed on live needle cohorts and senesced needles to determine the effect of site and fertilizer. Because live needle cohorts and senesced needles vary in nutrient concentration, but are not a central question in this study, the fixed effect of needle age was treated as a block (including senescent needles as one needle-age category) to account for the variability associated with needle age when assess needle nutrient response. Needle age was not analyzed as repeated-measures because each nutrient varies in unpredictable ways among needle ages due to differences in phloem mobility of nutrients, and no covariance structure was better than unstructured variance.

Second, vector diagrams were used to graphically examine plant foliar responses to fertilization. These diagrams show changes in nutrient concentrations and content in fertilized plots relative to controls (Timmer and Stone, 1978; Haase and Rose, 1995). In vector analysis, nutrients that are limiting or deficient display simultaneous increases in nutrient concentration, nutrient content and needle weight. Nutrients that are sufficient increase in content, but not in concentration or in needle weight. Toxic elements or nutrients at harmful levels increase in needle concentration, but decrease in content and needle weight. Only first year needles were used to control for variation arising from multiple growing seasons (Bowen, 1985), and data were normalized relative to the control to allow comparison of multiple elements (Haase and Rose, 1995). In order to investigate assays of foliar Ca that could be used as an indicator for nutrient deficiency, we determined if there was an effect of fertilization in two-year old needles in either active (soluble-Ca or structural-Ca) or inactive Ca (Ca-oxalate) in foliage across sites. The effect of fertilizer and site on concentrations of soluble Ca, structural Ca and Ca-oxalate were analyzed in the second needle cohort using a two-way ANOVA.

To meet the second objective, the influence of fertilization on N cycling in soils across sites was examined by performing three types of analyses. First, a two-way ANOVA was used to determine the effect of site and fertilizer treatment on net N mineralization, net nitrification, and soil inorganic N, percent nitrate, pH, C:N, C concentration and N concentration. Because forest

floor and mineral soil vary in N cycling rates, but differences between them are not a central question in this study, the fixed effect of horizon (forest floor or 0-10 cm mineral soil) on net N mineralization (net $NO_3^- + NH_4^+$), and net nitrification (net NO_3^-) was treated as a block to account for the variability associated with horizon when determining response to fertilization. Horizons were not added or averaged together since this may obscure the directionality of responses in N cycling rates, which may be in opposite directions in each horizon, and because of the large difference in magnitude of N cycling rates between horizons. Second, fertilizer treatment or site effects are difficult to detect from changes in soil N concentration, C concentration, and C:N ratios due to sampling and measurement challenges, spatial variability in soil properties, and slow changes in soil properties (Homann et al., 2001, 2008). Therefore, a minimal detectable difference (MDD) was calculated within each soil horizon using power analyses computed separately for each factor (fertilizer and site) of a 2-way ANOVA (Zar, 1999). The MDD is the amount of change in the nutrient (in kg C or N g^{-1} soil) between pre-treatment and sampling, specific to the sites in this study, which is necessary to detect a change in soil properties. Third, to identify the linear effect of various initial (pre-incubation) soil conditions on the response of net N mineralization and net nitrification, these rates were investigated blocked with the fixed effect of horizon to control for the variability associated with horizon differences. Across sites and fertilizer treatments, all factors that may affect N cycling were considered based on previous literature or correlations in the current dataset, and then were eliminated first by meeting the minimum detectable difference criteria (i.e. experienced a change in soil C or N greater than required by the MDD calculation), second by eliminating variables that co-vary in a correlation analysis ($r^2>0.50$), and third by using Bayesian information criterion (BIC) with the lowest BIC value identifying the best fitting model.

Models of needle nutrients and soil N cycling rates were fit to the data using PROC MIXED, correlations of soil factors and N cycling rates were fit to the data using PROC CORR, and models of soil factors as explanatory variables of soil N cycling rates and MDD were fit to the data using PROC GLM, in SAS v9.2 (SAS Institute Inc., Cary, NC). Because data were unbalanced, LSMEANS were used to estimate means and the differences between means.

Statistical significance for all models was at the level of α=0.05 except for multiple contrasts which were adjusted according to Bonferroni correction (Zar, 1999).

Results

For live and senesced foliar N, there was an overall significant interactive effect of site and fertilizer treatment (site*fertilizer interactive effect, two-way ANOVA, DF=117, *P*=0.040; Table 3.2). Live and senesced N concentrations were higher at GDH than at OSU by a mean of 2.0 mg N g^{-1} dry weight (S.E. = 0.02), which varied by fertilizer treatment. At OSU (high-Ca, low-N), across needle age classes, needle N concentrations in the urea treatment were 1.4 – 2.1 mg N g^{-1} greater than control and both Ca treatments (all P < 0.001), with no other significant differences observed (Table 3.2). At GDH (low-Ca, high-N), there was no effect of fertilizer treatment on needle N concentrations. There were no significant interactive effects of fertilizer treatment and needle age on needle N concentrations at either site.

There were no significant interactive effects of site, fertilizer treatment, or needle age on needle Ca concentrations at either site. There was an overall significant effect of site (P<0.001) on needle Ca concentrations (site main effects, DF=137, two-way ANOVA; Table 3.2). Across all age classes and fertilizer treatments, live and senesced foliar Ca concentrations were higher at OSU than at GDH by a mean of 8.1 mg Ca g^{-1} (S.E. = 0.6, *P*<0.001). $CaCl_2$ fertilization significantly reduced live and senesced foliar Ca by an average of 1.3 mg Ca g^{-1} (S.E. = 0.6) relative to controls (*P*=0.037) at both sites. At the high-Ca site (OSU) only, $CaCl_2$ fertilization reduced live and senesced foliar Ca by 2.5 mg Ca g^{-1} relative to controls (S.E.= 0.8, *P*=0.001) and by 1.5 mg Ca g^{-1} relative to lime treatments (S.E.= 0.8, *P*=0.054). The urea treatment reduced live and senesced foliar Ca by 1.5 mg Ca g^{-1} relative to control plots (S.E. = 0.8, *P*=0.055) at the high-Ca site. Foliar Ca concentrations at the low Ca site were not influenced by fertilizer treatments.

For live and senesced foliar weights, there was a significant interactive effect of site and fertilizer treatment (site*fertilizer interactive effect, DF=117, two-way ANOVA, P=0.018). Across all age classes and fertilizer treatments, live and senescent needle weights were higher at

OSU than at GDH by a mean of 0.11 g per 50 needles. OSU had two older needle age classes than GDH, which contributed to overall higher needle weight since needle weight increased with increasing needle age. OSU control needles had a greater weight across all age classes than urea (P=0.032), lime (P=0.020) and $CaCl_2$ (P=0.031) treatments (Table 3.2). GDH had significantly higher specific leaf area (data not displayed, 91.6 cm^2 g^{-1}; S.E. = 1.8) than OSU (82.2 cm^2 g^{-1}; S.E. = 1.2, P<0.001, DF=131, site main effect, two-way ANOVA), although specific leaf area decreased with increasing live needle age and GDH had two fewer live needle age classes than OSU. Across all age classes at the high-N site (GDH), specific leaf area was lower in both urea (mean = 87.9 cm^2 g^{-1}; S.E = 3.3, P=0.018) and $CaCl_2$ fertilized plots (mean = 89.2 cm^2 g^{-1}; S.E. = 3.0, P=0.032) than in control plots (98.0 cm^2 g^{-1}; S.E. = 3.0, P=0.018).

Vector analyses for OSU (high-Ca, low-N) indicated an N deficiency response to added urea, with increased N content and concentration (Figure 3.1). Nitrogen concentration remained the same in lime and $CaCl_2$ treatments, and nutrient content increased in $CaCl_2$ treatments, indicating N sufficiency with these fertilizer additions (Figure 3.1A). Analyses of GDH (low-Ca, high-N) did not indicate N deficiency in any fertilizer treatment, with N concentrations remaining the same despite N content increasing (Figure 3.1B). Both sites displayed sufficiency or dilution of Ca in all treatments (Figure 3.1B).

There was no effect of fertilizer on foliar soluble Ca, structural Ca or Ca-oxalate concentrations at either site (Figure 3.2). In addition, OSU (high-Ca, low-N) and GDH (low-Ca, high-N) had similar foliar concentrations of soluble and structural Ca. However, OSU had 20-60 times higher foliar Ca-oxalate concentrations than GDH (site main effect, two-way ANOVA, DF= 31, P<0.001; Figure 3.2).

Net N mineralization did not differ between the high and low-Ca sites, but differed marginally by treatment (P=0.078) with urea fertilization yielding increased net N mineralization relative to controls (P=0.012, DF=134, two-way ANOVA, Figure 3.3). Forest floor of control plots had essentially no net N mineralization at both sites, and control plots had no difference in

net N mineralization rates between sites (Figure 3.3C & 3D). At OSU urea fertilization resulted in high net N mineralization of the forest floor that exceeded control (P=0.001), lime (P=0.015) and $CaCl_2$ (P=0.047) treatments (Figure 3.3A). Fertilizer treatments had no effect on N mineralization rates at the high-N GDH site (Figure 3.3B and 3D).

Net nitrification displayed significant site*fertilizer interactions (P=0.045, DF=134, two-way ANOVA). At the low-N OSU site, urea increased net nitrification 8-fold relative to controls in the mineral soil (P=0.020, Figure 3.4C), and although not significantly different, urea was the only treatment with detectable nitrification in the forest floor (Figure 3.4A). At the high-N GDH site, forest floor net nitrification in both urea and lime treatments was greater than the control (P<0.01) and $CaCl_2$ treatments (P<0.001, Figure 3.4B), but fertilization did not significantly affect nitrification in the mineral soil (Figure 3.4D).

The minimal detectable difference (MDD) of soil N concentration, C concentration, and C:N exceeded observed differences for these variables in a majority of comparisons within treatments and soil horizons (Table 3.3). For example, the MDD for soil N between treatments was 3.9 kg N g^{-1} (Table 3.3) soil for organic horizon, and at OSU the difference between control and urea was lower (2.5 kg N g^{-1}, Table 3.5) and thus did not meet the criteria for MDD. Therefore, while statistical differences were observed between sites and horizons in soil N concentration, C concentration, and C:N (Table 3.5) they should be treated with caution as they may not be valid over regional spatial variability and long time-periods. However, these factors were included in models evaluating controls on soil net N mineralization and net nitrification, as they may still be informative in understanding soil N cycling rates. There were significant main effects of site (P=0.002) and treatment (P=0.027) on initial (pre-incubation) inorganic-N (initial NH_4^+-N plus initial NO_3^--N) (two-way ANOVA, Table 3.4), with generally higher N in urea fertilized plots. There were significant main effects of site on initial% NO_3^- (two-way ANOVA, P<0.001, Table 3.4), but no clear patterns in treatment effects. There were significant main effects of site and treatment on soil pH (two-way ANOVA, P<0.001, Table 3.4), with lime addition yielding increased soil pH in all measurements except mineral soil of the Ca-rich OSU site.

The candidate variables for predicting net N mineralization were total N concentration, total C concentration, C:N, initial extractable inorganic-N ($NO_3^- + NH_4^+$) (Booth et al., 2005), and soil pH, with site, horizon, and fertilizer treatment included in the model to control for their associated variance. However all these variables either did not meet the criteria for minimal detectable difference (i.e. experienced a change in soil C or N greater than required by the MDD calculation), or produced unsatisfactory models that explained less than 20% of the variance in mineralization rates (BIC, $R^2 < 0.20$).

The candidate variables for predicting net nitrification were net N mineralization rates ($NO_3^- + NH_4^+$ production), total N concentration, C:N, initial extractable inorganic-N ($NO_3^- + NH_4^+$), extractable inorganic-N percent NO_3^- and pH (Booth et al., 2005) with horizon included in the model to control for their associated variance. Candidate variables were reduced by either not meeting the criteria for minimal detectable difference (Table 3.3), or because of covariance. Final variables included net N mineralization, C:N, initial inorganic-N ($NO_3^- + NH_4^+$), initial inorganic-N percent NO_3^-, and soil pH. From these five variables the best one, two or three factor model was selected using BIC criterion. The final model (R^2=0.38, P<0.001) was:

$$\text{Net nitrification} = \beta_0 + \beta_1\text{horizon} + \beta_2\text{soilpH} + \beta_3\text{inorganic N*horizon} + \beta_4\text{netNmin*horizon} + \varepsilon$$

where netNmin is net N mineralization (mg N kg^{-1} soil $month^{-1}$); inorganic N is initial extractable inorganic N ($NO_3^- + NH_4^+$ mg N kg^{-1} soil); soilpH is soil pH; the β are parameters to estimated from the data; and ε is the random error term with ε~(ν0,σ2). Horizons were included in the model to account for the variability associated with each in terms of both a main effect (y-intercept) and interactive effect (slope). Generally, net nitrification increased with decreasing soil pH, and with increasing net N mineralization rates and initial extractable inorganic N (Table 3.4).

Discussion

Foliar N response to N fertilization reflects site fertility

We expected that responses to N fertilization at our sites, selected for contrasting N status, would reflect underlying N fertility. Foliar responses fit our expectations: when N was added at the low-N site (OSU), needle N concentration increased compared to unfertilized needles, and also increased in needle weight in first-year needles indicating limitation using vector analysis. In contrast, at the high-N site (GDH) there was no increase in needle N concentrations or needle weight in response to N addition across age classes indicating sufficiency using vector analysis. The graphical vector analysis approach agreed with the simpler approach of comparing N concentration to critical values. In coastal Pacific Northwest Douglas-fir forests foliar N levels of 14.5 – 15.0 mg N g^{-1} are typically cited as N critical values below which N additions would stimulate growth (Ballard et al., 1986; Hopmans and Chappell, 1994). Our first-year needle cohorts were over the critical value (15.8 mg N g^{-1}) at the high-N site, and below the critical value (14.3 mg N g^{-1}, Table 3.2) at the low-Ca site. Foliar N concentration is generally predicted to increase with increasing N supply (Aber et al., 1989, 1998) along atmospheric N deposition gradients or in response to experimental N additions (Magill et al., 1996, 2004; McNulty et al., 1996). The nonresponsiveness of foliar N concentration to added N is uncommon but has been observed where combined ambient and experimental N deposition is high (NITREX, ~50 kg N ha^{-1} yr^{-1}, (Boxman et al., 1998; Schleppi et al., 1999). In comparison, our sites have low N deposition (~3 kg N ha^{-1} yr^{-1}, Perakis and Sinkhorn, 2011) but relatively high soil N concentration (6 and 2 g N kg^{-1} at the high and low N sites compared to 2 g N kg^{-1} at one of the NITREX sites, (Gundersen et al., 1998). The high soil N concentration at these sites is a legacy of long-term disturbance cycles that promote early-successional N_2-fixing red alder (Perakis et al., 2011), which is capable of fixing 100-200 kg N ha^{-1} yr^{-1} (Binkley et al., 1994). In this study, the lack of foliar N response represents a unique case of sites that may have overcome N-limitation due to non-anthropogenic processes.

Despite the apparent clarity in foliar N diagnosis of responses to N fertilization at the low-N site, it is less clear how well foliar indicators represent actual tree growth response to N fertilization. Typically, trees respond to N fertilizer by increasing aboveground net primary productivity (ANPP) (Gower et al., 1992; LeBauer and Treseder, 2008), which can occur via increases in bole volume (as basal area and/or height) or total foliar mass. However, comprehensive analyses of tree growth response to fertilization at these and other sites found no significant stem volume growth response to added urea at either of these site, and general but non-significant decreases in growth in the urea treatment at the high-N site (GDH) (Mainwaring et al., in review). Increased ANPP implies increases in total foliar mass, which though unmeasured, may have occurred in response to fertilization. Appropriate surrogates for total foliar mass, which were measured include individual needle longevity and needle weight, which influence total tree-level needle biomass. Needle longevity (Mainwaring et al., in review) and needle weight did not respond to fertilizer treatments (Table 3.2), nor did specific leaf-area. Total needle number or mass to determine total canopy biomass, a stand attribute that has been found to respond positively to nitrogen fertilization in Douglas-fir (Brix and Ebell, 1969; Brix, 1981, 1983; Linder and Rook, 1984), was not measured. Typically, an increase in needle numbers was accompanied by other responses such as increases in stem volume growth, basal area increment, tree height growth, or needle width (Brix, 1981, 1983), implying that it is unlikely that needle numbers would increase in response to fertilization without other observable responses. These considerations overall suggest that canopy mass did not increase in response to fertilization.

It is unlikely that lags in growth response will explain the inconsistencies between positive live and senesced N response and negligible growth response at our low-N site. Other Pacific Northwest Douglas-fir forests did not have long time lags in stem volume or foliar responses to N fertilization (Brix, 1983; Edmonds and Hsiang, 1987; Carter et al., 1998). Peak response in needle production, net assimilation and foliar N occurred within 3 to 4 years after fertilization (Brix, 1981, 1983). Others have indicated significant basal area growth response in 3 years (Carter et al., 1998), volume growth response in 4 years (Edmonds and Hsiang, 1987), and cumulative volume and 4-year periodic annual increment in the first 4 years (Sucre et al., 2008)

suggesting that a response to N fertilization should have been detectable in the 3 year time-course of this study. The lack of Douglas-fir stem volume response at either site indicates that N was not a limitation for aboveground productivity (Mainwaring et al., in review). At the high-N site (GDH) the lack of live and senesced foliar N concentration and weight response to N fertilization further confirms that this site is not N limited. Nutrient imbalances have been proposed as causes of decreased Douglas-fir seedling productivity and photosynthetic capacity when foliar N exceeded 13 mg N g^{-1} (Manter et al., 2005). This foliar N concentration level was exceeded at the high-N site (GDH) in all treatments and at the low-N site (OSU) in the urea treatment (Table 3.2). This supports the idea that it is possible for some temperate terrestrial systems, unpolluted by repeated N fertilization or atmospheric deposition, to not be N limited (Perakis and Sinkhorn, 2011).

No live and senesced foliar response to lime and $CaCl_2$ fertilization

Calcium is a likely candidate for nutrient limitation at these sites, due to the overall high N that has been linked to ecosystem Ca loss (Perakis and Sinkhorn, 2011). We expected to observe Ca limitation particularly in the high-N (GDH) site where the first-year foliage contained 1.1 mg Ca g^{-1} before treatment (Mainwaring et al., in review), (well below the critical value for Douglas-fir: 1.5 mg Ca g^{-1} dry-weight, Carter et al., 1992), and potentially low enough to limit growth or reduce Ca-dependent physiological processes including stomatal function, cell division, cell wall synthesis, signaling function and repair of damage from stress and structural chemistry (McLaughlin and Wimmer, 1999; Lautner and Fromm, 2010). However, in the three years since fertilization neither site exhibited treatment-related changes to needle Ca concentrations, and vector analysis indicated Ca sufficiency and dilution (Table 3.2, Figure 3.1). It may be that foliar responses to Ca additions take longer than the 3-year time course of this study. Foliar calcium concentration has been shown to increase in response to Ca-addition within similar time frames: increases in sugar maple foliage Ca were recorded within 2 years of liming (Lea et al., 1980; Juice et al., 2006), in Douglas-fir seedling foliage after 6 months to two-years of liming (Heilman and Ekuan, 1973; Littke and Zabowski, 2007), and in mature Scots pine 4 years after liming (Tveite et al., 1990). However, the length of time necessary for foliar response to fertilization

may be greater than three years for mature Douglas-fir, due to a low calcium requirement relative to other species (Dauer et al., 2007) or due the limited mobility of calcium in plant tissues (Bangerth, 1979; Marschner, 1995; McLaughlin and Wimmer, 1999). Specifically, Ca moves slowly through tree stems, and may be stored in the bole before influencing leaf concentrations (Thomas, 1967; Gülpen et al., 1995; Boucher and Côté, 2002). Therefore, increased Ca uptake at the roots may not directly translate to increased foliar concentration or content if Ca^{2+} ions must first fill exchange sites in the xylem through the length of the stem (Bell and Biddulph, 1963; Ferguson and Bollard, 1976; Boucher and Côté, 2002). Most nutrient diagnosis techniques assume that under nutrient limitation there is a direct relationship between uptake of the limiting nutrient and plant growth (Ingestad and Agren, 1991; Haase and Rose, 1995). However, nutrients like Ca are less directly related to growth, making them less well suited to foliar diagnosis (Thelin et al., 1999). We expect that foliar Ca concentrations in both lime and $CaCl_2$ fertilized plots may increase in the future, although the potential lag in response highlights the difficulty in diagnosing tree response to Ca fertilizer treatments and the inadequacy of methods to detect tree deficiency.

We expected to be able to detect increases in physiologically active pools of Ca in foliage after fertilization. However, no response was found in different chemical pools of Ca; we observed no effect of Ca fertilization on extractable fractions of foliar Ca, soluble and structural Ca (physiologically active), and Ca-oxalate (physiologically inactive; Figure 3.2). Physiologically active chemical pools of Ca in tissues can be informative for interpreting Ca limitation (DeHayes et al., 1997; Borer et al., 2004; Littke and Zabowski, 2007). In our study, the combined physiologically active pools (soluble-Ca plus structural-Ca) measured at either site in 2 year-old, post-treatment needles exceeded the reported critical value (1.5 mg Ca^{-1}; Carter, 1992) and remained constant across sites and treatments (GDH mean 1.8 mg Ca g^{-1}, S.E. = 0.04; OSU mean 1.9 mg Ca g^{-1}, S.E. = 0.05; Figure 3.4). Deficiency may occur when physiologically active pools of Ca fall below critical levels. This may be the case in first-year needles at our sites where total Ca ranged from 1.6 to 0.9 mg Ca g^{-1} across fertilizer treatments in the low-Ca site (Table 3.2), although individual pools of Ca were not measured. Shifts in critical physiologically active Ca are likely overlooked with measurements of total Ca (DeHayes et al., 1997; Borer et al., 2004). For

example, at our sites, the differences in total Ca were due entirely to the 20- to 60-fold differences of the inert Ca-oxalate pool (Figure 3.2). Low Ca concentration in physiologically active pools may result in limited ability to use stored Ca to buffer growing foliar Ca needs especially if soil Ca-supply is low. Measuring multiple foliar Ca pools may allow greater detection of minor changes in physiological important Ca.

In addition to a lack of foliar Ca response, tree growth responses to both Ca fertilizers at both sites were not strong, with no significant increases over the control (Mainwaring et al., in review). However, average growth responses to lime addition were positive across the region (Table 3.6), and the comprehensive analysis of the entire fertilization study found significant growth response to Ca fertilizers in 2 out of 14 sites (Mainwaring et al., in review). These growth responses justify continued exploration of Ca additions to improve productivity in these high N forest sites. As has been speculated with foliar nutrient concentrations, growth responses to Ca may occur over longer time periods in mature conifer trees. Typically mature trees do not exhibit tree growth responses until 5-9 years after fertilization with lime and $CaCl_2$ (Tveite et al., 1990; Long et al., 1997; Juice et al., 2006). Mechanisms that contribute to Ca growth response on a longer time scale may include resistance to disease, stomatal regulation and cold tolerance (McLaughlin and Wimmer, 1999; Lautner and Fromm, 2010). It is possible that at both sites we could eventually observe substantial foliar and tree-growth responses, and some growth response in 3 years (Mainwaring et al., in review) suggests that time lags in Ca fertilization may be resolved in the near future.

Soil N cycling in fertilized soils

Additions of N to soil are expected to increase N mineralization in ecosystems thought to be N limited (Aber et al., 1993, 1998; Nave et al., 2009), so it was expected that at least the lower N-status site would respond to N-addition with increased soil N mineralization. This expectation was met only in the forest floor of the low-N site where net N mineralization was accelerated after N addition and exceeded all other soils whether fertilized or not (Figure 3.3). The likely conclusion is that the low-N site (OSU) had N limitation in the forest floor. In mineral soil at both

sites, there were no significant responses in N mineralization to N fertilization; in fact, adding N to high N mineral soils had a slightly negative effect on N mineralization rates. This type of negative response occurs in other high N temperate forests (Berg and Matzner, 1997; Aber et al., 1998; Corre et al., 2003), and the mechanism for N-additions dampening N mineralization has been attributed to stabilization of soil organic matter (Neff et al., 2002; Swanston et al., 2004), or to the suppression of extracellular enzymes that mineralize organic matter (Allison and Vitousek, 2005). Incubation of soil from similar young coastal Douglas-fir sites that had repeated N-fertilization had decreased cumulative respiration, suggesting increases in organic matter stability (Swanston et al., 2004). Likewise, Douglas-fir forests in Oregon and Washington had no sustained increases in forest floor mineralization after repeated N-fertilization on both N-rich and N-poor sites (Chappell et al., 1999) and decreases in potential N mineralization in the A-horizon after repeated N-fertilization (Fox, 2004). This suggests that it is not easy to predict whether N fertilization will result in an increase in soil N supply based on either soil N capital or soil N concentration, and that enhanced availability of soil inorganic N supply may not be sustained for several years after fertilization, particularly where soil N is high. Regardless, it appears that high N sites are not good candidates for N fertilization, given the lack of additional soil N mineralization and tree growth following fertilization (Mainwaring et al., in review).

Soil N capital has been suggested as a controlling factor of N cycling across a wide number of ecosystems (Binkley and Hart, 1989; Hart et al., 1997; Perakis and Sinkhorn, 2011). Therefore it was expected that the high-N site would generally have higher net N mineralization rates across fertilizer treatments. However, in contrast to these expectations, there was no overall statistical difference between sites in N mineralization rates. This was surprising considering that other sites in the Pacific Northwest coastal region have strong relationships between N-mineralization and site N-capital. For example, there was a 100-fold difference in forest floor N mineralization when conducted by laboratory incubation along a 4-fold N capital gradient (Chappell et al., 1999), and mineral soil yielded 10-fold differences in annual in-situ N mineralization among sites with a 2-fold difference in soil N concentration (Perakis and Sinkhorn, 2011). The lack of a strong site influence on how N mineralization responded to N fertilization

may be due to short sampling periods in our study. Because N mineralization was measured for only one month, more extreme values from inter-annual variability may not have been captured. In 2009, both sites were slightly warmer (+ 1.0 °C at both sites) but also drier (-5.4 cm at GDH and -1.5 cm at OSU) than the mean June temperature and precipitation between 1971 and 2000 (Daly et al., 1994), which could have increased and suppressed, respectively, net N mineralization rates.

In this study, significant increases in pH with lime addition in the forest floor (Table 3.4) did not influence net N mineralization rates, despite this being a common finding (Yavitt and Newton, 1990; Duggin et al., 1991; Nohrstedt, 2002). Increases in pH with lime addition could directly influence the microbial community or alter organic matter, also making it more susceptible to mineralization (Andersson et al., 2000; Andersson and Nilsson, 2001). Mechanisms by which Ca would directly stimulate N mineralization are less clear, as Ca is typically thought to reduce N mineralization rates through the stabilization of organic matter via cation bridging (Muneer and Oades, 1989; Chan and Heenan, 1999). In this study, $CaCl_2$ addition also did not influence N mineralization, in agreement with other studies of non-lime Ca additions (Groffman et al., 2006), suggesting that lime may have stronger influences on soil N cycling than Ca-addition alone.

In addition to soil N capital as a predictor of N fertilization response, soil pH (Smallidge et al., 1993) or soil C:N (Smallidge et al., 1993; Gundersen et al., 1998; Booth et al., 2005) are potential indicators or mediators of N mineralization response to fertilization. Nearby Douglas-fir sites along a native soil N gradient exhibited net N mineralization decreases with increasing C:N in the forest floor (Prescott et al., 2000). We explored these two soil factors, along with total soil N concentrations, as explanations for differences in N mineralization across all fertilization treatments and sites. Differences in soil total N and C concentrations and C:N between sites and fertilizers should be treated with caution as they were lower than the difference required to meet a minimal detectable difference (Table 3.3). However, these measurements may be useful when compared to other soil measurements such as net N mineralization. None of the soil factors, i.e.

soil N status (either as initial inorganic-N concentration or total N concentration), C:N and pH, were significant factors in a model explaining net N mineralization rates at these sites. The fact that no measured soil factors explained net N mineralization, suggests static site characteristics like soil texture, soil moisture, and site history may control soil N cycling at these sites (Prescott, 1995; Chappell et al., 1999). Moreover, prediction of net N mineralization as a response to N-addition may be difficult given the limited response to N-fertilization or Ca-fertilization observed across sites and treatments.

Nitrification rates were higher overall in the high-N site (GDH), and also were influenced by fertilizer treatment in ways that varied between each site (Figure 3.4). Unfertilized mineral soil displayed 8-times higher NO_3 production in the high-N (GDH) than low-N site (OSU) despite only a 2-fold difference in net N mineralization, suggesting an overall stronger potential for nitrification and possibly nitrate leaching loss at the high-N site. The addition of urea fertilizer increased nitrification at both sites, by 10-fold relative to controls in forest floor at the high-N site (Figure 3.4B) and 7-fold relative to controls in the mineral soil at the low-N site (Figure 3.4D). Surprisingly, the mineral soil did not increase in nitrification at the high-N site, highlighting the complexity of predicting nitrification responses to urea.

Nitrate production at both sites was influenced when Ca was added as lime, but less so when Ca was added as $CaCl_2$. Nitrification increased in all limed treatments, except for the low-N site mineral soil (Table 3.4). An increase in nitrification with liming of acid soils has been observed widely (De Boer and Kowalchuk, 2001; Yao et al., 2011), and is likely due to changes in pH-sensitive soil communities (Zar, 1999; Baath and Anderson, 2003; Rousk et al., 2010). The forest floor at the high-N site had especially amplified nitrification due to lime addition, i.e. 11-fold greater nitrification as compared to the control, whereas in contrast, the response to $CaCl_2$ was minimal. Because we observed significant increases in pH with added lime but no changes in pH with added $CaCl_2$ (Table 3.4), we concluded that the response of increased nitrification following lime treatment was likely an indication of sensitivity to pH rather than increased Ca ions.

Controls on autotrophic nitrification are relatively well studied, and nitrification is expected to increase with increased pH (Persson and Wirén, 1995; De Boer and Kowalchuk, 2001) and the availability of NH_4^+, the initial substrate for nitrification (Vitousek et al., 1982; Avrahami et al., 2002). Likewise, it was found that pH, net N mineralization rates, and initial extractable inorganic N were most important in explaining nitrification across sites (R^2=0.39, P<0.001). Interestingly, despite an increase in nitrate production with lime fertilization in forest floor of the high-N site, we found that across both sites soil pH had an overall negative relationship with net nitrification. This was consistent with previous findings at sites in the Oregon Coast Ranges (Perakis and Sinkhorn, 2011), which became increasingly acidified and had high NO_3 production along a gradient of increasing soil mineralization. This result contrasts with long-standing ideas that nitrifiers are stimulated at higher or moderate pH (Tietema et al., 1992; Persson and Wirén, 1995; Ste-Marie and Paré, 1999), but supports a more recent finding that important nitrifying communities are acid tolerant archaebacteria (Leininger et al., 2006; Francis et al., 2007) and that their abundance makes *in situ* nitrification less predictable than previous studies suggest. It is possible that factors like pH control nitrification only when N availability is high. At our low-N site, we found a positive relationship between inorganic N and nitrification, suggesting substrate limitation of autotrophic nitrification. In contrast, both urea addition and liming increased nitrification in forest floor at the high-N site, suggesting positive effects of both N supply and pH on nitrification where N is relatively abundant. Although lime is frequently used to ameliorate acid soils, especially in areas with soil acidification due to acid deposition and N pollution, nutrient management in limed forests must be carefully considered. Fertilization with lime may also influence N cycling, affecting long-term site fertility and soil nutrient losses. In N-rich sites, fertilization with $CaCl_2$ may be an alternative to lime in providing supplemental Ca without stimulating nitrification. Nevertheless, more studies are necessary to fully elucidate long-term N and Ca relationships.

The long-term balance of soil nutrients after fertilization is unknown, but could be influenced by several mechanisms of coupled Ca and N uptake or leaching loss. At the high-N

site, trees do not appear to be increasing their N uptake, based on a lack of increase in foliar N concentration, and despite an increase in potential available inorganic N supply with N fertilization (Table 3.2 and Figure 3.3B & 3.4B). It is therefore possible that the additional nitrification from fertilization at these sites would result in a net loss of NO_3^- from soils. Nitrate leaching is especially problematic because it may contribute to further calcium depletion of soils (Perakis et al., 2006), which already are low in base cation concentration. Interestingly, liming increased total standing inorganic N pools in the forest floor at the high-N site, suggesting increases in long-term N mineralization and/or less N immobilization may increase N supply to trees. Because of the favorable electrochemical balance between cation and anion during mass flow delivery (Marschner et al., 1991), increased supply of soil nitrate may influence tree Ca accretion as the uptake of nitrate (relative to ammonium) enhances the uptake of Ca (Macklon and Sim, 1980; Jentschke et al., 2001). But it is unknown if additional nitrate from nitrification will stimulate Ca uptake in the long-term in areas with low Ca in tree biomass. Nitrogen fertilization remains a common practice across the region, and the idea of interplantings with N_2-fixing red alder is often discussed, both with the intention to increase forest productivity (Chappell et al., 1991; Sucre et al., 2008; Footen et al., 2009), but it is possible that some forest soils would equally benefit from added lime, either alone or in conjunction with urea, as long as nitrification rates are better understood and managed.

Conclusions

Understanding of N and Ca fertilization effects on N-rich sites needs to be improved to sustain productivity in the north-central Oregon Coast Range. Additions of N are highly common to increase forest productivity, however, the influence of N additions on long-term nutrient balances is not well understood for high-N sites. The processes underlying the interaction of nutrients in both unfertilized and fertilized soil systems need to be better identified across multiple ecosystem and soil types. Additions of Ca may be necessary to alleviate nutrient imbalances in high-N sites or in conjunction with N fertilization, and may be successful at increasing tree growth in the long-term. Nutrient deficiency diagnoses using tree foliage has been

applied to N and Ca, but they could be broadened to predict long-term productivity under both common N-based fertilization and Ca-fertilization or blended fertilization. Calcium nutrient deficiency may not be immediately related to foliar growth or to bole growth, especially compared to nutrients like N on which foliar diagnosis techniques are based. Evaluation of Ca-status and response to fertilization may require novel approaches relating health and productivity to a non-growth measurement. Because these forests are intensively managed, nutrient balances over the long-term must be carefully considered in order to maintain sustainable soil nutrient systems.

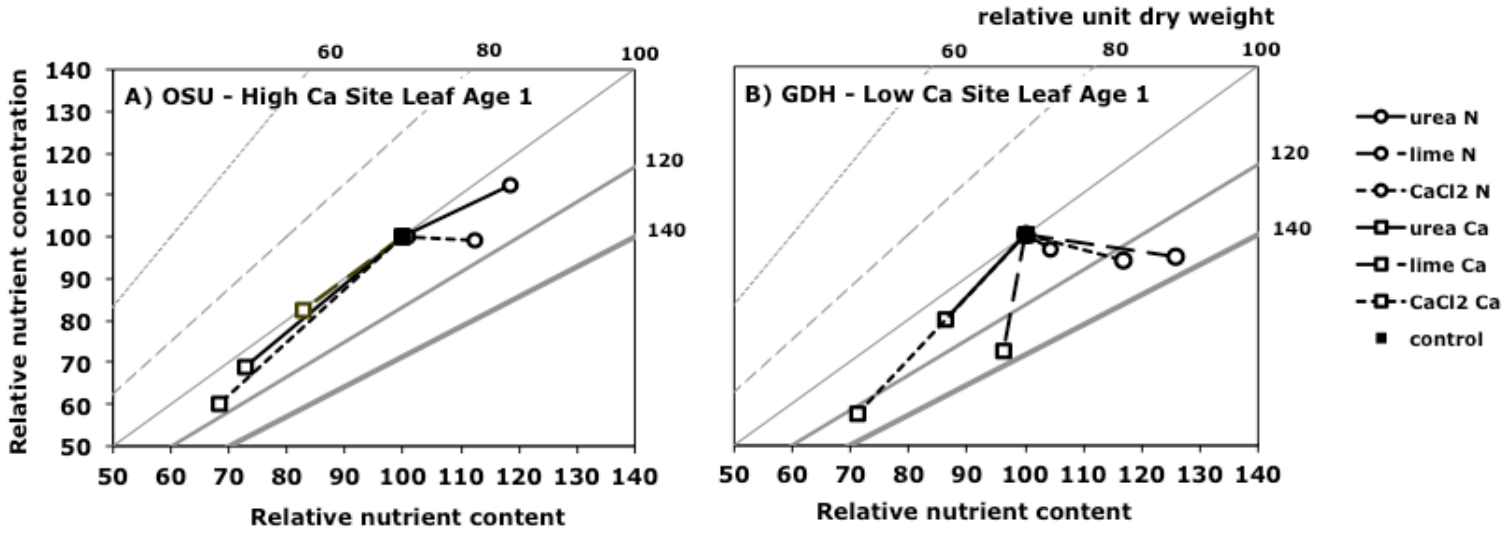

Figure 3.1. A) OSU high-Ca site and B) GDH low-Ca site vector diagram of foliar nutrients N and Ca for first year age class difference between control and treatment fertilized with urea, lime and $CaCl_2$. Units are displayed relative to the control treatment, which is at 100% of nutrient concentration, nutrient content and unit dry weight.

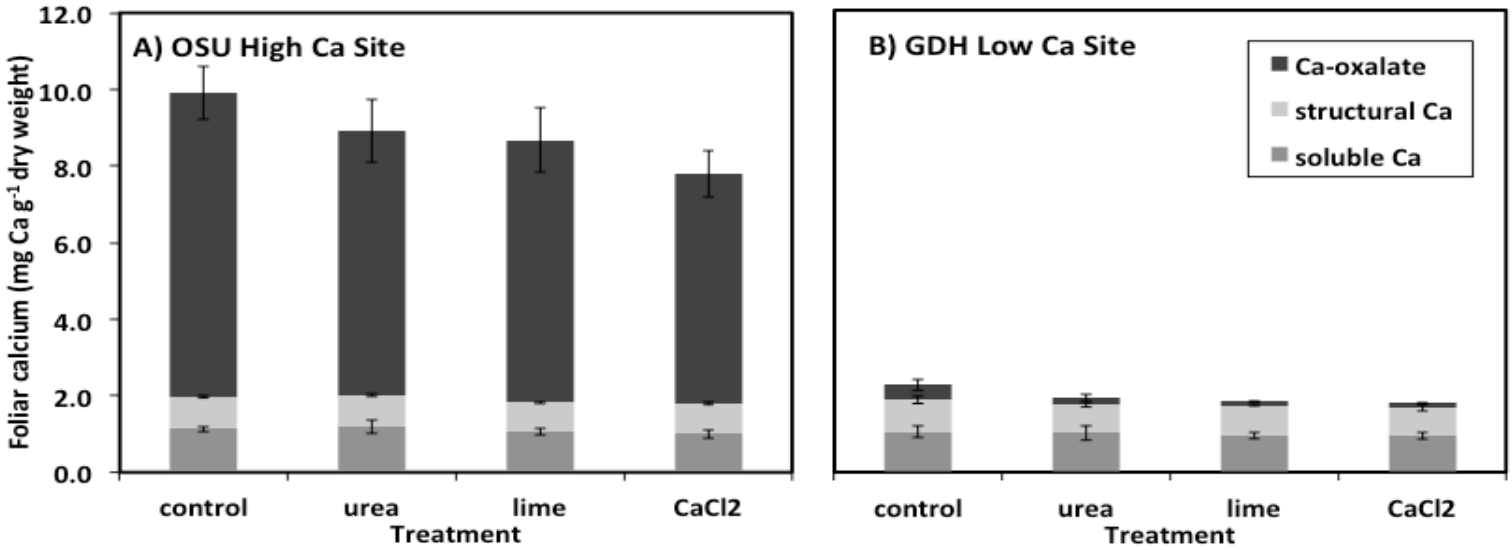

Figure 3.2. Mean soluble Ca, structural Ca and Ca-oxalate (water, acetic acid and HCl extractable, respectively) in second-year foliage at A) OSU low N/high Ca site and at B) GDH high C/low Ca site three years after fertilization with urea, lime and $CaCl_2$. Error bars represent standard error.

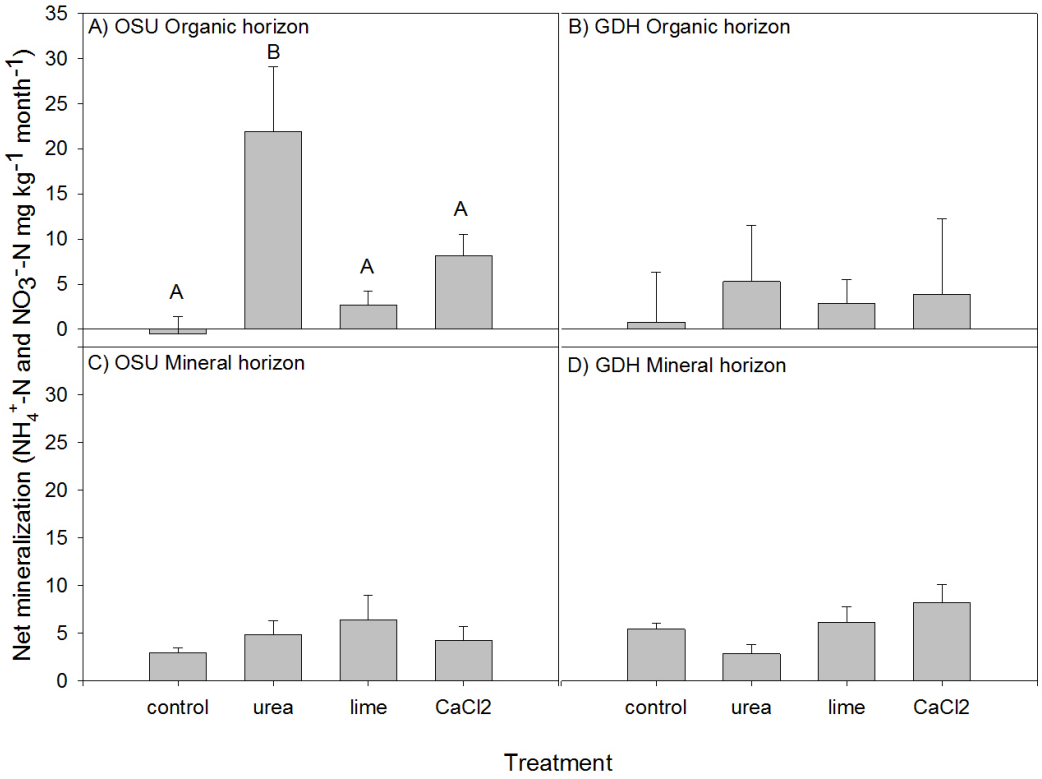

Figure 3.3. Mean net N mineralization in organic (A&B) and 0-10 cm mineral (C&D) soil horizons in control and fertilized plots at OSU, low N/high Ca site (A&C) and at GDH, high N/low Ca site (B&D) during field incubation June 2009. Upper-case letters indicate significant differences (two-way ANOVA, $P<0.05$) among treatments within site and horizon. Error bars represent standard error.

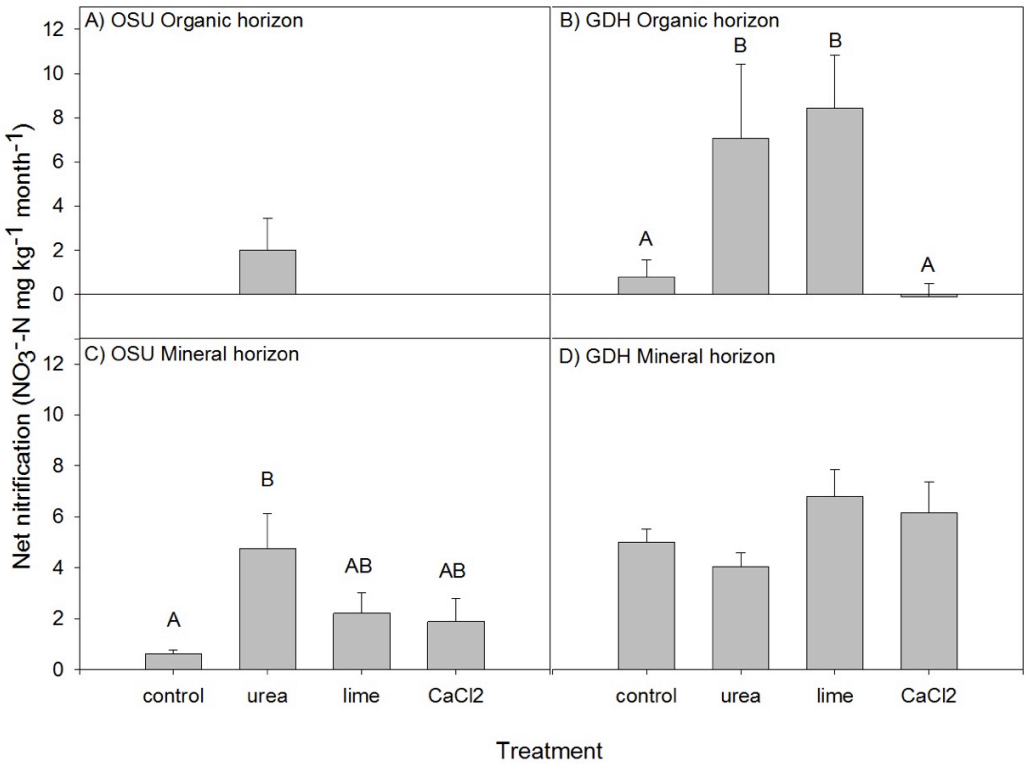

Figure 3.4. Mean net nitrification in organic (A&B) and mineral (C&D) horizons at OSU, low N/high Ca site (A&C) and at GDH, high N/low Ca site (B&D) in fertilized plots during field incubation June 2009. Upper-case letters indicate significant differences (two-way ANOVA, $P<0.05$) among treatments within site and horizon. Error bars represent standard error.

Table 3.1. Average stand, tree and soil attributes for the two sites before treatment (2006) from Mainwaring et al (in review). Chemistry for first-year foliage and mineral soil characteristics (0-10 cm) is a mean of 5 composited samples, standard error in parenthesis. Soil Ca:N is expressed as g Ca kg^{-1} exchangeable Ca by mass relative to total soil g N kg^{-1} by mass.

	High-Ca, Low-N OSU Site	**Low-Ca, High N GDH Site**
Plot attributes		
Site Index (m @ 50 years)	46.9	41.3
Douglas-fir stem density (trees ha^{-1})	819	724
Douglas-fir basal area (m^2 ha^{-1})	32.6	32.6
Tree Attributes		
Foliar Ca (mg Ca g^{-1} tissue dry weight)	6.3 (0.2)	1.7 (0.1)
Foliar N (mg N g^{-1} tissue dry weight)	12.8 (0.2)	14.9 (0.3)
Foliar Ca:N	0.49	0.11
Quadratic mean DBH (cm)	25.9	29.2
Height (m)	18.0	21.1
Crown Ratio	0.67	0.64
Foliar retention (years)	3.31	1.62
Age at breast height (years)	14.8	19.8
Soil attributes		
pH (H_2O)	6.29 (0.08)	4.68 (0.03)
Ca (g Ca kg^{-1} soil)	2.68 (0.19)	0.11 (<0.01)
C (g C kg^{-1} soil)	30.8 (0.8)	114.6 (3.8)
N (g N kg^{-1} soil)	2.0 (<0.1)	6.0 (0.2)
C:N	15.2 (0.2)	19.2 (0.2)
Ca:N	1.32	0.02

Table 3.2. Live and senesced foliar calcium and nitrogen concentrations (mg g^{-1} dry weight) and leaf weight (g per 50 needles dry weight at OSU (high Ca, low N) and GDH (low Ca, high N) sites in the Coast Range of Oregon after three years of fertilization by urea, lime and $CaCl_2$. Foliage data are reported as means by annual cohort for live needles and senesced needles. There was an effect of site*fertilization (P=0.040) on N concentration; of site (P<0.001) on Ca concentration; and of site*fertilization (P=0.003) on needle weight (two-way ANOVA). Upper-case letters represent differences across fertilizer treatments within site (two-way ANOVA, P<0.05). Standard errors in parentheses.

	Nitrogen mg N g^{-1} dry weight				Calcium mg Ca g^{-1} dry weight				Needle Weight g per 50 needles dry weight			
Signficant effect:	*site*fertilizer*				*site*				*site*fertilizer*			
Treat-ment:	**Control**	**Urea**	**Lime**	**$CaCl_2$**	**Control**	**Urea**	**Lime**	**$CaCl_2$**	**Control**	**Urea**	**Lime**	**$CaCl_2$**
OSU Site	**B**	**A**	**B**	**B**	**A**	**BC**	**AB**	**C**	**A**	**B**	**B**	**B**
1 yr	14.3 (0.3)	16.0 (0.7)	14.3 (0.7)	14.1 (0.4)	9.0 (1.3)	6.2 (0.4)	7.4 (0.7)	5.4 (0.4)	0.17 (0.02)	0.18 (0.02)	0.17 (0.02)	0.20 (0.01)
2 yr	14.4 (0.4)	14.9 (0.1)	14.3 (0.6)	13.1 (0.3)	12.0 (0.9)	9.4 (0.9)	9.7 (1.1)	7.7 (0.7)	0.25 (0.03)	0.23 (0.02)	0.24 (0.02)	0.24 (0.01)
3 yr	13.1 (0.2)	14.8 (0.5)	13.1 (0.7)	12.3 (0.5)	12.0 (0.8)	11.0 (1.4)	10.7 (1.4)	8.0 (1.9)	0.32 (0.03)	0.28 (0.02)	0.28 (0.02)	0.28 (0.02)
4 yr	11.7 (0.4)	12.8 (0.6)	11.5 (0.5)	9.8 (n=1)	10.4 (0.6)	11.0 (1.8)	9.8 (1.0)	7.3 (0.4)	0.58 (0.04)	0.46 (0.03)	0.46 (0.02)	0.40 (0.01)
senesced needles	6.9 (0.2)	9.3 (0.7)	7.0 (0.3)	7.0 (0.3)	16.5 (2.1)	13.7 (2.2)	15.3 (2.3)	15.9 (2.1)	n.d	n.d.	n.d.	n.d.
GDH Site												
1 yr	15.8 (1.0)	15.3 (0.6)	15.0 (0.6)	14.9 (0.6)	1.6 (0.3)	1.3 (0.1)	1.2 (0.1)	0.9 (0.1)	0.14 (0.01)	0.15 (0.03)	0.18 (0.03)	0.17 (0.01)
2 yr	15.8 (0.3)	17.1 (1.1)	15.5 (0.8)	15.3 (0.5)	2.0 (0.4)	1.6 (0.5)	1.6 (0.1)	1.5 (0.2)	0.18 (0.01)	0.18 (0.01)	0.20 (0.02)	0.22 (0.02)
senesced needles	11.6 (0.4)	11.8 (0.6)	10.7 (0.2)	11.9 (0.1)	3.8 (0.9)	4.2 (0.4)	4.0 (0.4)	4.5 (0.3)	0.14 (0.01)	0.17 (0.01)	0.16 (0.01)	0.15 (0.01)

Table 3.3. Minimal detectable difference in units of soil nitrogen (kg N g^{-1} soil), carbon (kg C g^{-1} soil) and C:N ratio for organic and mineral horizons by comparisons between sites and between treatments. The MDD is the amount of change in the nutrient (in kg C or N g^{-1} soil) between pre-treatment and sampling, specific to the sites in this study, which is necessary to detect a change in soil properties.

	Minimum Detectable Difference		
	Nitrogen (kg N g^{-1} soil)	**Carbon (kg C g^{-1} soil)**	**Carbon:Nitrogen**
Organic horizon			
Site	6.6	80.8	9.9
Treatment	3.9	83.3	3.6
Mineral horizon			
Site	7.8	167.7	9.4
Treatment	0.5	83.3	0.9

Table 3.4. Parameter estimates for model explaining net N nitrification rates (mg N kg^{-1} soil $month^{-1}$) in June 2009 across two sites and four fertilization treatments. This model (R^2=0.38) was selected using BIC criterion.

parameter	**Estimate**	**Error**	**P-value**
Intercept	6.66	2.98	0.027
horizon	0.56	1.19	0.638
soilpH	-1.22	0.47	0.010
inorganic N *horizon (organic)	0.12	0.20	<0.001
inorganic N *horizon (mineral)	0.37	0.21	0.087
netNmin*horizon (organic)	0.11	0.03	<0.001
netNmin*horizon (mineral)	0.42	0.09	<0.001

Table 3.5. Post-fertilization (2009) of soil inorganic N (NH_4^+ and NO_3^-), percent of extractable inorganic N that exists as NO_3^-, pH, C:N, C concentration and N concentration of organic horizon and 0-10 cm mineral soil for control, urea, lime and $CaCl_2$ plots. Differences in lower-case letters indicate treatments significantly different from one another within sites and horizons (means comparisons). Standard error in parentheses.

		site	fertilizer	site*fertilizer	OSU High Ca Site		GDH Low Ca Site	
Inorganic N (mg kg-^{1})		*P*=0.002	*P*=0.027		**organic**	**mineral**	**organic**	**mineral**
	control				15.3 (2.5)ab	1.9 (0.2)	20.9 (5.4)b	4.0 (0.2)
	urea				23.2 (3.7)a	3.6 (0.3)	29.9 (6.1)ab	6.1 (1.2)
	lime				16.4 (2.0)ab	2.9 (0.3)	35.5 (12.0)a	5.9 (1.0)
	$CaCl_2$				9.1 (0.8)b	2.7 (0.3)	18.8 (3.9)b	3.3 (0.3)
% NO_3		*P*<0.001						
	control				0.0 (0.0)	1.5 (1.0)ab	4.3 (4.3)	31.1 (4.5)
	urea				5.1 (4.2)	10.6 (2.6)a	5.3 (1.9)	25.9 (5.1)
	lime				0.0 (0.0)	1.6 (1.1)ab	10.3 (3.2)	30.2 (6.8)
	$CaCl_2$				0.0 (0.0)	0.0 (0.0)b	6.2 (4.7)	25.7 (3.5)
pH (H_2O)		*P*<0.001	*P*<0.001					
	control				6.28 (0.12)c	6.61 (0.08)a	5.54 (0.12)b	4.83 (0.05)ab
	urea				6.53 (0.07)b	6.28 (0.08)b	5.56 (0.10)b	4.69 (0.06)b
	lime				6.79 (0.06)a	6.67 (0.04)a	6.25 (0.10)a	5.04 (0.10)a
	$CaCl_2$				6.28 (0.08)c	6.62 (0.20)a	5.61 (0.13)b	4.84 (0.06)ab
C:N				*P*=0.028				
	control				36.4 (1.1)a	13.1 (0.2)	30.2 (0.9)	18.3 (0.5)
	urea				32.1 (0.9)b	13.7 (0.4)	29.4 (0.7)	19.5 (0.7)
	lime				35.5 (0.8)a	14.4 (0.5)	31.1 (0.8)	19.0 (0.6)
	$CaCl_2$				35.9 (1.1)a	15.0 (0.7)	29.0 (1.1)	18.3 (0.6)
C concentration (kg C g^{-1})		*P*=0.003	*P*<0.001					
	control				433 (20)a	27.7 (1.3)	472 (20)ab	109 (4.2)
	urea				459 (11)a	31.6 (2.1)	491 (5)a	131 (8.0)
	lime				385 (23)b	31.9 (2.0)	453 (9)b	114 (8.4)
	$CaCl_2$				416 (15)b	35.9 (2.5)	449 (20)b	113 (5.0)
N concentration (kg N g^{-1})		*P*<0.001	*P*<0.001					
	control				12.0 (0.7)a	2.1 (0.1)	15.5 (0.4)a	5.9 (0.2)
	urea				14.5 (0.6)b	2.3 (0.1)	16.8 (0.4)b	6.7 (0.2)
	lime				10.9 (0.7)a	2.2 (0.1)	14.6 (0.4)a	6.0 (0.4)
	$CaCl_2$				11.7 (0.5)a	2.4 (0.1)	15.5 (0.6)a	6.2 (0.3)

Table 3.6. Volume growth response, basal area growth response and height growth response (%; 100*[treatment –control]/control) of measurement trees to fertilizer treatments. Treatments were not statistically different from the control. Reproduced from Mainwaring et al. in review.

		OSU High Ca Site	GDH Low Ca Site
Volume growth response (%)			
	Urea	0.1	-11.3
	Lime	6.8	2.4
	$CaCl_2$	7.4	0.5
Basal area growth response (%)			
	Urea	4.8	-5.4
	Lime	11.9	4.0
	$CaCl_2$	10.5	6.4
Height growth response (%)			
	Urea	-3.8	-16.0
	Lime	0.4	4.2
	$CaCl_2$	1.2	-7.9

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CHAPTER 4 – ROLE OF CA-OX IN CONTROLLING CA/SR DISCRIMINATION AND ^{44}CA/^{40}CA FRACTIONATION

Abstract

Cation tracers such as calcium and strontium (Ca/Sr) ratios and Ca-isotopes (^{44}Ca/^{40}Ca) are gaining interest as tools to identify sources and pathways of Ca cycling in ecosystem studies. The mechanisms that influence these elemental and stable isotope ratios however remain poorly resolved during cycling through ecosystem pools and especially among different soluble, structural and crystalline forms of cations. We synthesized Ca-oxalate (Ca-ox), a ubiquitous biomineral found in terrestrial ecosystems, and determined that crystal formation exhibited preference for Ca over Sr with a discrimination factor of 5.4, and for ^{40}Ca over ^{44}Ca with a^{44}Ca-enrichment factor of -1.46‰ for the precipitation of Ca-ox. Inorganic synthesis of Ca-ox may be comparable to *in situ* Ca/Sr bulk values in plants, but less so for ^{44}Ca/^{40}Ca due to complex physiological processes during Ca-ox crystal formation in plants. At high and low-Ca status sites with differences in Ca-ox accumulation in Douglas-fir tissues in the Coast Range of Oregon we investigated plant pools and soils to compare whole-plant level, organ-level and tissue-level Ca/Sr discrimination and ^{44}Ca/^{40}Ca separation. Tissue-level Ca/Sr discrimination factors were higher in magnitude than whole-plant or plant organ-level discrimination at both sites, primarily due to Ca-ox accumulation. There was a positive linear relationship between Ca-ox concentrations and Ca/Sr values (R^2 = 0.55) across all tree tissue types and sites indicating that Ca-ox accumulation can have a significant impact on bulk tree tissue Ca/Sr values. Organ-level ^{44}Ca/^{40}Ca separation factors were equal or greater in magnitude than whole-plant and plant tissue-level separation factors, indicating that movement of Ca between plant organs on xylem exchange sites is likely more important than Ca-ox formation in shaping bulk ^{44}Ca/^{40}Ca values. Using a multi-tracer approach, the ecosystem pools at two sites were comparable in directionality on bivariate plots of Ca/Sr and ^{44}Ca/^{40}Ca suggesting consistent patterns within this species across sites with different Ca-status. Continued mechanistic understanding of discrimination and fractionation is necessary in order to use Ca/Sr and ^{44}Ca/^{40}Ca as tracers of cations in terrestrial ecosystems.

Introduction

Calcium (Ca) affects a wide range of ecosystem processes and functions (McLaughlin and Wimmer, 1999) including buffering the effects of acid deposition (Johnson et al., 1991), stability and structural integrity of trees (Lautner and Fromm, 2010), plant cellular signaling in response to environmental stimuli (McAinsh and Schroeder, 2009) and water flow regulation (Gilliham et al., 2011). There has been significant progress in identifying sources and pathways of Ca cycling using cation tracers such as Ca and strontium (Sr) ratios (Blum et al., 2000; Drouet et al., 2005) and Ca isotopes (Holmden and Bélanger, 2010), but the mechanisms that influence these elemental and stable isotope ratios during cycling among different soluble, structural and crystalline forms and ecosystem pools remain poorly resolved.

Ca-oxalate (Ca-ox) is an insoluble crystal that is ubiquitous in the biosphere and accumulates in tissues of plants (Hudgins et al., 2003; Franceschi and Nakata, 2005) and fungi (Cromack et al., 1979; Connolly and Jellison, 1995; Dutton and Evans, 1996; Tait et al., 1999), as a way to detoxify cytoplasmic Ca and as a defense from herbivory (Franceschi and Nakata, 2005). Calcium-oxalate can be found in many different tissue types (Franceschi and Horner, 1980) in all major groups of photosynthetic organisms, including angiosperms and gymnosperm tree species. Soils may also accumulate Ca-ox (Fox and Comerford, 1990; Lilieholm et al., 1992; Certini et al., 2000), which precipitates as a byproduct of oxalic acid secretion on or near fungal hyphae or tree roots (Connolly and Jellison, 1995; Dutton and Evans, 1996; Gadd, 1999; Casarin et al., 2003). Indeed, Ca-ox accounts for up to 20% of total ecosystem Ca in Douglas-fir forests of Oregon, and up to 70% of total Ca in older leaf tissues (Dauer, Chapter 2), yet despite this importance Ca-ox is rarely considered as a potential factor shaping Ca and Sr behavior, as well as Ca isotope fractionation, in terrestrial ecosystems.

Ratios of Ca/Sr are used to distinguish among various sources of Ca in plant and soil systems since both are alkaline earth elements with similar chemical and biological properties (Blum et al., 2000; Pett-Ridge et al., 2009; Nezat et al., 2010). However, use of Ca/Sr to trace Ca sources remains problematic because of the preferential uptake and translocation of Ca over Sr by plants (Poszwa et al., 2000; Dasch et al., 2006; Drouet and Herbauts, 2008), which may arise because of mass differences in Ca versus Sr (Ca atomic weight: 40.1, Sr atomic weight: 87.6) or biological selectivity for essential Ca over non-essential Sr.

Calcium/strontium discrimination may occur during the passage of cations through the endoderm, which separates conducting vessels from leaf mesophyll (Gierth et al., 1998; Drouet and Herbauts, 2008), via preferential leaching of Sr from leaves relative to Ca (Poszwa et al., 2000) or via ionic exchange as cations ascend in the xylem (Blum et al., 2012). Empirical evidence shows that Ca versus Sr discrimination occurs primarily between the stem and foliage of trees on a plant organ level rather than between soil and roots on a whole-plant level, and is more influenced by species than by different soil types or cation supply, allowing calculation of a species-specific discrimination factor (Dasch et al., 2006; Blum et al., 2008, 2012; Drouet and Herbauts, 2008). Another possible mechanism for Ca/Sr discrimination could be preferential precipitation of Ca over Sr during crystal formation with oxalate, although this possibility is yet unstudied. Strontium competes with Ca for precipitation with oxalate anions in both biogenic and aqueous reactions (Franceschi and Schueren, 1986; Singer et al., 2008), and incorporation of Sr in Ca-ox crystals has been observed in plants (Franceschi and Schueren, 1986; Storey and Leigh, 2004) and fungi (Sayer et al., 1997; Connolly et al., 1999). The accretion of Ca-ox crystals may dominate over Sr-oxalate, as Ca is a smaller molecule (1 Å) compared to Sr (1.3 Å) which binds more strongly to oxalate, and is less soluble than Sr-oxalate (Benefield and Morgan, 1990; Connolly et al., 1999). An ultimate result may be less Sr incorporation in plant crystal idioblasts and more susceptibility for Sr-oxalate dissolution and loss from needle tissues or from soils. Unknowns include the degree to which Ca-ox formation discriminates for Ca, how Ca-ox concentrations in plants and soil influence bulk Ca/Sr values, and on what scale (whole-plant, organs, or tissues) potential discrimination could occur.

Naturally occurring stable Ca isotopes ($^{44}Ca/^{40}Ca$) have the potential to provide information on process controlling Ca sources and dynamics in terrestrial ecosystems (Bullen and Bailey, 2005; Wiegand et al., 2005; Page et al., 2008). Ca isotopes are beginning to be used in terrestrial ecosystem Ca studies (Perakis et al., 2006; Cenki-Tok et al., 2009; Holmden and Bélanger, 2010), building on information derived from marine ecosystems on the factors controlling Ca isotope fractionation (De La Rocha and DePaolo, 2000; Sime et al., 2007; Eisenhauer et al., 2009). Vegetation is thought to drive terrestrial patterns of Ca isotopes as evidenced by a span of ~ 4‰ fractionation in forest ecosystems (Wiegand et al., 2005; Holmden and Bélanger, 2006; Page et al., 2008), which is much larger than the ~ 1 ‰

fractionation effects associated with abiotic and biotic precipitation of Ca-bearing minerals (Skulan et al., 1997; Fantle and DePaolo, 2005; Gussone et al., 2005). There is limited information about processes controlling Ca isotope fractionation in plants, though patterns across forest ecosystems are consistent across all studies (von Blanckenburg et al., 2009; Nielsen et al., 2011) indicating that likely sources of Ca isotopic fractionation in trees are at an whole-plant level during Ca uptake at the root favoring light ^{40}Ca, and on a plant-organ level during kinetic ion-exchange reactions within cell walls of the conducting xylem sapwood favoring heavy ^{44}Ca moving in the transpiration stream (Schmitt et al., 2003; Wiegand et al., 2005; Page et al., 2008; Cenki-Tok et al., 2009; Holmden and Bélanger, 2010). Experiments using pea plants in hydroponics confirm fractionation in these processes, both driven by physio-chemical cation-exchange processes with pectins on cell walls (Russell and Papanastassiou, 1978; Cobert et al., 2011). Pea leaf Ca-ox was ^{44}Ca depleted compared to bulk Ca (Cobert et al., 2011), although possible fractionating mechanisms were not explored further. This process may be similar to Ca-isotope fractionation that occurs in marine organisms during the biomineralization of Ca-carbonate, which preferentially precipitates light ^{40}Ca (Lemarchand et al., 2004; Gussone et al., 2006; Griffith et al., 2008). It is unknown whether other mechanisms for fractionation, like Ca-ox biomineralization in plant tissues, may shape ^{44}Ca/^{40}Ca patterns along the soil-to-plant continuum, or whether these patterns exist only on plant tissue-scale. In soils, Ca-ox accumulation may also influence ^{44}Ca/^{40}Ca patterns. Both soil exchangeable-Ca and acid leaches of soil (Ca-ox) show depletion in δ^{44}Ca relative to HF-digests (residual soil) at the soil surface (Perakis et al., 2006). If the formation of Ca-ox is a fractionating process that prefers ^{40}Ca, then these data are evidence of cycling of Ca through a Ca-ox soil fraction. Information on fractionation associated with Ca-ox formation and dynamics, therefore, has potential to greatly resolve interpretation of ^{44}Ca/^{40}Ca variations in plants and soils.

The objectives of this study were three-fold; first we synthesized Ca-ox in the laboratory to determine the degree of Ca/Sr discrimination and ^{44}Ca/^{40}Ca fractionation during oxalate crystal formation. Secondly, from contrasting Ca-rich and Ca-poor forest sites we examined Ca/Sr and ^{44}Ca/^{40}Ca of needle, bole-wood and bark of Douglas-fir tissues in bulk and physiological fractions of Ca (soluble-Ca, structural-Ca and Ca-oxalate), bulk values in twig xylem sap, and in soil exchangeable and leachable-Ca fractions to determine Ca/Sr

discrimination and $^{44}Ca/^{40}Ca$ fractionation at whole-plant, plant-organ and tissue levels. Thirdly, we used Ca/Sr and $^{44}Ca/^{40}Ca$ together in a multi-tracer approach to determine if we could identify sources of Ca for plant uptake by soil depth.

Methods

Fractionation in Ca-ox formation

We synthesized calcium oxalate under controlled laboratory conditions to examine the kinetics of Ca/Sr and $^{44}Ca/^{40}Ca$ fractionation. Calcium chloride and sodium oxalate solutions were mixed at 23°C to yield 10 mL of solution with a final concentration of 1mM of both calcium and oxalate ion. To achieve this concentration, 8 mL of Nanopure water and 1mL 10 mM calcium chloride were added to a serum vial and shaken. At the start of the precipitation reaction, 1 mL of 10 mM sodium oxalate was added to the serum vial and shaken, then rested unstirred until filtering. Samples were poured into a syringe, filtered with 13 mm Whatman Nucelopore polycarbonate Track-etch membranes (0.8 μm), to separate solid Ca-ox crystals, and syringe filters were rinsed 2 times with 3 mL of Nanopure water. After the initial sampling at t = 0 minutes, repeat sampling occurred at 5 min, 10 min, 20 min, 40 min, 60 min, 120 min, 180 min, 1440 min, and 2880 min (a total of 10 samples). Filters were cut in half for chemical and isotopic analysis, and for crystal inspection. Solutions were acidified with 0.5 mL 12 N HCl. Both the solutions and half-filters were analyzed at U. S. Geological Survey in Menlo Park, CA for Ca and strontium (Sr) by inductively-coupled plasma optical emission spectrometer (ICP), Perkin Elmer Optima 3000DV (Norwalk, CT, USA), and for $^{44}Ca/^{40}Ca$ isotopes using a thermal-ionization mass spectrophotometer (TIMS) and a "double spike" mass spectrometry approach (Perakis et al. 2006, Page et al. 2008). Scanning transmission electron microscope (STEM) images of the remaining half filters were taken to track crystal formation using a Philips CM-12 microscope.

Ca/Sr ratios and Ca natural abundance stable isotopes at contrasting sites

We evaluated plant and soil patterns in Ca/Sr and $^{44}Ca/^{40}Ca$ in two 25-year-old Douglas-fir forests in 2 sites of contrasting Ca availability in the Oregon Coast Range. These sites were part of a larger study of nine Douglas-fir dominated sites along a nutrient gradient

in the north-central Oregon Coast Range described by (Perakis and Sinkhorn, 2011). These two sites are located at 44° 38'N, 123° 48'W (high-Ca site, Site 5) and 45° 10'N, 123° 55'W (low-Ca site, Site 16). Soils at both sites are classified as Andic Dystrudepts (http://soils.usda.gov, Benton and Tillamook County Soil Surveys), developed from sedimentary parent material from uplifted ocean floor during the tertiary period (Orr et al., 1992). Both sites experience maritime, temperate climates with cool, wet winters and warm, dry summers. The average annual rainfall is 173 and 196 cm yr^{-1}, and mean annual temperature was 11.4 and 11.2°C at the high-Ca and low-Ca site respectively (Daly et al., 1994).

We collected multiple foliar age classes and dead needles as needle litterfall to represent a time continuum of needle aging. At each site, in 2007, sun needles and needle-litterfall were collected from three trees at the end of the growing season (Sept/Oct). Live foliage was separated into age classes (5 age classes at the high-Ca site and 4 age classes at the low-Ca site), and needle-litterfall was collected without age-class separation. In May 2010 three trees from each site were cored twice with an increment borer and separated into bark and bole-wood (sap-wood only) tissues. Four additional bark samples were taken with a 2 cm diameter x 2 cm deep corer. To separately analyze phloem chemistry, the inner-bark with moist, light-colored tissues approximately 2 mm deep was removed from the rest of the bark. Samples from each site were composited into a single sample per site of inner-bark, outer-bark and bole-wood. Live and dead needles, inner-bark, outer-bark and bole-wood samples were flash frozen in an 80°C cooler and subsequently freeze-dried (Virtis 35L Genesis Super XL, SP Industries, Warminster, PA, USA). Sap was collected at two time points by removing a large branch from three trees at each site in February 2010 then again in June 2010. Several small twigs, <4 mm diameter, from each of the three larger branches were placed in a pressure bomb (PMS Instrument Company, Albany OR, USA) and twig xylem sap was extracted for less than 5 minutes for each twig and composited until at least 3 ml were obtained from each site. Pressure was applied with increasing increments up to 3.5 MPa, and no higher than 1 MPa above the point at which twig xylem sap appeared on the cut end of each stem (Dambrine et al., 1995).

To determine the total Ca concentrations in tissues of live needles, dead needles, outer-bark, inner-bark and bole-wood samples, we combusted samples at 500° C and acid digested them with 12 N HCl to measure total Ca concentrations. To determine the Ca-ox in plant tissues, we measured a subsample of tissue and performed a sequential chemical extraction technique (Fink 1991a) that separates plant tissue Ca into 3 fractions: (1) water soluble Ca^{+2} ions, (2) acetic acid soluble, structurally bound Ca-pectin and Ca-lignin, and (3) HCl soluble Ca-ox. Calcium concentrations were measured using atomic absorption spectrophotometry on an AAnalyst 200 Spectrometer, Perkin Elmer (Waltham, MA, USA).

Soils from each site were collected in June 2010 by removing the forest floor layer, sampling 0-10 cm deep with a 5.1 cm diameter soil corer, followed by 10-20 cm and 70-100 cm deep with an 4 cm diameter slide hammer. Soils were sieved to 2 mm to remove rocks and debris and to homogenize the soil, composited by site, and air-dried at room temperature. These soils received a sequential treatment, yielding exchangeable and leachable fractions (Bullen and Bailey, 2005; Perakis et al., 2006). The exchangeable fraction was assayed by equilibrating 5 g of soil with 50 mL of 1 N NH4OAc for 24 h. The leachable fraction was assayed by thoroughly rinsing 1 g of soil remaining from the exchange procedure with de-ionized water and combining with 10 mL of 1 N HNO3 for 24 h at 30° C. Total Ca and Sr was determined on whole rock digests for soil depths 0-10 cm and 70-100 cm only via lithium metaborate/tetraborate fusion and dilute nitric digestion, with analysis by inductively coupled plasma spectroscopy, at Acme Labs, Vancouver BC.

On each plant tissue total digest and sequential extraction sample, xylem sap sample and soil exchangeable and leachable sample stable isotope $^{44}Ca/^{40}Ca$ were analyzed using TIMS and Ca/Sr ratios were analyzed using ICP with the methods previously described. Due to cost associated with measurements of $\delta^{44}Ca$, there was limited replication of samples, including measurements of only two needle age-classes, and patterns in data are not evaluated statistically. The same dried tissue samples were run in duplicate for Ca by sequential extraction and digest Ca and analyzed using atomic absorption spectrophotometry. The difference between duplicate extractions of a single tissue was a mean of 2.3 ± 2.4%. Duplicates of 12 samples of extraction fluid samples were analyzed with TIMS for $^{44}Ca/^{40}Ca$. Difference in $^{44}Ca/^{40}Ca$ between duplicates of samples all agreed within 0.18‰ (mean

difference = 0.10 ± 0.06‰ SD), consistent with the reproducibility of 0.15‰ performed in this laboratory in previous Ca isotope studies (T. Bullen, personal communication). Calcium and strontium measurement on ICP reproducibility of a single sample was typically within 2% (T. Bullen, personal communication).

Isotope and statistical calculations

The Ca/Sr discrimination for the creation of Ca-ox in the solution experiment was calculated as a discrimination factor (DF) where values > 1 indicate preferential precipitation of Ca-ox over Sr-oxalate (Dasch et al., 2006; Drouet and Herbauts, 2008):

$$DF_{Ca-ox} = \frac{Ca / Sr_{solid}}{Ca / Sr_{aq}} .$$

Discrimination in Ca-ox versus Sr-oxalate precipitation was also assessed using a partition coefficient (K_d), as commonly applied in the geochemical literature (Gabitov and Watson, 2006):

$$K_{dCa-ox} = \frac{Sr / Ca_{solid}}{Sr / Ca_{aq}} ,$$

where the discrimination factor and the partition coefficient are related in that $1/DF = K_d$.

Calcium $^{44}Ca/^{40}Ca$ isotope ratios are presented using the $\delta^{44}Ca$ notation calculated as:

$$‰\delta^{44}\text{Ca} = \frac{\text{sample}^{44}\text{Ca}/^{40}\text{Ca} - \text{standard}^{44}\text{Ca}/^{40}\text{Ca}}{\text{standard}^{44}\text{Ca}/^{40}\text{Ca}} \text{ x } 1{,}000 ,$$

where the standard (sea water) $^{44}Ca/^{40}Ca$ ratio was 0.021713 on this mass spectrometer (Page et al., 2008).

The inorganic synthesis of Ca-ox was assumed to exhibit Rayleigh type behavior (Balci et al., 2006), which can be described by the following natural log function:

$$\delta^{44}Ca_{aq} = \varepsilon \ln (f),$$

where $\delta^{44}Ca_{aq}$ is the isotopic composition of the remaining aqueous Ca at a given time during the reaction and *f* is the proportion of Ca_{aq} remaining after a given time. Epsilon (ε) is the isotopic enrichment factor that is described by (ε) = 1000*(α-1), where α =

$(^{44}Ca/^{40}Ca_{solid})/(^{44}Ca/^{40}Ca_{aq})$. If the precipitation reaction follows Rayleigh behavior, a plot of $\delta^{44}Ca_{aq}$ versus ln (*f*) will yield a straight line, the slope of which is equal to ε (Mariotti et al., 1981).

To estimate the preference for Ca versus Sr during plant uptake, movement and deposition we estimated Ca/Sr discrimination factors (DF) where a DF > 1 indicates preferential uptake, movement and deposition for Ca over Sr in a flow-path and a DF < 1 indicates preference for Sr over Ca (Dasch et al., 2006). To preference for ^{40}Ca over ^{44}Ca along a flow-path, we estimated $^{44}Ca/^{40}Ca$ separation factors (SF) where a positive SF indicates preferential uptake, movement and deposition of ^{44}Ca (heavy isotope) over ^{40}Ca (light isotope) and a negative SF means the preference for ^{40}Ca over ^{44}Ca (Holmden and Bélanger, 2010). In order to compare overall foliage we used the averaged of the total digest Ca/Sr values across live needle age classes (high-Ca site age class 1 through 5, low-Ca site age class 1 through 4) and the mean $\delta^{44}Ca$ across all the live needle age classes (high-Ca site age class 1 and 5 only, low-Ca site age class 1 and 4 only). In order to compare overall soil we used concentration-weighted soil values for forest floor and mineral soil (0-10 cm deep). For Ca/Sr values we calculated for each soil depth:

$$(Ca_{exch} + Ca_{leach}) / (Sr_{exch} + Sr_{leach}),$$

where Ca_{exch} is the concentration of exchangeable Ca and Ca_{leach} is the concentration of leachable Ca (respectively) at a given soil depth, and Sr_{exch} is the concentration of exchangeable Sr and Sr_{leach} is the concentration of leachable Sr (respectively) at a given soil depth. For $\delta^{44}Ca$ values we calculated for each depth:

$$[(\delta^{44}Ca_{exch} * Ca_{exch}) + (\delta^{44}Ca_{leach} * Ca_{leach})] / (Ca_{exch} + Ca_{leach}),$$

where $\delta^{44}Ca_{exch}$ is the exchangeable and $\delta^{44}Ca_{leach}$ is the leachable extraction Ca-isotope value (respectively) at a given soil depth, and Ca_{exch} is the exchangeable Ca concentration and Ca_{leach} is the leachable Ca concentration (respectively) at a given soil depth. Discrimination factors of foliar Ca-ox were calculated by division of sinks of cations by sources of cations, for example, forest floor source cations compared to foliage sinks of cations were calculated as:

$$DF_{forestfloor->foliage} = \frac{Ca / Sr_{meanfoliage}}{Ca / Sr_{forestfloor}},$$

where $Ca/Sr_{meanfoliage}$ = average Ca/Sr of all needle age classes, and $Ca/Sr_{forestfloor}$ = Ca/Sr of the forest floor weighted by exchangeable and leachable cation concentration. Separation factors

were calculated by subtraction between sources of Ca and sinks of Ca, for example between mineral soil (0-10 cm depth) sources and foliage sinks were calculated as:

$$SF_{0\text{-}10\text{mineral soil}\rightarrow\text{meanfoliage}} = \delta^{44}Ca_{\text{meanfoliage}} - \delta^{44}Ca_{0\text{-}10\text{mineralsoil}},$$

where $\delta^{44}Ca_{\text{meanfoliage}}$ = average $\delta^{44}Ca$ of all needle age classes, and $\delta^{44}Ca_{0\text{-}10\text{mineralsoil}}$ = $\delta^{44}Ca$ of the 0-10 cm depth soil weighted by exchangeable and leachable Ca concentration.

We estimated DFs and SFs along several different flow-paths that varied in cation source and sink compartments in order to contrast whole-plant, organ-level and tissue-level discrimination. Whole-plant level discrimination was considered to be any flow path between soil cation sources and plant compartments, for example between forest floor and needles (Dasch et al., 2006; Blum et al., 2008). Plant organ-level discrimination was considered to be any flow path between plant compartments, for example between bole-wood and needles. Plant tissue-level discrimination was estimated using chemical sequential extraction fractions of cations for all plant tissues, and we considered soluble-Ca as the source of cations for each tissue, and structural-Ca and Ca-oxalate fractions as sinks of cations. Inner- and outer-bark were similar in Ca/Sr and $^{44}Ca/^{40}Ca$ so only outer-bark DFs and SFs were calculated.

To use both Ca/Sr and $^{44}Ca/^{40}Ca$ in a multi-tracer approach, we plotted Ca/Sr and $^{44}Ca/^{40}Ca$ on the same graph. Areas where soil and plant compartments intersect suggest a source of cations for that plant compartment (Blum et al., 2002; Bullen and Bailey, 2005). All regression statistics were conducted using the GLM procedure in SAS 9.2 statistical software (SAS Institute Inc., Cary, NC). We used linear regression to examine the relationship of Ca concentrations with Ca-ox concentrations, needle age, Ca/Sr and $^{44}Ca/^{40}Ca$.

Results

Laboratory synthesis of calcium oxalate

The STEM images showed formation of both mono-hydrate and di-hydrate Ca-ox crystals throughout the time course of the experiment (Figure 4.1). The majority of crystals formed (70 – 98%) were mono-hydrate, which precipitated in monoclinic prismatic, agglomerate or hexagonal forms. The remaining Ca-ox dihydrate crystals formed tetragonal

bipyramidal crystals (weddellite). The relative percentage of each crystal type did not change over the time course of the experiment (Figure 4.2).

Calcium oxalate formation in our experiment exhibited discrimination with respect to both Ca/Sr and $^{44}Ca/^{40}Ca$. Calcium was removed from the aqueous phase and incorporated into crystals at a faster rate than Sr, and likewise ^{40}Ca was incorporated into crystals at a faster rate than ^{44}Ca (Figure 4.3). The precipitation reaction followed a two-stage 2nd order model with first a rapid crystallization phase, and second, a slow dissolution-reprecipitation phase that exhibited less Ca/Sr discrimination and $^{44}Ca/^{40}Ca$ fractionation. Two Ca/Sr discrimination factors were calculated for both phases of the reaction; the stage of rapid crystallization yielded DF = 5.4 (by mass), and the phase of dissolution-reprecipitation yielded DF = 14.7 (by mass) (Figure 4.3A). The isotopic enrichment factor of Ca-ox was calculated based on the rapid-crystallization phase of the experiment as ε = -1.46‰ (α= 0.99854) (Figure 4.3B). Calculations were based on a regression through all points between f = 1 and f = 0.25 when the first stage of rapid crystallization occurred and describe a strong linear relationship between $\delta^{44}Ca_{aq}$ and ln (f) (R^2= 0.96, P<0.001, Figure 4.3B). The slope was used to define a value for ε of -1.46‰ (-1.82 to -1.10‰ at the 95% confidence interval), which could be interpreted to reflect an apparent Rayleigh fractionation associated with the precipitation of Ca-ox. As the experiment proceeded, ε and α decreased substantially (to ~ 0) during the slower dissolution and precipitation phase (f = 0.26 to f = 0.05).

Ca concentrations in tree and soil fractions

The plant tissue with the highest total digest Ca concentrations was needle age class 4 and 5 at the high-Ca site and dead needles both sites (Table 4.2). Overall, the high-Ca site had 2-fold to 4-fold higher concentrations of total digest Ca as compared to the low-Ca site among all plant tissues, except outer-bark which had similar Ca-concentrations between sites (Table 4.2). Within live needles only, Ca-ox accounted for 43 – 52% of total digest Ca at the high-Ca site and 10 – 30% of total digest Ca at the low-Ca site. The high-Ca site had a 2- to 10-fold higher Ca-ox than the low-Ca site (Table 4.2). Bole-wood, inner-bark and outer-bark tissues were low in total digest Ca and Ca-ox concentration compared to foliar tissues within both sites (Table 4.2). Across all plant tissue types, Ca-ox was an average of 40% of the total digest

Ca at the high-Ca site, but only an average of 23% of the total digest Ca at the low-Ca site. Across both sites within all plant tissues Ca-ox concentrations increased with total digest Ca concentrations ($R^2 = 0.99$, $P < 0.001$). Xylem sap Ca concentration was higher in the winter than in the early summer and slightly more concentrated at the low-Ca site compared to the high-Ca site (high-Ca site: 14.1 mg L^{-1} in February and 9.2 mg L^{-1} in June; low-Ca site: 17.2 mg L^{-1} in February and 10.0 mg L^{-1} in June). The sums of the three sequential extractions of Ca were compared to total digest Ca to determine overall extraction efficiency. The extraction of Ca was a mean of 85 (± 8% SD) complete in high-Ca site live and dead foliage, whereas the low-Ca site was 86% (± 4% SD) complete. In bole (wood, inner-bark, outer-bark) tissues, the sums of extractions were a mean of 103% (± 12% SD) and 97% (± 3% SD) complete at the high and low-Ca site, respectively.

Total extractable soil Ca concentrations in the sum of NH4OAc exchangeable (i.e., electrostatically bound) and HNO_3-leachable (i.e., Ca-ox bound) fractions decreased with depth (Table 4.2). The forest floor had at least twice as much extractable-Ca (exchangeable and leachable-Ca combined) as the 0-10 cm deep mineral soil at each site. The high-Ca site had more than twice as much extractable soil Ca than the low-Ca site in the forest floor, and site differences increased in lower depths to up to 10 times more extractable soil Ca in the high-Ca site. At both sites, Ca in the soil HNO_3-leachable fraction dominated the Ca concentration of forest floors. At the high-Ca site, leachable-Ca was 3-times higher than exchangeable-Ca in the forest floor, but concentrations of leachable Ca were similar (0-10 and 10-20 cm depth soils), or lower (deep 70-100 cm soils) than the exchangeable-Ca fraction. In comparison, at the low-Ca site, leachable-Ca was 4-times higher than exchangeable-Ca in the forest floor and remained higher (0-10 and 10-20 cm) or equal (70-100 cm) in lower soil depths.

Ca/Sr and Ca isotopes in tree foliar and bole tissues and in xylem sap

For all plant tissues we compared Ca/Sr values and $^{44}Ca/^{40}Ca$ values between concentration-weighted mass balance totals of the three extractable cation fractions with an independent digest total sample as a control. Unity between mass balance and digest total Ca/Sr or $^{44}Ca/^{40}Ca$ values would indicate that sequential extractions did not miss a substantial

fraction of Ca (or Sr), that concentrations of Ca (and Sr) were accurately partitioned between sequential extractions and that Ca/Sr or $^{44}Ca/^{40}Ca$ values correctly reflect the extracted fraction. We found reasonable agreement between mass balance totals and independent digest totals of Ca/Sr, which varied by a mean absolute value of 22 (± 17 SD) within tree tissues across both sites (Table 4.3). We found good agreement between mass balance totals and independent digest totals of $^{44}Ca/^{40}Ca$ values within tree tissues across both sites, which varied by a mean absolute value of 0.08‰ (± 0.08 SD), lower than our estimate of reproducibility of the analysis (~ 0.18‰) except for outer-bark at the high-Ca site (-0.22‰, Table 4.3).

In foliage, Ca/Sr of the total digest at the high-Ca site generally increased with needle age from first year needles (Ca/Sr = 146) to 5 year-old needles (Ca/Sr = 232) and dead needles (Ca/Sr = 237; mean Ca/Sr = 189 ± 37 SD across all live age classes, Figure 4.4A). The low-Ca site total digest foliage displayed slight decreases from first year needles (Ca/Sr = 163) to 4 year-old needles (Ca/Sr = 153) and dead needles (Ca/Sr = 120, mean Ca/Sr = 164 ± 8 SD across all live age classes, Figure 4.4B). In the high-Ca site, the Ca-ox extraction showed a marked pattern of increasing Ca/Sr from 232 to 523 with live leaf age, whereas the soluble-Ca and structural-Ca extractions were less variable, (mean soluble-Ca: Ca/Sr = 99 ± 6.8 SD; mean structural-Ca: Ca/Sr = 100 ± 22 SD) across all live age classes (Figure 4.4A). The Ca-oxalate fraction in the low-Ca site also increased with each live age class, from 54 to 206, which was a lower magnitude of change in comparison to the high-Ca site (Figure 4.4B). The Ca/Sr of both the soluble-Ca and structural-Ca fractions at the low-Ca site had the opposite pattern of decreasing from first year age class (soluble-Ca: Ca/Sr = 107; structural-Ca: Ca/Sr =159) to the 4th year age class (soluble-Ca: Ca/Sr = 249; structural-Ca: Ca/Sr = 308, Figure 4.4B). Leaf life span was estimated to be 2.7 years at the high-Ca site, and 1.5 years at the low-Ca site. In both sites, the Ca/Sr mass balance of the dead needles was lower than the oldest age class, and may reflect a mixing of multiple aged needles post-senescence.

Comparison of two $^{44}Ca/^{40}Ca$ values that were greater than the reproducibility of our analysis (~ 0.18‰) were considered large enough to be differences. In foliage, both the high and low-Ca sites displayed similar total digest Ca-isotope values among live needle-age class in older needles (high-Ca site, 5th year needles: $\delta^{44}Ca$= -1.01‰; low-Ca site, 4th year needles: -$\delta^{44}Ca$= 1.15‰) but ^{44}Ca enriched 1st year needles at the high-Ca site compared to the low-Ca

site (high-Ca site: δ^{44}Ca= -0.88‰; low Ca site: δ^{44}Ca= -1.20‰; Table 4.3, Figure 4.5). The Ca-ox fraction was the most ^{44}Ca-depleted at both the high-Ca site (age-class 1: δ^{44}Ca= -1.36‰; age-class 5: δ^{44}Ca= -1.06‰) and low-Ca site (age-class 1: δ^{44}Ca= -1.50‰; age-class 4: δ^{44}Ca= -1.52‰; Figure 4.5). Also, the soluble-Ca fraction was the most ^{44}Ca-enriched at both the high-Ca site (age-class 1: δ^{44}Ca= -0.55‰; age-class 5: δ^{44}Ca= -0.74‰) and low-Ca site (age-class 1: δ^{44}Ca= -0.81‰; age-class 4: δ^{44}Ca= -0.81‰; Figure 4.5)

Bole-wood tissues at both sites displayed lower total digest Ca/Sr (high-Ca site: Ca/Sr = 73, low-Ca site: Ca/Sr = 94) than inner-bark (high-Ca site: Ca/Sr = 136, low-Ca site: Ca/Sr = 152) and outer-bark tissues (high-Ca site: Ca/Sr =166, low-Ca site: Ca/Sr = 158, Figure 4.4). Ca/Sr sequential extraction patterns were similar between inner-bark and outer-bark whereas bole-wood values had more separation between soluble-Ca and the other fractions. Soluble-Ca fractions in bole-wood tissues had high Ca/Sr values at both sites compared to other Ca fractions (high-Ca site: Ca/Sr = 282, low-Ca site: Ca/Sr = 388, Figure 4.4). Soluble-Ca fractions in bark tissues also generally had higher Ca/Sr values than other fractions (mean inner- and outer-bark, high-Ca site: Ca/Sr = 256 ± 37 SD; low-Ca site: Ca/Sr = 334 ± 37 SD, Figure 4.4). Ca-ox fractions in bark tissues were lower than other Ca-fractions at the low-Ca site (low-Ca site mean inner- and outer-bark, Ca/Sr = 99 ±15 SD, Figure 4.4B). This was opposite from the general trend of the foliage where Ca-ox Ca/Sr values were Ca-enriched compared to other Ca fractions, and soluble Ca fractions were among the most Ca-depleted. In foliar and bole tissues across both sites, there was a positive relationship between Ca-ox concentrations and total digest Ca/Sr ratios (R^2 = 0.55, P<0.001, Figure 4.6A).

Bark tissues were the most ^{44}Ca-depleted of the plant tissue Ca-fractions at both sites in total digest Ca (inner-bark high-Ca site: δ^{44}Ca = -2.63‰; inner-bark low-Ca site: δ^{44}Ca = -2.95‰; outer-bark high-Ca site: δ^{44}Ca = -2.67‰; outer-bark low-Ca site: δ^{44}Ca = -2.90‰; Figure 4.5). There was small separation between inner-bark and outer-bark tissues sequential extraction Ca-isotope values at both sites, though Ca-ox was among more ^{44}Ca-depeted Ca-fractions at the high-Ca site (Figure 4.5A). Bole-wood tissues were ^{44}Ca-enriched in comparison to inner and outer-bark, and had minor separation between sequential extraction Ca fractions except for ^{44}Ca-enriched Ca-ox at the high-Ca site (δ^{44}Ca = -1.01‰; Figure

4.5A). Across all foliar and bole tree tissues, there was a positive yet non-significant relationship between Ca-ox concentrations and $^{44}Ca/^{40}Ca$ (R^2=0.08; *P*=0.22; Figure 4.5B).

In order to trace cations along a flow path from soils to foliage, we measured Ca/Sr in twig xylem sap, and to investigate seasonal differences in needle cation sources we measured during the winter (February) and during bud burst (June). Ca/Sr in xylem sap at the high-Ca site (Ca/Sr_{Feb} = 88, Ca/Sr_{June} = 122) had values similar to mineral soil (0-10 cm deep leachable: Ca/Sr = 81, 0-10 cm deep exchangeable: Ca/Sr = 91). In comparison, xylem sap was Ca-depleted compared to 1st age class total digest foliar values (Ca/Sr =146). However at the low-Ca site, the xylem sap was enriched in Ca (Ca/Sr_{Feb} = 176, Ca/Sr_{June} = 171) and more similar to 1st age class total digest foliar values (Ca/Sr =163) compared to soil fractions (0-10 cm deep leachable soil Ca/Sr = 93; 0-10 cm deep exchangeable soil Ca/Sr = 96, Figure 4.4A). Ca/Sr displayed little seasonal variation in the xylem sap, especially at the low-Ca site (Figure 4.4).

The high-Ca site displayed slightly more ^{44}Ca-depleted xylem sap in June ($\delta^{44}Ca$ = -1.57‰) compared to February ($\delta^{44}Ca$ = -1.38‰; Figure 4.5B). There was more seasonal variability in the low-Ca site xylem sap, which also displayed ^{44}Ca-depletion in June ($\delta^{44}Ca$ = -2.07‰) compared to February ($\delta^{44}Ca$ = -1.06‰). At the high-Ca site, first year needles ($\delta^{44}Ca$ = -1.52‰) reflected the xylem sap during bud burst in June. However, at the low-Ca site first year needles ($\delta^{44}Ca$ = -1.47‰) were intermediate to the seasonal values of the xylem sap. At the high-Ca site, forest floor and 0-10 cm depth soil fractions of Ca were ^{44}Ca-depleted compared to both measures of xylem sap (Figure 4.5B). At the low-Ca site, June xylem sap isotope values were similar to, and February xylem sap isotope values were ^{44}Ca-depleted, as compared to forest floor and 0-10 cm depth soil isotope values (Figure 4.5B).

Ca/Sr and Ca isotopes in soil depths

Calcium/strontium values were similar between exchangeable and leachable fractions of cations (Figure 4.4A) both within and across sites. At the high-Ca site, Ca/Sr decreased in forest floor (Ca/Sr =310) to 70-100 cm depth (Ca/Sr = 69) in exchangeable-cations, and decreased from forest floor (Ca/Sr = 144) to 70-100 cm depth (Ca/Sr = 66) in leachable-

cations (Figure 4.4A). There was a similar pattern of decreasing Ca/Sr with increasing soil depth at the low-Ca site in both soil extractions; Ca/Sr decreased from forest floor (Ca/Sr = 147) to 70-100 cm depth (Ca/Sr = 80) in exchangeable-cations, and decreased from forest floor (Ca/Sr = 115) to 70-100 cm depth (Ca/Sr = 102) in leachable-cations (Figure 4.4A). The whole-soil digest Ca/Sr values were lower than either exchangeable or leachable-Ca fractions at both sites at 0-10 cm depth (Ca/Sr = 35, high-Ca site; Ca/Sr = 33, low-Ca site) and 70-100 cm depth (high-Ca site: Ca/Sr = 29; low-Ca site: Ca/Sr = 25, data not displayed).

There was variation in exchangeable and leachable $^{44}Ca/^{40}Ca$ with depth that differed by site. At the high-Ca site, leachable-Ca was ^{44}Ca-enriched in upper (forest floor: $\delta^{44}Ca$ = -1.89‰; 0-10 cm depth: $\delta^{44}Ca$ = -1.89‰; 10-20 cm depth: $\delta^{44}Ca$ = -1.89‰) and ^{44}Ca-depleted in lower (70-100 cm depth: $\delta^{44}Ca$ = -1.43‰) depths (Figure 4.5B). In comparison, exchangeable Ca was more ^{44}Ca-depleted in upper depths (forest floor: $\delta^{44}Ca$ = -2.26‰; 0-10 cm depth: $\delta^{44}Ca$ = -2.21‰) and more ^{44}Ca-enriched in lower depths (10-20 cm depth: $\delta^{44}Ca$ = -1.38‰; 70-100 cm depth: $\delta^{44}Ca$ = -1.11‰) than leachable-Ca at the high-Ca site (Figure 4B). At the low-Ca site, leachable-Ca was ^{44}Ca-depleted only in upper horizons (forest floor: $\delta^{44}Ca$ = -2.30‰; 0-10 cm depth: $\delta^{44}Ca$ = -1.75‰) compared to exchangeable-Ca (forest floor: $\delta^{44}Ca$ = -1.80‰; 0-10 cm depth: $\delta^{44}Ca$ = -1.89‰; Figure 4.5B). Overall at both sites, as depth increased, $^{44}Ca/^{40}Ca$ also increased, with the exception of exchangeable-Ca in 10-20 cm soil, which displayed unusually high $^{44}Ca/^{40}Ca$ ($\delta^{44}Ca$ = -0.51‰).

Ca/Sr discrimination and $^{44}Ca/^{40}Ca$ separation factors in the plant-soil system

To estimate the preference for cations during uptake, movement and deposition, we calculated Ca/Sr discrimination factors (DF) where a DF > 1 indicates preference for Ca over Sr in the cation sink relative to the cation source. Whole-plant estimates of DF were < 1 (preferential movement of Sr) between either forest floor or 0-10 mineral soil and bole-wood (range: 0.52 to 1.00) but were > 1 (preferential movement of Ca) between either forest floor or 0-10 mineral soil and twig xylem sap or needles at both sites (range: 1.03 to 2.22) with the exception of forest floor to twig xylem sap in the high Ca site (February: 0.63, June: 0.87, Table 4.4). Overall, plant organ-level Ca/Sr DF indicated a preference for Ca movement from bole-wood to outer-bark (high-Ca site: DF = 2.26, low-Ca site: DF = 1.68), bole-wood to twig

xylem sap (high-Ca site: DF = 1.20, low-Ca site: DF =1.87) and bole-wood needles (high-Ca site: DF = 1.74, low-Ca site: DF = 2.58, Table 4.4). Twig xylem sap flow path to needles had DF >1 at the high Ca site (February: DF = 2.15, June: DF = 1.55), but DF < 1 at the low Ca site (February: DF = 0.93, June: DF = 0.96, Table 4.4). Plant tissue-level DF in needles indicated a preference for Ca deposition in both structural-Ca and Ca-ox at both sites (range: DF = 1.24 to 6.01), with the exception of young needles at the low-Ca site (DF = 0.22, Table 4.4). The opposite pattern was true for bole-wood and outer-bark which all indicated preference for deposition of Sr in both structural-Ca and Ca-ox (range DF = 0.21 to 0.70, Table 4.4). The highest DF by far was at the tissue-level between soluble-Ca and Ca-ox fraction flow path in older needles (age class 5) at the high-Ca site (DF = 6.01, Table 4.4).

To estimate the preference for Ca-isotopes during uptake, movement and deposition, we calculated $^{44}Ca/^{40}Ca$ separation factors (SF) where a positive value indicates preference for ^{44}Ca over ^{40}Ca in cation sinks compared to sources. Whole-plant SF between soil pools (either forest floor or mineral soil) and plant pools (bole-wood, foliage or twig xylem sap) were all positive (range: SF= 0.09 to 1.14‰) indicating overall preference for ^{44}Ca (heavy-isotope) uptake and movement along most flow paths except for between mineral soil and twig xylem sap in June at the low-Ca site (SF = -0.28‰, Table 4.4). Plant organ-level SF were also generally positive with the exception of radial movement of cations from bole-wood to outer-bark (high-Ca site: SF = -1.15‰, low-Ca site: SF = -1.20‰), which had negative SFs indicating preferential movement of ^{40}Ca (light-isotope) and from bole-wood to twig xylem sap at both sites in June (high-Ca site: SF = -0.05‰, low-Ca site: SF = -0.37‰). When needles are the sink for cations, separation factors all indicated preference for movement of ^{44}Ca on a whole plant-level from soil pools, or from plant organ-level from bole-wood and twig xylem sap, with the exception of February twig xylem sap at the low-Ca site (SF= -0.11‰, Table 4.4). Plant tissue-level SFs indicated preferential movement of ^{40}Ca (light isotope) to structural-Ca fractions from soluble-Ca in all tissues (range: SF = -0.04 to -0.55‰) except bole-wood at the low-Ca site (SF = 0.09‰) and outer-bark at both sites (high-Ca site: SF = 0.37‰, low Ca site: SF = 0.09‰, Table 4.4). Likewise, SFs indicated preferential movement of ^{40}Ca from soluble-Ca to Ca-ox within all tissues (range: SF = -0.05 to -0.83‰) except within bole-wood at both sites (high-Ca site: SF = 0.46‰, low-Ca site: SF = 0.23‰, Table 4.4).

Multi-tracer approach to identifying Ca sources

We observed qualitatively similar patterns between sites in bivariate plots of Ca/Sr versus $^{44}Ca/^{40}Ca$ of various ecosystem compartments (Figure 4.7). Separate Ca/Sr or $^{44}Ca/^{40}Ca$ measurements often yielded overlap among tissue, detrital, and soil compartments, but considered together, Ca/Sr and $^{44}Ca/^{40}Ca$ provided more clear separation among compartments. All leaf tissues were enriched in Ca/Sr compared to soils, whereas bark tissues were ^{44}Ca depleted compared to soils. Deeper soils were ^{44}Ca enriched and Ca/Sr depleted compared to upper soils. At both sites bole-wood had a similar signature to lower soil depths (>30 cm). At the high-Ca site February xylem sap was also similar to lower soil depths (>30 cm). In contrast, at the low-Ca site xylem sap was closer to plant signatures than to soil signatures. Overall the low-Ca site leaves were closest in Ca/Sr and $^{44}Ca/^{40}Ca$ to exchangeable cations in the forest floor.

Discussion

Oxalate crystal formation in the laboratory exhibited preference for Ca over Sr, and for lighter Ca (^{40}Ca) over heavier Ca (^{44}Ca). The Ca/Sr discrimination factor and $^{44}Ca/^{40}Ca$ enrichment factor were large enough to influence patterns of bulk natural abundance Ca/Sr and $^{44}Ca/^{40}Ca$, although the comparability between batch Ca-ox synthesis and plant tissues and systems is unknown. The discrimination factor for Ca during synthesis of Ca-ox was estimated to be 5.4 during the first rapid-crystallization phase, which was greater than *in situ* Ca/Sr discrimination factors reported across ecosystems ranging from 1.14 to 1.91 between vegetation and cation sources (Blum et al., 2000, 2012; Dasch et al., 2006). The isotope enrichment factor for the precipitation of Ca-ox during the first rapid-crystallization phase was -1.46‰, which was within the range (-0.22 to -1.73‰) reported for biological fractionation across ecosystems between Ca sources and vegetation (Schmitt et al., 2003; Wiegand et al., 2005; Page et al., 2008; Cenki-Tok et al., 2009; Holmden and Bélanger, 2010).

To explore how inorganic Ca-ox precipitation discrimination factors (Ca/Sr) and enrichment factors ($^{44}Ca/^{40}Ca$) compare with that of plant and soil Ca-ox creation, here we

discuss known mechanisms for discrimination and fractionation in both inorganic and organic Ca systems. Studies of discrimination and fractionation of Ca-ox are rare in the literature, but Ca-ox has many similarities with Ca-carbonate, a comparatively well-studied biomineral created with ionic bonding between Ca^{2+} ions and carbonate (CO_3^{-2}). Inorganic precipitation of both Ca-carbonate and Ca-ox is controlled similarly by kinetic reactions and spiral step crystal growth (Nielsen and Toft, 1984; Qiu and Orme, 2008; Njegić-Džakula et al., 2009), so it is possible that Ca/Sr discrimination or $^{44}Ca/^{40}Ca$ fractionation is influenced by similar mechanisms. We therefore provisionally draw upon studies of Ca/Sr discrimination and $^{44}Ca/^{40}Ca$ fractionation in Ca-carbonate as a first-step in evaluating these processes in Ca-ox.

Kinetic processes likely dominated both discrimination (Ca/Sr) and enrichment ($^{44}Ca/^{40}Ca$) factors in our inorganic Ca-ox synthesis experiment due to its short-term nature, with the caveat that equilibrium processes of recrystallization and exchange during back reactions were not measured and cannot be distinguished from kinetic effects. Kinetic effects dominate Ca/Sr discrimination during Ca-carbonate precipitation (Paquette and Reeder, 1995; Tesoriero and Pankow, 1996; Gabitov and Watson, 2006; Tang et al., 2008b) and are generally assumed to dominate $^{44}Ca/^{40}Ca$ fractionation (Tang et al., 2008a; Nielsen et al., 2011). In our Ca-ox study, equilibrium effects could have occurred in the second, slow part of the reaction if preferential dissolution caused either Ca/Sr discrimination or $^{44}Ca/^{40}Ca$ fractionation. Of these, Ca/Sr discrimination seems more likely due to considerably higher solubility of Sr-oxalate (K_{sp} [at 25 ^{0}C] 1.6 x 10^{-7}) than Ca-ox (K_{sp} 2.6 x 10^{-9}, monohydrate) (Benefield and Morgan, 1990). In Ca-carbonate systems, dissolution can cause Ca/Sr discrimination (Hönisch, 2002), but is not thought to cause $^{44}Ca/^{40}Ca$ isotopic fractionation (Hönisch, 2002; Fantle and DePaolo, 2007; Griffith et al., 2008). Ultimately, equilibrium effects were probably minimal during the short time course of our experiment and here we emphasize discrimination and fractionation during the first part of the experiment with rapid crystallization where kinetic effects appear to dominate.

Discrimination between Ca^{2+} and Sr^{2+} during precipitation occurs due to the greater ionic radius of Sr. Both Ca-ox and Ca-carbonate precipitation prefer Ca over Sr, though more Sr is incorporated in tissues when the precipitation rate is high (Gabitov and Watson, 2006; Tang et al., 2008b). An expression of the amount of Sr uptake is the Sr/Ca partition coefficient

(K_{dSr}), which for Ca-carbonate decreases from 0.35 to 0.12 as precipitation rates decrease (Gabitov and Watson, 2006). Similarly, we calculate K_{dSr} (1/DF) for Ca-ox of 0.20 (Ca/Sr DF = 5.4) during an initial rapid precipitation phase (0 to 360 min) and 0.07 (Ca/Sr DF = 14.7) during a second slow reaction phase (1440 to 2880 min). In natural systems, if kinetic reactions dominate Ca/Sr discrimination, then discrimination differences may be strongly related to precipitation rates of Ca-ox, and precipitation rates *in situ* may vary due to many factors that shape Ca and oxalate supply. Variation in Ca/Sr in marine Ca-carbonate created can also reflect equilibrium and biological controls (Stoll et al., 2002; Lorrain et al., 2005), which may be related to both species and age of the organism (Schöne et al., 2011). A conceptual model has been suggested that bases Ca/Sr separation on thermodynamic constraints of the channel/carrier-mediated transport of Ca and Sr ions into a cell (Langer et al., 2006), rather than crystal precipitation kinetics. Strong physiological effects are likely also true for biogenic Ca-ox in plants and soils, given tree species differences in discrimination factors (Dasch et al., 2006). Coupling Ca/Sr discrimination information to physiological information about Ca channels, Ca concentrations or Ca-ox formation rates may provide some mechanistic understanding of this result.

Differences in weights and atomic radii of ^{44}Ca and ^{40}Ca that influence fractionation during Ca-carbonate precipitation may also shape fractionation during Ca-ox precipitation. The enrichment factor we estimate for Ca-ox formation (-1.46‰) is near the range of enrichment factors reported for the inorganic formation of calcite and aragonite (~0 to -1.3‰), which varies with Ca-carbonate precipitation rate (Gussone et al., 2003; Lemarchand et al., 2004; Marriott et al., 2004; Tang et al., 2008a). Mechanisms for discrimination could be due to kinetic or equilibrium processes, which has been a subject of much debate (Marriott et al., 2004; Fantle and DePaolo, 2007; Nielsen et al., 2011). It is likely that large fractionation effects of Ca-carbonate are controlled by kinetic reactions, because long-term equilibration of Ca-carbonate in marine organisms and aquifers produces equilibrium fractionation of ~0‰ (Fantle and DePaolo, 2007; Jacobson and Holmden, 2008; Nielsen et al., 2011). In kinetic controlled fractionation, high rates of precipitation will have greater $^{44}Ca/^{40}Ca$ separation whereas low precipitation rates will be closer to equilibrium with fractionation approaching 0‰ (Tang et al., 2008a). Our Ca-ox data also indicate an increase in $^{44}Ca/^{40}Ca$ fractionation with increased precipitation rate, then as the experiment proceeded, the fractionation

decreased substantially (to ~ 0‰) during the slower dissolution and precipitation phase (f = 0.26 to f = 0.05), as has been observed in similar systems (Bullen et al., 2001; Baesman et al., 2007).

The process(es) causing $^{44}Ca/^{40}Ca$ fractionation in Ca-carbonate precipitation are generally modeled by either preference for the light isotope via diffusion of Ca from bulk solution to the crystal surface, or surface reaction processes influenced by differing ^{44}Ca versus ^{40}Ca bonding strength during dehydration of $Ca(H_2O)_6^{2+}$ water complexes (Tang, et al., 2008a; DePaolo, 2011; Gussone et al., 2011). In the surface reaction model, covalent bonding within a Ca-aquocomplex is stronger than in a carbonate mineral, causing heavier ^{44}Ca to preferentially remain in a Ca-aquocomplex that yields ^{44}Ca-depleted solids (Marriott et al., 2004). In contrast to Ca-carbonate, water molecules in Ca-ox exist within the crystal structure; consequently, Ca^{2+} may not need to be completely dehydrated during the precipitation process. Incomplete dehydration is assumed to influence kinetics of crystal growth (Nancollas and Gardner, 1974; Curreri et al., 1979; Heijnen, 1982; Heijnen and Van Duijneveldt, 1984), but has not been directly studied. If Ca^{2+} ions are not completely dehydrated, then fractionation may be minimized between $^{44}Ca(H_2O)_2^{2+}$ and $^{40}Ca(H_2O)_2^{2+}$ and the observed fractionation may be primarily due to diffusion differences between ^{44}Ca and ^{40}Ca. Fractionation during formation of ikaite ($CaCO_3(H_2O)_6$) was less ^{44}Ca depleted compared to water-free Ca carbonate precipitated at the same temperature (Gussone et al., 2011). Our fractionation factor was larger (ε = -1.46 per mil) compared to the range of synthetic ikaite (ε = -0.75 to -0.42‰). The difference may be due to Ca-ox dehydration to monohydrate versus ikaite precipitation with 6 water molecules per molecule of $CaCO_3$, supporting the idea of a dehydration mechanism. We note, however, that Ca-ox crystals in plant tissues are subject to processes that make it difficult to directly relate inorganic precipitation fractionation to *in situ* processes, particularly because Ca-ox precipitation in plants is highly regulated by matrix proteins and is not stochastic (Webb, 1999; Bouropoulos et al., 2001; Li et al., 2003).

Dehydration levels may be higher in mono-hydrate versus di-hydrate or tri-hydrate Ca-ox although these are less stable forms (Baran and Monje, 2008; Echigo and Kimata, 2011) and are more difficult to observe. In our study, both mono-hydrate and di-hydrate crystals were formed throughout the experiment, and the percentage of each crystal type did

not change over the time course of the experiment so it is unlikely that different crystal types influenced the Ca/Sr or $^{44}Ca/^{40}Ca$ variations in our precipitation reaction. Therefore, we assume that diffusion processes dominated $^{44}Ca/^{40}Ca$ fractionation during inorganic precipitation of Ca-ox, although this may provide an opportunity for future studies to compare fractionation during different levels of Ca hydration as particular crystal morphologies can be formed as a factor of experimental variables like stirring and temperature (Doherty et al., 1994; Thongboonkerd et al., 2006).

In summary, the formation of Ca-carbonate and Ca-ox are two common biominerals whose formation discriminates for Ca over Sr and fractionating for Ca isotopes. Whereas Ca-carbonate can be of great importance in shaping $\delta^{44}Ca$ and Ca/Sr of marine and arid terrestrial systems, it is likely that Ca-ox is similarly important in acid forest soils, since Ca-carbonate more readily dissolves with weak acidity.

Ca/Sr ratios and Ca-ox in forest ecosystems

The Ca/Sr ratios that we observed were within the range of other ecosystems, although our foliage (range: Ca/Sr = 81 to 523) was low compared to most (Watmough and Dillon, 2003; Dasch et al., 2006; Pett-Ridge et al., 2009). It is unknown if this is due to species differences as this is the first study to report Ca/Sr in Douglas-fir, or to differences in mineral cation sources. Generally conifer are reported to have foliar Ca/Sr values that were higher than deciduous species (Watmough and Dillon, 2003; Dasch et al., 2006) so there is no indication that Douglas-fir would be lower in Ca/Sr than other tree species. Our whole-soil digest Ca/Sr values were low (Ca/Sr ~25 to 35) compared to others reported in literature (mean across sites: Ca/Sr = 90, Pett-Ridge et al. 2009) suggesting that mineral cation sources low in Ca/Sr may be related to low foliar Ca/Sr values at these sites. Regardless, we found higher values of Ca/Sr in tree tissues than soils, as in other forest ecosystems (Poszwa et al., 2000; Drouet and Herbauts, 2008; Blum et al., 2012). Preference for Ca over Sr during plant uptake, movement and deposition could occur on different scales. Whole-plant level discrimination could occur during root uptake. Cation uptake in roots could occur via a passive apoplastic uptake pathway, which is non-selective between divalent cations, or an active symplastic pathway is more regulated in terms of both controlling the rate of Ca supplied, and excluding Sr ions via

Ca specific channels (White and Broadley, 2003). The symplastic pathway could be a mechanism for Sr discrimination, although the relative contribution of each pathway is unknown (White and Broadley, 2003). Organ-level discrimination could occur as Ca moves along apoplastic exchange sites in the xylem resulting in different Ca/Sr values. Tissue-level discrimination could occur due to Ca-ox accumulation, caused by a preference for Ca in crystal formation, especially across needles of different ages that vary widely in Ca-ox concentration. We explore these types of discrimination below.

Ca-ox fractions in foliage were high in Ca/Sr compared to all other plant tissues (Figure 4.4) and across all plant tissues (bole-wood, inner and outer-bark, and needle tissues) at both sites we found a positive relationship between Ca-ox concentrations and Ca/Sr ratios (Figure 4.6A), supporting the idea the Ca-ox accumulation can create tissue-level fractionation that plays a role in bulk Ca/Sr patterns. Ca/Sr increases with needle age in conifers (Poszwa et al. 2000), likewise at our sites Ca/Sr increased with needle age as Ca-ox accumulated, which influenced the overall positive relationship between Ca/Sr and Ca-ox concentrations. Elevated Ca/Sr in needles with high Ca-ox is consistent with preference for Ca over Sr during oxalate crystal formation. This raises the possibility that Ca/Sr variation in forests may reflect differences not only among tree species (Dasch et al., 2006), but also Ca-ox status. We suggest that while inherent difference between species' physiology may be linked to Ca/Sr discrimination, cation supply can also have a determinate bearing on bulk Ca/Sr values. Furthermore, environmental conditions that influence physiological processes involved in Ca uptake or Ca-oxalate crystal formation may influence overall Ca/Sr.

Mechanisms for Ca/Sr discrimination in Ca-ox accumulation could occur in several ways. First, preferential movement of Ca over Sr to leaves could occur, as well as preferential movement of Ca to oxalate idioblasts after arrival in leaves. Strontium has been observed to accumulate in endodermal cells preferential to mesophyll and oxalate idioblasts in *Larix* needles (Gierth et al., 1998), which supports this idea. Secondly, preferential precipitation of Ca into oxalate crystals could cause Ca/Sr discrimination. Observations show that Sr is incorporated into plant Ca-ox crystals in idioblasts, although to a lesser extent than Ca, and primarily when Sr supply is high in experimental conditions (Franceschi and Schueren, 1986; Kim and Heinrich, 1995; Gierth et al., 1998). Thirdly, Ca/Sr discrimination could be due to

increased solubility and loss of Sr-oxalate over time. Oxalate crystals in the mesophyll of spruce needles dissolve in leaching experiments (Fink, 1991b), and generally Sr is detected as a component of foliar leachate (Gosz and Moore, 1989). Within leaves Sr-oxalate could preferentially dissolve and leach from foliage as Sr-oxalate is more soluble than Ca-ox. Our data do not resolve which mechanism is most active in Ca/Sr fractionation or on what scale.

Xylem sap from twigs had a higher Ca/Sr than bole-wood tissue within both sites, which indicates that Ca is preferentially transported to leaves over Sr. In bole-wood and bark tissue, the soluble cation fraction was higher in Ca/Sr than other fractions suggesting that Ca is more mobile than Sr in these tissues, and bole-wood had low total digest Ca/Sr suggesting that Sr preferentially accumulates in woody tissues of the bole. Low Ca/Sr ratios occur in wood in several others species (Watmough and Dillon, 2003; Pett-Ridge et al., 2009), suggesting exchange on xylem apoplastic sites or deposition in endodermal cells to be the primary Ca/Sr fractionation mechanism on a plant organ-level. Tissue-level discrimination may also occur in Ca-ox deposition; DF were estimated to be < 1 between soluble Ca and Ca-ox in bole-wood at both sites (Table 4.4). Although, Ca-ox was only ~15% of the total digest Ca in the bole-wood at both sites compared to higher percent of Ca in Ca-ox in other measured tissues (high-Ca site foliage mean: 71%, low-Ca site foliage mean: 37%, high-Ca inner and outer-bark mean: 27%, low-Ca site inner-and outer-bark mean: 15%, Table 4.2).

Ca in the transpiration stream is likely a mix of enriched Ca that has moved slower up the tree due to interaction with xylem exchange sites, and free Ca ions from more direct soil sources (Clarkson, 1984; Atkinson et al., 1992). Trees redistribute Ca seasonally (Stark et al., 1985; Kazda and Weilgony, 1988; Dambrine et al., 1995), suggesting that the ratio of Ca from internal sources (xylem bound Ca) to external sources (Ca from soil via root uptake) may also change seasonally. However, xylem sap Ca/Sr was fairly similar across our two sample dates, especially at the low-Ca site, consistent with soil water Ca/Sr values across coastal Douglas-fir forests (Ca/Sr_{Feb}: 75 versus Ca/Sr_{June}: 70, Perakis *unpublished data*). In contrast, xylem sap Ca/Sr differed between our two study sites. The low-Ca site had slightly higher Ca concentrations, and higher Ca/Sr (Ca/Sr_{Feb}: 176, Ca/Sr_{June}: 171) compared to the high-Ca site (Ca/Sr_{Feb}: 88, Ca/Sr_{June}: 122) despite similar soil Ca/Sr (Figure 4.4A), suggesting that root uptake discrimination for Ca/Sr differed by site. Discrimination factors for a whole-plant flow

path between soil sources and twig xylem sap indicate an overall higher discrimination at the low-Ca site (Table 4.4) which supports this idea, although because we measured twig rather than root or bole xylem sap the Ca/Sr ratio may be a product of both root-level discrimination as well as movement in the xylem throughout the entire tree. Because the low-Ca site had higher concentrations of Ca in the xylem sap despite 7-fold lower Ca concentrations in 0-10 mineral soil, trees at the low-Ca site may be actively taking up and transporting more Ca to resolve Ca deficiency.

Similar whole-plant discrimination factors existed between forest floor cations and foliage (needles) in Douglas-fir across sites of differing Ca supply (high-Ca site: DF= 1.35, low-Ca site: DF = 1.36), similar to the range in other tree species (range: DF = 1.14 to 1.91, Dasch et al., 2006; Blum et al., 2008, 2012). Estimations of species-specific whole-plant level discrimination factors using foliage are only accurate if discrimination at an organ-level and tissue-level either do not occur, or are consistent within a species across sites and levels of cation supply. At our sites, plant organ-level discrimination was as high or higher than whole-plant discrimination, with DF from bole-wood to foliage among the highest, in agreement with patterns found in plant organs of *Fagus sylvatica* and *Quercus robur* (Drouet and Herbauts, 2008). However, unlike previous studies that found consistent DFs within species across sites (Dasch et al., 2006; Drouet and Herbauts, 2008; Blum et al., 2012) there were strong site differences in DF from bole-wood to foliage with the high-Ca site (DF = 2.58) indicating a stronger preference for Ca compared to the low Ca site (DF = 1.74, Table 4.4), and suggesting that a species-specific DF may not be accurate across these sites with differing cation supply. Interestingly, DF estimates of whole-plant fractionation were similar between sites using forest floor Ca/Sr values, but not using mineral soil Ca/Sr values, so estimating whole-plant DF depends on integrated measurement of cations available for plant uptake which may vary with cation supply status. Differences that occur in cation movement between the two sites were also illustrated by differences in xylem sap Ca/Sr DFs. Despite higher-concentrations of Ca in the low-Ca site xylem sap and overall higher preference for Ca in the flow path from soil sources to xylem sap as compared to the high-Ca site (Table 4.4), there were site differences in the flow-path from twig xylem sap to needles, with greater Sr relative to Ca movement (DF slightly <1) at the low Ca site. This could be an indication of Ca

impoverishment and Ca depletion during cation movement along the transpiration stream if bole, branches or twigs contain strong sinks for Ca.

We found that Ca/Sr discrimination factors between different chemical fractions in plant tissues were equal or greater than discrimination factors on a whole-plant or plant-organ level. This suggests that mechanisms for cation discrimination may be complex enough to be influenced not only by species differences (Dasch et al., 2006; Blum et al., 2012), or plant organ differences (Drouet and Herbauts, 2008), but also environmental differences that influence tree physiology, for example, nutrient status between sites. Additionally, needle age was important for Ca/Sr discrimination factors. For example, DFs between foliage and exchangeable cations in forest floor at the high-Ca site in our study could range from 0.9 to 1.6 depending on which needle age class is chosen (we reported an average across needle ages). This is likely a product of strong discrimination of needles for Ca in Ca-ox pools especially in older needles (e.g. high-Ca site age-class 5: DF = 6.01, Table 4.4) that accumulate Ca-ox (high-Ca site age-class 5: 51% of tissue Ca in Ca-ox, Table 4.2). Discrimination factors calculated using foliage or any single plant organ may not accurately represent whole-plant nutrient sources. Therefore, care should be taken when choosing plant compartments for Ca/Sr indicators. Additionally, results of our study suggest that Ca/Sr values that vary by cation status or needle age may account for the large variation in Ca/Sr values observed within species (foliar Ca/Sr range: SD= 64 to 1858, Watmough and Dillon, 2003).

We found that within sites, exchangeable- and leachable-Ca fractions had surprisingly similar Ca/Sr at all depths (mean difference: 12 ± 11) in contrast to Ca/Sr differences in exchangeable (Ca/Sr = 270 ± 156 SD) and leachable-Ca (Ca/Sr = 309 ± 181 SD) in forest floor across four sites in New Hampshire (absolute value mean difference: Ca/Sr = 119 ± 98 SD, Bullen and Bailey, 2005). That we did not observe a preference for Ca in either fraction may indicate that there was 1) minimal discrimination between cations in soil fractions or 2) sampling of various chemical forms of Ca including Ca-ox in both exchangeable and leachable soil extractions (Dauer, Chapter 5), although our data do not resolve these possible explanations. The similarity between the two soil extractions indicates there is little power to distinguish plant uptake of different soil cation pools using Ca/Sr at these sites, however overall patterns of Ca/Sr in both extractions may still reveal information about cation cycling.

Soil Ca/Sr values were greatest in shallow soil depths, possibly due to preferential biotic uptake and recycling of Ca through upper soil horizons (Poszwa et al., 2000; Dasch et al., 2006; Drouet and Herbauts, 2008). The high degree of variation in internal-plant Ca/Sr relative to variation among soil chemical fractions and depths provides further indirect evidence that biological Ca recycling may influence Ca/Sr in soils. However, the similarity in soil Ca/Sr at both sites despite a 3- to 30-fold difference in forest floor and mineral soil Ca raises the possibility that biological selectivity for Ca over Sr is more important than ecosystem inputs via atmospheric deposition and/or weathering as factors shaping whole-ecosystem Ca/Sr. Indeed, whole-soil digest Ca/Sr were fairly similar at both sites in 70-100 cm soil (Ca/Sr = 29 versus 25 in high and low-Ca sites, respectively), with slightly higher values in 0-10 cm soil at both sites (Ca/Sr = 35 versus 33 in high- and low-Ca sites, respectively), indicative of preferential biotic recycling of Ca in upper soil horizons without strong differences in Ca/Sr supplied by weathering.

Ca-isotopes and Ca-ox in forest ecosystem

The range of $^{44}Ca/^{40}Ca$ that we estimated for plant tissues across sites and ages was within the range of values observed in plants in terrestrial systems (Nielsen et al., 2011). However, inner-bark ($\delta^{44}Ca$= –2.95‰) and outer-bark ($\delta^{44}Ca$= –2.90‰) at the low-Ca site are among the lowest reported value in terrestrial living tissues thus far (the lowest is tree roots: $\delta^{44}Ca$= –3.95‰, Page et al., 2008). Highly ^{44}Ca-depleted Ca isotope values such as these is evidence that plant Ca cycling is the driver of terrestrial $\delta^{44}Ca$ isotope plant-soil separation (Skulan et al., 1997; Holmden and Bélanger, 2010; Nielsen et al., 2011), although mechanisms for separation are not well studied. Fractionation of $^{44}Ca/^{40}Ca$ could occur on a whole-plant level during root uptake, on a plant organ level during Ca movement along apoplastic exchange sites in the xylem, or on a plant tissue-level during accumulation of ^{40}Ca preferring Ca-ox crystals, which may drive patterns across needle-ages that vary widely in Ca-ox concentration. We explore fractionation at a whole-plant level, organ-level and tissue-level below.

In our study, fractionation occurred on a plant-organ level during Ca movement from bole-wood to twig xylem sap and needles, and from twig xylem sap to needles (range: SF = -

1.20 to 0.64‰) was similar in magnitude to a whole-plant level fractionation during Ca uptake between forest floor or mineral soil and plant (range: SF= -0.28 to 1.14‰). Both were slightly higher in magnitude than tissue-level processes of fractionation like Ca-ox accretion (range: SF= -0.83 to 0.46‰, Table 4.4). For example, we observed greater separation in isotope values on a plant-organ level that preferred light ^{40}Ca movement in a flow-path from bole-wood to outer-bark (high-Ca site δ^{44}Ca: SF = -1.15‰; low-Ca site δ^{44}Ca: SF= -1.20‰, Table 4.4), compared to tissue-level fractionation in needles that preferred light ^{40}Ca deposition in a flow-path between soluble-Ca and both structural-Ca and Ca-ox (range: SF= -0.32 to -0.83‰, Table 4.4).

Whole-plant scale fractionation between soil sources and plant compartments generally preferred heavy ^{44}Ca. Mechanisms for whole-plant Ca stable isotope fractionation are likely driven by fractionation at the root level, which has been suggested to be the primary method for biological fractionation in trees, though this assumption is without clear mechanisms (Holmden and Bélanger, 2010). Root uptake via a passive apoplastic pathway that is indiscriminant of cations (White and Broadley, 2003), so passive processes must mediate Ca fractionation during uptake and may include dehydration of Ca aquocomplexes before entering root Ca^{2+} channels.

Large isotope separation on a plant organ-level has been observed between bole and foliar tissues as enrichment of ^{44}Ca isotopes as Ca^{2+} ions move from branches to stems, twigs and leaves (Schmitt et al., 2003; Wiegand et al., 2005; Page et al., 2008; Cenki-Tok et al., 2009). The process of exchange of Ca ions on xylem sites and removal again into the transpiration stream (Bangerth, 1979; Clarkson, 1984) may be an opportunity for fractionation; Ca-isotope fractionation in trees is commonly observed as ^{44}Ca-depletion in woody tissues relative to soil solution or exchangeable-Ca (Schmitt et al., 2003; Wiegand et al., 2005; Page et al., 2008; Cenki-Tok et al., 2009; Holmden and Bélanger, 2010). Ca moving through woody tissues to leaves could undergo fractionation via many different processes including binding to xylem exchange sites, chelation with organic acids in sap (Bradfield, 1976; White et al., 1981), displacement by other competing cations, and variation in quantity and quality of xylem exchange sites (Ferguson and Bollard, 1976) or passage of cations from xylem vessels into the leaf mesophyll (Gierth, 1998; Drouet and Herbauts, 2008).

We found tissue-level fractionation in foliage in that Ca-ox was ^{44}Ca depleted compared to soluble Ca at both sites and needle-ages (δ^{44}Ca: SF= -0.32 to -0.83‰). However, we did not find a strong relationship between Ca-ox and bulk δ^{44}Ca values among all plant tissues (Figure 4.5B), suggesting that factors other than Ca-ox accumulation were important in shaping Ca-isotope fractionation. Due to the preference for ^{40}Ca over ^{44}Ca in Ca-ox crystal formation in our synthesis experiment, we expected to see ^{44}Ca-depleted plant tissues in older and dead needles at the high-Ca site that are particularly concentrated in Ca-ox. However, Ca-isotopes in old needles at the high-Ca site were particularly rich in Ca-ox (79% of the total Ca), although the Ca-ox fraction was not ^{44}Ca-depleted compared to other needle ages at either site. Overall, needles at the high-Ca site were either similar to low-Ca site tissues of the same age class, or slightly ^{44}Ca-enriched (i.e. total digest needle age 1 year class, Figure 4.5). The conflicting δ^{44}Ca patterns observed with Ca oxalate accumulation in our needles versus inorganic data suggest that the formation of Ca-ox *in vivo* is more complex than a simple precipitation reaction.

Calcium biomineral precipitation in living organism has been studied for Ca-carbonate, which offers a useful comparison. Physiological similarities between some marine organisms and tree Ca biomineral precipitation exist in that they are both multi-step processes requiring active Ca pumping through numerous tissues, in which each step may fractionate stable Ca isotopes (Nielsen et al., 2011). For example, coral may exhibit Ca isotope fractionation during Ca diffusion into tissue, during active transport via attachment to proteins like ATPases, or in surface kinetic reactions during Ca dehydration for Ca-carbonate precipitation (Böhm et al., 2006). Trees may have similar fractionation processes including diffusion from xylem vessels, active transport through Ca ion channels across cell membranes, and crystal surface kinetic reactions. On a cellular scale, a parallel may be drawn between Ca-ox deposition in idioblasts and Ca-carbonate precipitation in coccoliths of phytoplankton. Terrestrial plant cells are similar to phytoplankton in that Ca is kept at μM concentrations in the cytoplasm, and actively pumped via Ca channels into vacuoles or apoplastic spaces where it may precipitate as Ca-ox. In marine organisms, fractionation may occur via endocytosis (for tree tissues, uptake or arrival in tissue mesophyll) or at the plasmas membrane as Ca^{2+} in aquocomplexes are dehydrated then attached to proteins of Ca channels (Gussone et al., 2006);

a physiological fractionation mechanism that could be controlled by both species differences and environmental factors such as Ca supply. We expect that, like Ca-carbonate precipitation, the processes in needle tissues involved in Ca^{2+} sequestration in Ca-ox crystal have multiple points for fractionation, and that Ca-ox physiology and accumulation at a tissue-level has the potential to influence bulk $\delta^{44}Ca$ values, although we did not observe clear patterns in Douglas-fir. Because Ca-ox accumulation in plant tissues is related to different levels of Ca-supply, it may be a useful to for predicting forest $^{44}Ca/^{40}Ca$ values across sites with different nutrient status, although site differences were not observed in this study.

We expected that sites where Ca is less abundant might be more ^{44}Ca-depleted overall because a greater proportion of Ca at the site will have cycled through biological tissues, allowing heavier isotopes to preferentially be lost during leaching processes. However, live needles at both sites displayed similar $^{44}Ca/^{40}Ca$ values, and soils did not indicate clear ^{44}Ca depletion at the low-Ca site (Figure 4.5B). Overall, exchangeable and leachable Ca fraction in soils were similar across soil depths and sites (absolute mean difference, high-Ca site: $\delta^{44}Ca$ = 0.38 ± 0.09‰; low-Ca site: $\delta^{44}Ca$ = 0.52 ±0.61‰), except the leachable fraction of Ca in soils was ^{44}Ca-depleted compared to the exchangeable fraction in deep mineral soils (10-20 cm and 70-100 cm deep) at the high-Ca site, and in forest floor and 10-20 cm deep in the low-Ca site. A nearby Oregon Coast Range site had a similar range ($\delta^{44}Ca$ = -1.8 to -0.4‰) for exchangeable and leachable-Ca fractions from 0 to 60 cm deep mineral, and also equal $\delta^{44}Ca$ of exchangeable and leachable fractions (Perakis et al., 2006). Exchangeable and leachable Ca isotope values do not seem to vary to a great extent at other sites (differences ≤ 0.50‰, Page et al., 2008; Holmden and Bélanger, 2010) nor to vary in predictable patterns by depth, for example some sites indicate slightly ^{44}Ca-enriched exchangeable Ca compared to leachable Ca (Page et al., 2008), and others report the opposite (Holmden and Bélanger, 2010). It may be that interchange of cations between the exchangeable and leachable pool minimizes isotope fractionation.

Using Ca/Sr and $^{44}Ca/^{40}Ca$ in multi-tracer approaches

Biological discrimination for Ca over Sr and fractionation between ^{44}Ca and ^{40}Ca complicates use these tracers for identifying soil source of cations. We found that in bivariate

plots of Ca/Sr and $^{44}Ca/^{40}Ca$ both sites had predictable directionality for plant compartments and for soil depths, for example, similarity in wide separation between foliage versus bole-wood versus bark (Figure 4.7). Bole-wood at both sites was closer to soil Ca/Sr and $^{44}Ca/^{40}Ca$, whereas foliage was high in Ca/Sr and ^{44}Ca-enriched compared to bark. The sites were also similar in directionality of Ca/Sr and $^{44}Ca/^{40}Ca$ with soil depth. While bivariate plots like these are typically used to indicate areas of overlap to identify soil sources of cations, large discrimination and separation factors obscure our ability to use plots in this way. Instead the plots could potentially help identify discrimination and separation mechanisms among ecosystem pools that vary with site. The separation in Ca/Sr and $^{44}Ca/^{40}Ca$ by plant tissue type suggests that mechanisms for discrimination or fractionation may be predictable if the mechanisms and the scales at which discrimination and fractionation occurs are more fully understood. Noticeable differences between the sites are that the low Ca site foliage is closer to overlap with forest floor exchangeable cations (Figure 4.7). This may reflect the tighter cycling of cations through biological pools at the low Ca site, which has a greater portion of ecosystem Ca in plant tissues (Perakis and Sinkhorn, in progress). In order for Ca/Sr and $^{44}Ca/^{40}Ca$ to be used as tracers of plant cation uptake, application of whole-plant discrimination factors and plant-organ discrimination factors between roots and the remaining plant, which we did not measure, should be applied. This would elucidate plant-soil system patterns without the confounding of biological uptake processes.

Conclusions

This study identifies a new mechanism for Ca/Sr discrimination and $^{44}Ca/^{40}Ca$ fractionation. Ca-ox precipitation was found to favor Ca over Sr and lighter ^{40}Ca over ^{44}Ca. Because Ca-ox is ubiquitous in terrestrial ecosystems, there is the potential for the accumulation, creation or dissolution of the crystal to result in predictable patterns of Ca/Sr and $^{44}Ca/^{40}Ca$. Observations in the field demonstrated that bulk Ca/Sr values can be influenced by Ca-ox concentrations, and that Ca-ox accumulation related to tree Ca supply status can have a significant impact on bulk tree tissue Ca/Sr values. This study provided mechanistic understanding of Ca/Sr and $^{44}Ca/^{40}Ca$ patterns, although more investigations across multiple ecosystems are needed.

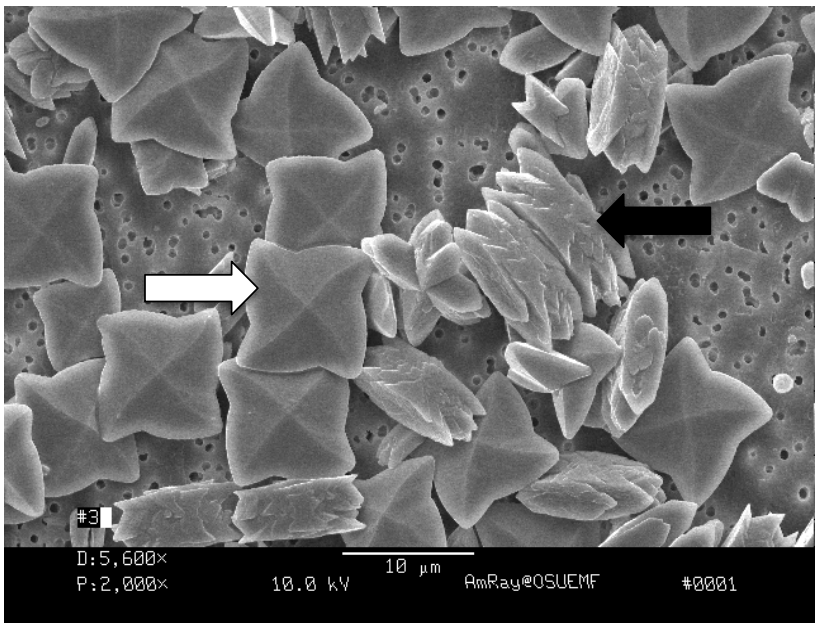

Figure 4.1. An example of the STEM images taken of filters after Ca-oxalate synthesis. Both mono-hydrate (raphide, black arrow) and di-hydrate (druse, white arrow) crystals were present.

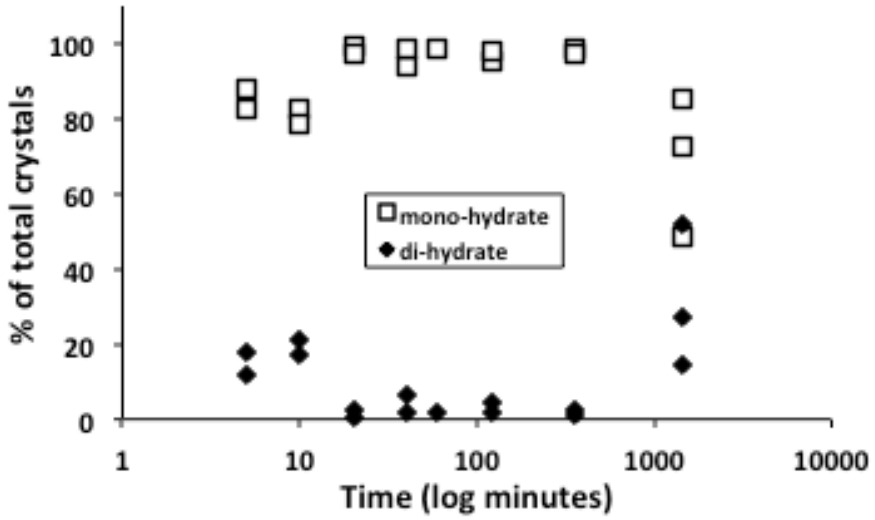

Figure 4.2. The percent of crystal types observed in STEM images of solid precipitates on filters after Ca-oxalate synthesis.

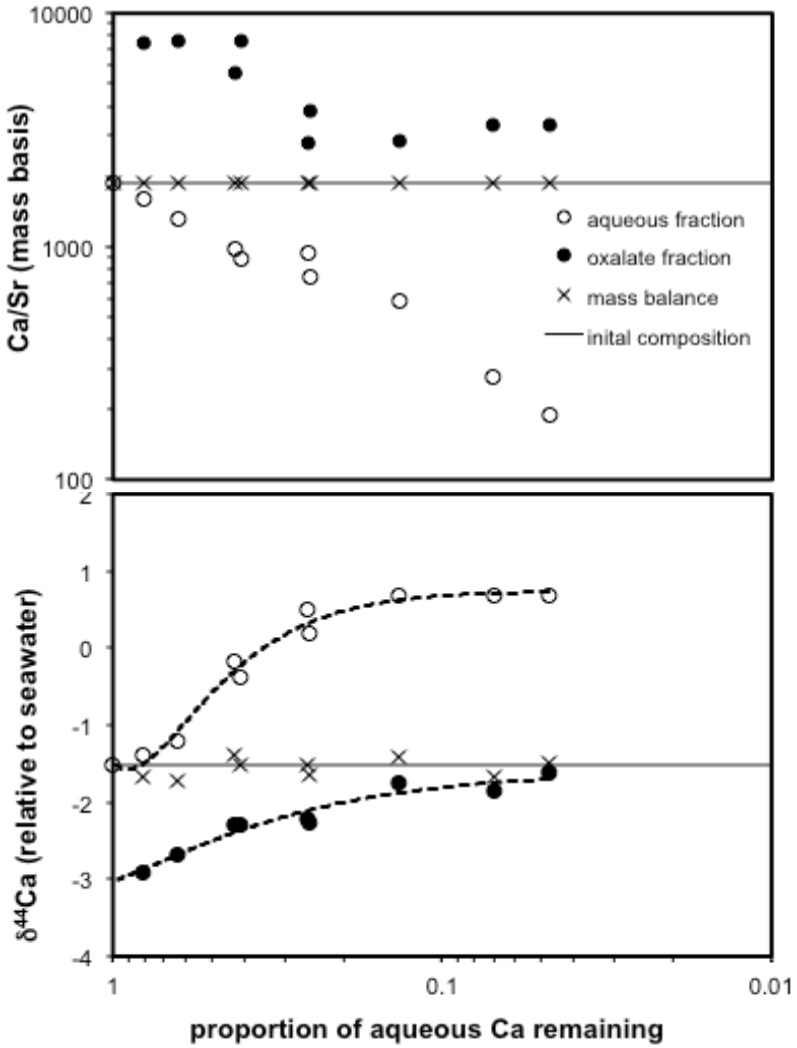

Figure 4.3. The A) Ca/Sr and B) stable isotope fraction relative to seawater of δ^{44}Ca of the aqueous filtered solution (open circles) and total Ca oxalate (solid circles) on filters as a function of the proportion of Ca remaining in the solution, representing a time course of 0 to 2880 minutes. The mass balance of Ca/Sr are (aqueous Ca + Ca-oxalate)/(aqueous Sr + Sr-oxalate) and for δ^{44}Ca are the fraction remaining*δ^{44}Ca of aqueous Ca + (1-fraction remaining)*δ^{44}Ca of Ca oxalate.

Figure 4.4. Ca/Sr ratios for ecosystem pools at a A) high-Ca and B) low-Ca site, including of sequential extraction (soluble Ca, structural Ca and Ca-oxalate) and total fractions for live leaves at two age classes, xylem, phloem and bark tissues, and xylem sap collected in two seasons, as well as, soil extractions of Ca using an exchangeable (1 N NH_4OAc) and leachable (1 N HNO_3) procedure. Total Ca and Sr in plant tissues was determined by whole-tissue digestion. Mass balances for foliage, bole and soil were calculated weighted by concentration of each fraction.

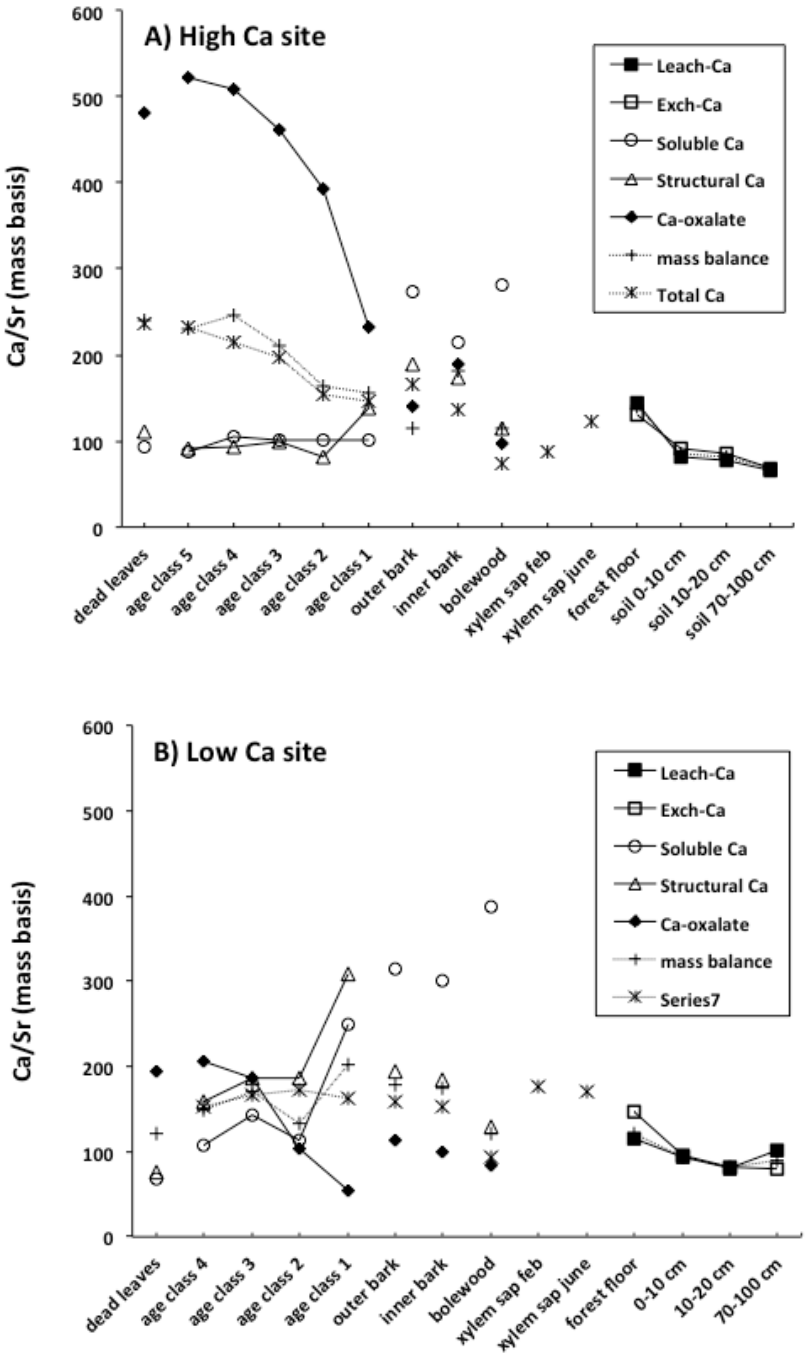
A) High Ca site
Ca/Sr (mass basis)
600
500
400
300
200
100
0
Leach-Ca
Exch-Ca
Soluble Ca
Structural Ca
Ca-oxalate
mass balance
Total Ca
dead leaves
age class 5
age class 4
age class 3
age class 2
age class 1
outer bark
inner bark
bolewood
xylem sap feb
xylem sap june
forest floor
soil 0-10 cm
soil 10-20 cm
soil 70-100 cm
B) Low Ca site
Ca/Sr (mass basis)
600
500
400
300
200
100
0
Leach-Ca
Exch-Ca
Soluble Ca
Structural Ca
Ca-oxalate
mass balance
Series7
dead leaves
age class 4
age class 3
age class 2
age class 1
outer bark
inner bark
bolewood
xylem sap feb
xylem sap june
forest floor
0-10 cm
10-20 cm
70-100 cm

Figure 4.4

Figure 4.5. δ^{44}Ca (relative to seawater) for ecosystem pools at an A) high-Ca and B) low-Ca site, including of sequential extraction (soluble Ca, structural Ca and Ca-oxalate) and total fractions for live needles at two age classes, xylem, phloem and bark tissues, and xylem sap collected in two seasons, as well as, soil extractions of Ca using an exchangeable (1 N NH4OAc) and leachable (1 N HNO3) procedure. Total Ca in plant tissues was determined by whole-tissue digestion. Mass balances for foliage, bole and soil were calculated weighted by concentration of each fraction.

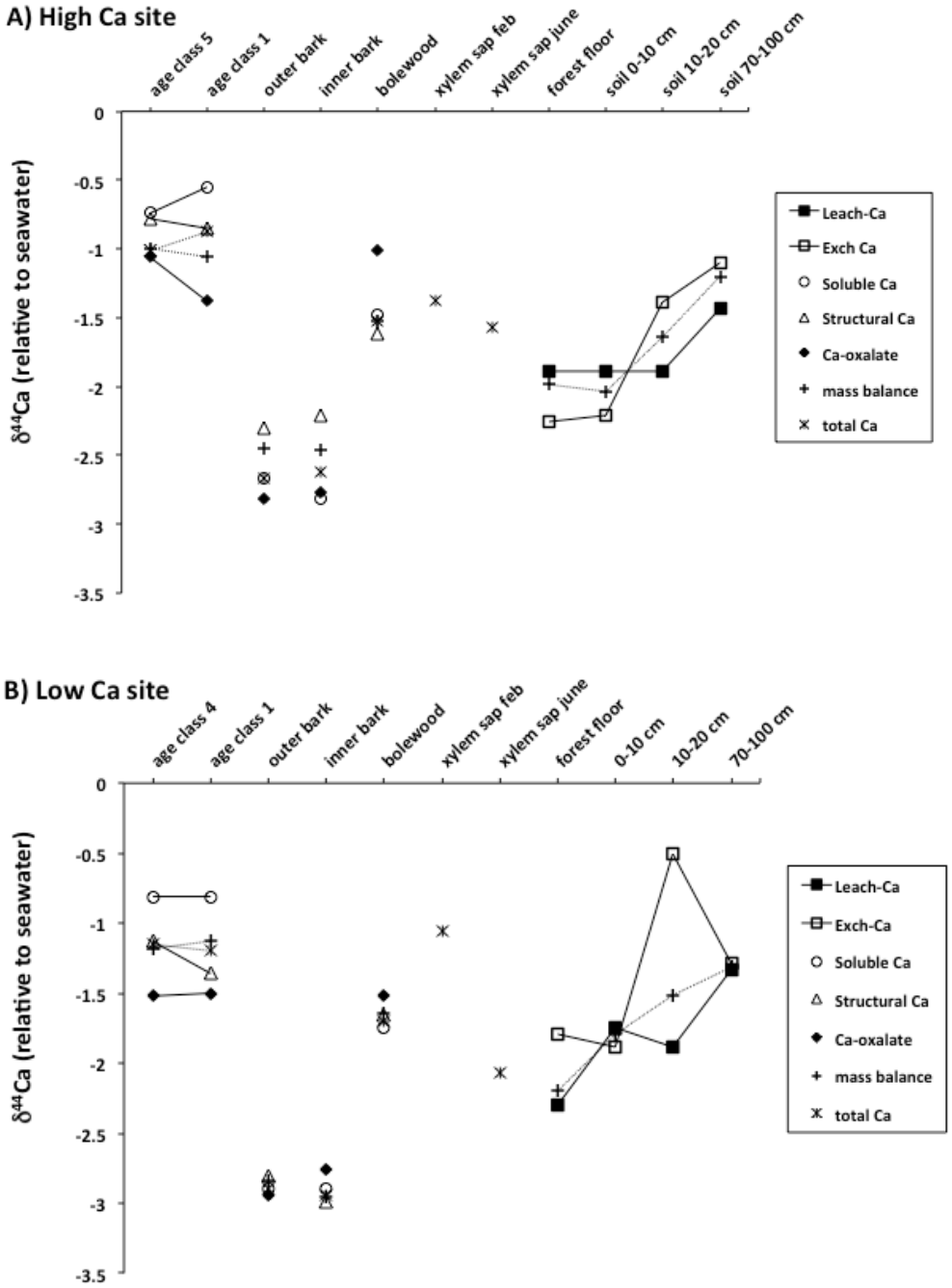
A) High Ca site
age class 5
age class 1
outer bark
inner bark
bolewood
xylem sap feb
xylem sap june
forest floor
soil 0-10 cm
soil 10-20 cm
soil 70-100 cm
δ44Ca (relative to seawater)
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
Leach-Ca
Exch Ca
Soluble Ca
Structural Ca
Ca-oxalate
mass balance
total Ca
B) Low Ca site
age class 4
age class 1
outer bark
inner bark
bolewood
xylem sap feb
xylem sap june
forest floor
0-10 cm
10-20 cm
70-100 cm
δ44Ca (relative to seawater)
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
Leach-Ca
Exch-Ca
Soluble Ca
Structural Ca
Ca-oxalate
mass balance
total Ca

Figure 4.5

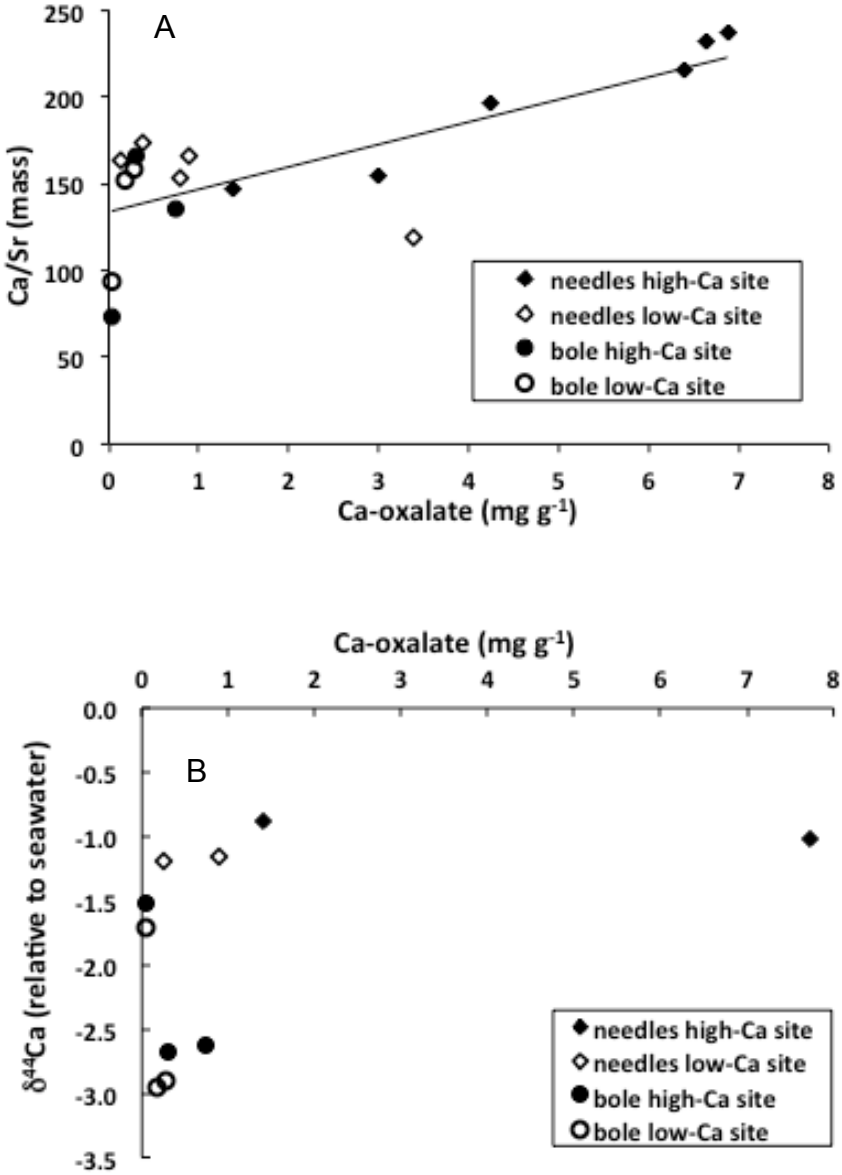

Figure 4.6. The relationship between A) Ca-oxalate concentration (mg g^{-1}) and Ca/Sr (R^2= 0.55, P<0.001) and between B) Ca-oxalate concentration (mg g^{-1}) and δ^{44}Ca (relative to seawater) in all plant tissues in a high-Ca and low-Ca site. All plant tissues include live (age class 1-5) and dead needles, and bole-wood, inner-bark and outer-bark tissues for Ca/Sr and live (age class 1 and 4 or 5) and bole-wood, inner-bark and outer-bark tissues for δ^{44}Ca.

Table 4.1. Characteristics of two sites in the Coast Range of Oregon including soil exchangeable Ca pools to 100 cm depth, soil pH and texture in surface soils, Ca in plant biomass, tree age in 2007 and total aboveground biomass. ANPP calculated as the net annual aboveground in stems, branches, foliage mass plus litterfall (Perakis and Sinkhorn, 2011).

Site	Soil exchangeable Ca 0-100 cm depth	Soil $pH_{(H2O)}$ 0-10 cm depth	Soil texture (%) 0-10 cm depth			Ca in aboveground plant biomass	Tree age	Total aboveground biomass	ANPP
	kg ha^{-1}		sand	silt	clay	kg ha^{-1}	years	Mg ha^{-1}	Mg ha^{-1} yr^{-1}
high-Ca (5)	5680	5.50	53	23	25	172	30	163	21.5
low-Ca (16)	280	4.61	30	31	39	98	27	122	14.3

Table 4.2. Concentrations of total Ca (mg g^{-1} tissue) and Ca-oxalate (mg g^{-1} tissue) for live needles at different age classes, dead needles, bole-wood, inner-bark and outer-bark tissues at high-Ca status and low Ca status sites. Soil Ca concentrations in exchangeable (1 N NH_4OAc) and leachable (1 N HNO_3) fractions at high-Ca status and low Ca status sites, n.s. indicates no samples.

	High Ca site		Low Ca site	
	Total Ca mg Ca g^{-1} tissue	Ca-oxalate mg Ca g^{-1} tissue	Total Ca mg Ca g^{-1} tissue	Ca-oxalate mg Ca g^{-1} tissue
Needle age 1	3.2	1.4	1.5	0.1
Needle age 2	7.1	3.0	2.3	0.4
Needle age 3	8.5	4.2	2.9	0.9
Needle age 4	11.5	6.4	2.7	0.8
Needle age 5	12.9	6.6	n.s.	n.s.
Dead needle	12.1	6.9	6.5	3.4
Bole-wood	0.3	0.0	0.2	0.0
Inner-bark	2.2	0.7	1.3	0.2
Outer-bark	1.5	0.3	1.6	0.3
	Exchangeable Ca mg g^{-1} soil	Leachable Ca mg g^{-1} soil	Exchangeable Ca mg g^{-1} soil	Leachable Ca mg g^{-1} soil
Forest floor	974	2738	327	1255
0-10 cm	591	711	57	133
10-20 cm	516	522	18	49
70-100 cm	322	131	21	24

Table 4.3. Mass balance and digest total Ca/Sr and δ^{44}Ca (relative to seawater) values for live needles at different age classes, dead needles, bole-wood, inner-bark and outer-bark tissues at high-Ca status and low Ca status sites. Absolute mean values across plant pools at each site for both Ca/Sr and δ^{44}Ca are reported. Parenthesis indicates standard deviation. n.s. indicates no samples.

	High Ca site			**Low Ca site**		
	Total digest Ca/Sr	Mass balance Ca/Sr	Difference (total digest-mass bal) Ca/Sr	Total digest Ca/Sr	Mass balance Ca/Sr	Difference (total digest-mass bal) Ca/Sr
Needle age 1	146	156	-9	163	202	-39
Needle age 2	155	164	-9	173	132	41
Needle age 3	197	211	-15	166	170	-4
Needle age 4	215	245	-30	153	149	4
Needle age 5	232	230	2	n.s.	n.s.	n.s.
Dead needle	237	241	-4	120	122	-2
Bole-wood	73	115	51	94	121	-27
Inner-bark	136	182	-46	152	174	-22
Outer-bark	166	115	-42	158	179	-21
Abs value mean:			**23 (19)**			**20 (16)**
	Total digest δ^{44}Ca ‰	Mass balance δ^{44}Ca ‰	Difference (total digest-mass bal) δ^{44}Ca ‰	Total digest δ^{44}Ca ‰	Mass balance δ^{44}Ca ‰	Difference (total digest-mass bal) δ^{44}Ca ‰
Needle age 1	-0.88	-1.06	0.18	-1.20	-1.13	-0.07
Needle age 4	n.s.	n.s.	n.s.	-1.15	-1.18	0.00
Needle age 5	-1.01	-1.00	-0.02	n.s.	n.s.	n.s.
Bole-wood	-1.52	-1.52	0.00	-1.70	-1.63	-0.07
Inner-bark	-2.63	-2.47	-0.16	-2.95	-2.95	0.00
Outer-bark	-2.67	-2.45	-0.22	-2.90	-2.84	-0.06
Abs value mean:			**0.12 (0.10)**			**0.04 (0.04)**

Table 4.4. Discrimination factors for Ca/Sr between source and sink (for example, DF = $Ca/Sr_{foliage}$ / $Ca/Sr_{forestfloor}$) and separation factors for $\delta^{44}Ca$ between source and sink (for example, SF = $\delta^{44}Ca_{foliage} - \delta^{44}Ca_{forest\ floor}$) at a high-Ca and low-Ca site in the Coast Range of Oregon. Foliage digest total means across live age classes, bole-wood, outer-bark, xylem sap (February, June) and Ca-concentration-weighted extractions (exchangeable and leachable) for forest floor and 0-10 cm depth mineral soil were used to calculate discrimination factors and separation factors. Soluble Ca (water extraction) was considered the source of Ca while structural Ca (acetic-acid) and Ca-oxalate (HCl) were sinks of Ca in each plant tissue type, needles of young and older classes (year 1 at both sites, year 5 at the high-Ca site, year 4 at the low-Ca site), bole-wood and outer-bark.

	Ca/Sr Discrimination factors (DF)		δ^{44}Ca Separation factors (SF) ‰	
	High-Ca Site	Low-Ca Site	High-Ca Site	Low-Ca Site
Whole-plant:				
Forest floor to bole-wood	0.52	0.78	0.47	0.49
0-10 soil to bole-wood	0.86	1.00	0.51	0.09
Forest floor to twig xylem sap	0.63, 0.87	1.46, 1.42	0.60, 0.41	1.14, 0.13
0-10 soil to twig xylem sap	1.03, 1.43	1.88, 1.82	0.65, 0.46	0.73, -0.28
Forest floor to needles	1.35	1.36	1.04	1.02
0-10 soil to needles	2.22	1.75	1.09	0.62
Plant organ-level:				
Bole-wood to outer-bark	2.26	1.68	-1.15	-1.20
Bole-wood to twig xylem sap	1.20, 1.67	1.87, 1.82	0.14, -0.05	0.64, -0.37
Bole-wood to needles	2.58	1.74	0.58	0.53
Twig xylem sap to needles	2.15, 1.55	0.93, 0.96	0.44, 0.63	-0.11, 0.90
Plant tissue-level:				
Soluble Ca to structural Ca				
Young needles (age 1)	1.37	1.24	-0.30	-0.55
Older needles (age 5 or 4)	1.04	1.49	-0.04	-0.31
Bole-wood	0.41	0.33	-0.14	0.09
Outer-bark	0.70	0.62	0.37	0.09
Soluble Ca to Ca-oxalate				
Young needles (age 1)	2.30	0.22	-0.83	-0.69
Older needles (age 5 or 4)	6.01	1.93	-0.32	-0.71
Bole-wood	0.34	0.21	0.46	0.23
Outer-bark	0.51	0.36	-0.14	-0.05

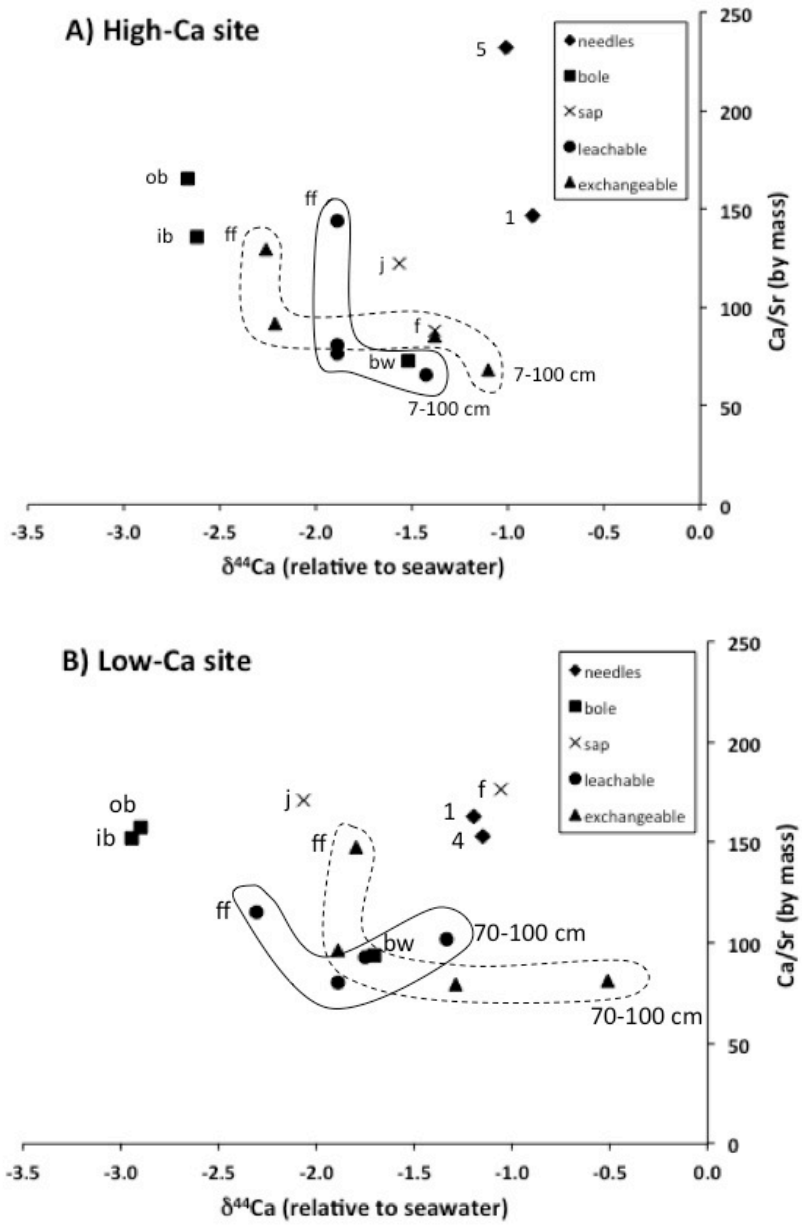

Figure 4.7. Ca/Sr versus $^{44}Ca/^{40}Ca$ at a A) high-Ca and B) low-Ca site as a multi-tracer approach to identifying soil sources of cations for trees. Plant tissues include: needle live age class (1, 4 or 5), bole tissues including bole-wood (bw), inner-bark (ib), and outer-bark (ob), xylem sap collected in February (f) and June (j). Soils include the forest floor (ff), 10-20 cm depth, 20-30 cm depth and 70-100 cm depth (70-100 cm) and are grouped by exchangeable Ca (dashed line) and leachable Ca (solid line) with decreasing soil depth in sequence from forest floor to 70-100 cm depth.

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CHAPTER 5 – CONTRIBUTION OF CALCIUM OXALATE TO SOIL EXCHANGEABLE-CALCIUM

Abstract

Standard methods for the determination of soil calcium (Ca) often focus on the exchangeable pool as the most dynamic available soil Ca pool. The exchangeable Ca pool is typically characterized using strong salts that purport to displace Ca^{2+} from clay and soil organic matter surfaces into solution for subsequent measurement. However, it is unknown to what degree other forms of soil Ca may contribute to exchangeable Ca measurement, and it has been suggested that biomineral Ca-oxalate does not contribute appreciably to exchangeable Ca measurements due to its inherent insolubility. We added calcium oxalate crystals to pure solutions of typical soil extractants in the laboratory, and found that Ca-oxalate partially dissolves in unbuffered salt solutions typically used to extract exchangeable-Ca, and differs depending on the type of salt extraction and concentration of the extractant solution. We also added calcium oxalate crystals to two forested Andisols of contrasting low- and high-Ca status, and found greater recovery of these spikes in exchangeable solutions than expected from pure solution studies. We found that in soil extractions, a 10-fold difference in ionic concentration of the extractant has more effect on Ca-ox dissolution than extraction pH, which ranged from 5 to 7. Recovery of spikes in soil extraction with 1 M ammonium acetate allowed us to estimate that extraction-dissolved Ca-ox could potentially contribute an additional 52% to standard measurements of exchangeable-Ca pools in a low-Ca site. However, Ca-ox was more difficult to detect against the large background pool of exchangeable Ca at a high-Ca site. Ca-oxalate is subject to different soil chemical and biological processes than other chemical Ca pools, and could be a substantial portion of soil Ca. Methods to independently quantify Ca-oxalate need more development but could be effective when both Ca and oxalate ions are measured.

Introduction

Calcium (Ca) is a macronutrient that undergoes intense recycling between plants and soils in terrestrial ecosystems (McLaughlin and Wimmer 1999). In the soil, Ca may exist in multiple chemical forms including soluble Ca, exchangeable Ca, Ca bound in organic matter, mineral-Ca, or biomineral-Ca. Approaches to investigating various chemical forms of Ca and their soil dynamics and plant-availability are lacking, despite considerable interest in understanding various chemical forms of other macronutrients such as nitrogen and phosphorous (Schlesinger, 1997). Nearly all ecosystem studies measure soils via exchangeable-Ca, which can be defined two ways. Mechanistically exchangeable-Ca is defined as hydrated Ca^{2+} ions that are held by negatively charged soil surfaces through relatively weak electrostatic and nonspecific interactions (Essington, 2004). Or, operationally, exchangeable-Ca can be defined as it is measured; an ion that is removed from the soil by a solution containing a neutral salt, as opposed to soluble-Ca, for example, which can be removed by water. Methodological and operational definitions of exchangeable-Ca are only equivalent if exchangeable-Ca procedures target only cations that are nonspecifically adsorbed to negative soil surfaces, with no additional contribution from other potential soil Ca sources (Essington, 2004).

Calcium-oxalate (hereafter Ca-ox in reference to Ca and oxalate bound as a molecule) is a ubiquitous biomineral that is by far the most abundant ionic organic mineral in natural environments, and commonly occurs in living plants, animals and fungi as well as non-living carbonate concretions, marine and lake sediments, hydrothermal veins and lignite coal (Echigo and Kimata, 2011; Baran and Monje, 2008). In terrestrial ecosystems the presence of Ca-oxalates are attributed primarily to formation within tissues of plants and fungi (Franceschi et al. 2005; Arnott, 1995) and in soil via Ca^{2+} precipitation with oxalate anion exudates from plants and fungi (Jones, 1998; Ryan et al. 2001; Dutton and Evans, 1996). Calcium-oxalate has been shown to accumulate in several soil types (Certini et al. 2000; Graustein et al. 1977; Verrecchia and Dumont, 1996), and is particularly common in association with ectomycorrhizal fungal mats (Cromack et al. 1979), and in detritus with wood-rot fungi (Dutton and Evans, 1996). Quantification of Ca-ox in soils has been attempted by only a few studies (Dauer, Chapter 2), yet it has been suggested that Ca-ox may contribute substantially

to ecosystem Ca budgets (Bailey et al. 2003) and play a significant role in Ca cycling (Smith et al. 2009).

Ca-ox crystals have no charge and are not held electrostatically to negatively charged soil surfaces, and so would not fit within the mechanistic definition of soil exchangeable-Ca. Routine extractions of soil exchangeable-Ca occur at the native pH of the soil, or buffered at a higher pH (Sparks, 1996), which implicitly assumes that Ca originating as Ca-ox crystals (hereafter Ca_{ox} in reference to Ca ions once bound in Ca-ox) is omitted because it is soluble only in very acidic conditions (pH <2) (Cromack et al. 1979). However, solubility may increase with increasing ionic strength of the solution due to higher entropy, known as the neutral salt effect. Accordingly, the solubility of Ca-ox has been observed to increase when subjected to high ionic strength tissue cultures (Hoover and Wijesinha, 1945; Belliveau and Griffin, 2001), suggesting that some portion of Ca_{ox} may dissolve during salt extraction and be measured as exchangeable-Ca, though this has not been tested explicitly. The potential inclusion of Ca_{ox} in measurements of exchangeable-Ca may be problematic in soil and ecosystem studies because the dynamics of crystalline Ca-ox are likely to differ from the ionic form of exchangeable-Ca. For example, it is commonly implied that the soil pool of exchangeable-Ca is available for plant uptake by delivering Ca ions to the soil solution (White, 2001; Sparks, 2003), whereas crystalline Ca-ox is unlikely to diffuse readily and be directly absorbed by plant roots due to its large size.

The objectives of this study were three-fold: 1) Determine the effect of ionic strength on the dissolution of Ca-ox by estimating the equilibrium constant of Ca-ox in solutions commonly used to extract exchangeable-Ca. We expected Ca-ox to partially dissolve in solutions that are used to extract exchangeable-Ca, and that dissolution would increase with higher ionic concentrations; 2) Determine the recovery of Ca_{ox} in both standard and modified soil extractions of exchangeable-Ca; 3) Compare the quantity of Ca in different Ca pools in soil, including estimates of exchangeable-Ca, Ca_{ox}, Ca bound in organic matter and whole-rock Ca digest, to more fully understand the comparative importance of exchangeable-Ca and Ca-ox in soil Ca availability.

Materials and Methods

Ca-ox solubility in salt solutions

To investigate how much Ca-ox is dissolved by salt solutions typically used to measure soil exchangeable-Ca we determined the equilibrium constants of Ca-ox in unbuffered ammonium acetate (NH_4OAc), ammonium chloride (NH_4Cl) and barium chloride ($BaCl_2$). We used a range of molarity (0.1 M, 1 M and 2 M) of a common extractant, NH_4OAc, to test how extractant concentration influences calcium oxalate dissolution. We determined Ca-ox solubility in solutions of hydrochloric acid (HCl) and nitric acid (HNO_3) as controls intended to dissolve/dissociate the majority of Ca-ox crystals (Lilieholm et al. 1992; Bullen and Bailey, 2005). We also determined Ca-ox solubility in solutions of acetic acid (HOAc) that dissolves only small amounts of Ca-ox for interest as potential soil extractants of Ca-ox. Ca-ox crystals (mono-hydrate crystals 99.9% pure, Sigma-Aldrich) were prepared by drying for 12 hours at 45°C, and weighed in three replicates of 0 (control), 0.5, 1, 10, 50 mg and added to 50 ml Falcon tubes. Subsequently, 20 ml of either 0.1 M NH_4OAc, 1 M NH_4OAc, 2 M NH_4OAc, 1 M NH_4Cl, 0.1 M $BaCl_2$, 0.5 *N* HCl, 1 *N* HNO_3, 2 *N* HOAc and nanopure water (H_2O) were added to the sample. If Ca-ox was completely dissolved, solutions would contain 0, 0.365, 7.3, 73, 365 mol Ca L^{-1}, which encompass the range of estimated Ca_{ox} in forest soils (Dauer, Chapter 2). Samples were shaken at ~23°C for 20 hours, then filtered through disposable 25 mm Acrodisc 1μm Glass Fiber filters attached to 60 ml syringes. Calcium concentrations were measured using atomic absorption spectrophotometry on an AAnalyst 200 Spectrometer (AA), Perkin Elmer (Waltham, MA, USA). The solubility product of Ca-ox in water (K_{sp}) was calculated as $\gamma^2[Ca^{2+}][C_2O_4^{2-}]$ where γ = activity coefficients of ions and γ=1 for water. For all other solutions, equilibrium concentration products were calculated as $[Ca^{2+}][C_2O_4^{2-}]$. The molarity of oxalate anions was assumed via stoichiometry where within Ca-ox molecules, one mol of Ca^{2+} binds to one mol of oxalate. After the dissolution, solution pH was measured with a pH probe (Fisher Scientific Accumet AR20 meter, with an AccuFast pH combination electrode), except for solutions of 0.1 M NH_4OAc and 0.1 M $BaCl_2$, which were estimated based on original solution. The ionic strength of the extractant solution (*I*) was estimated with the equation:

$$I = \frac{1}{2}\sum_{i=1}^{n} c_i z_i^2$$

Where c_i is the molar concentration of ion *i*, z_i is the charge number of ion *i*, and the sum is taken over all the ions (*n*) in the solution.

Soil exchangeable analysis of Ca-ox spike-recovery

We tested the dissolution of Ca-ox crystals during routine extractions of exchangeable-Ca from soils. To understand how soil Ca concentration may influence the dissolution of calcium oxalate crystals, we used two forest soils representing high and low levels of exchangeable-Ca. Both sites were Andic Dystrudepts located in the north-central Oregon Coast Range, with 1 M NH_4OAc mineral soil exchangeable-Ca (0-10 cm depth) of 564 kg Ca ha^{-1} at the high-Ca site (Site 5), and 93 kg Ca ha^{-1} at the low-Ca site (Site 16) (Perakis et al. in prep). Soils at the high-Ca site had 53% sand, 23% silt, 25% clay and a $pH_{(H2O)}$ of 5.50 in 0-10 mineral soils. Soils at the low-Ca site had 30% sand, 31% silt and 39% clay and a $pH_{(H2O)}$ of 4.61 in 0-10 mineral soil. In June 2010 at each site three samples of mineral soils were removed from 0-10 cm depth with a 5.1 cm diameter soil corer after brushing aside the forest floor layer. Soils were sieved to 2 mm to remove rocks and debris and to homogenize the soil, samples were composited by site, and stored for less than one week at 4°C before analysis.

We examined the dissolution of Ca-ox in mineral soil extractions in response to several extraction concentrations and temperatures. We used unbuffered 1 M NH_4OAc for these tests, which is among the most common extractants used for exchangeable Ca in forest soils (Sparks, 1996). To investigate the amount of Ca-ox that could be dissolved during analysis with soil, we used a Ca-ox spike-recovery technique calculated as the difference in Ca concentration between a soil spiked with Ca-ox and a control-unspiked sample. The percent-recovery indicates the amount of the spike dissolved during the extraction. We used 10-fold higher Ca-ox spikes at the high-Ca site, commensurate with approximate differences in exchangeable-Ca between the two sites. We tested the potential for a lower-ionic strength extraction solution to remove exchangeable-Ca without dissolving a large amount of Ca-ox,

and therefore offer a more accurate estimate of exchangeable-Ca, by examining 0.1M NH_4OAc as an extractant. To determine if temperature may play a role in dissolving Ca-ox during extraction, either by changes in entropy or changes in enzymatic activity of microbes, we compared a cold treatment (4°C) to extraction at room temperature (23°C). We used $CaCl_2$ as a highly soluble spike to examine the inherent reproducibility and efficacy of spike-recovery tests in these soils, and also to determine if Ca precipitation or adsorption occurs subsequent to extraction, which would interfere with the ability to relate Ca-ox dissolution in solution experiments to those with soil. We also determined the specificity of our analysis to concentrations of Ca-ox in the soil by adding two extremely high Ca-ox spikes. These treatments for both high- and low-Ca soils are detailed in Table 1 and included: 1) control (i.e., no Ca-ox spike added) with 1 M NH_4OAc extractant, 2) control with 0.1 M NH_4OAc extractant, 3) a Ca-ox spike at one-third the exchangeable-Ca concentration for each soil with 1 M NH_4OAc extractant, 4) a Ca-ox spike at one-third the exchangeable-Ca concentration for each soil with 0.1 M NH_4OAc extractant, 5) a Ca-ox spike and extraction equivalent to treatment 3 performed at 4°C, 6) a $CaCl_2$ spike at one-third the exchangeable-Ca concentration for each site with 1 M NH_4OAc extractant, 7) a five-times and 8) ten-times higher Ca-ox spike with 1 M NH_4OAc extractant. Each treatment was replicated three times (2 soils x 8 treatments x 3 reps = 48 samples total). For the above treatments, we adapted standard methods of soil Ca extraction (Sparks, 1996) by weighing 10 g (dry weight) of moist soil into Falcon filters fitted with 0.7 μ particle retention Whatman GF/F filters, adding 25 mL of extracting solution, gently stirring with a glass rod, then suction filtering after 10 minutes. Subsequently, 25 mL of extractant were added, and left at 23°C for 12 hours (except cold treatment samples which were left at 4°C), then suction filtered. Calcium concentrations were measured on all extractants by atomic adsorption spectrophotometry as above with the caveat that this technique cannot discern free Ca^{2+} ions from dissolved complexed Ca. After the extraction, extractant solution pH was measured with a pH probe.

To compare the importance of various soil Ca fractions to total Ca, we estimated concentrations of Ca in exchangeable Ca, Ca-ox, Ca bound in organic matter and total-Ca in 0-10 cm soil collected from the low-Ca site only. Calcium fractions were measured in both field-collected soil and in soil spiked with 10 mg calcium oxalate in the laboratory.

Exchangeable Ca was determined by extracting 8 g of soil using 50 ml of 0.1 M NH_4OAc, as above. Calcium oxalate was determined by extracting 2.5 g of soil with 20 ml 0.5 M HCl for 5 hours (Lilieholm et al. 1992). Calcium bound in organic matter water estimated by combusting 2.5 g of soil at 350°C (12 hrs), followed by extraction with 20 ml 0.5 M HCl for 5 hours, and then subtracting Ca_{ox} determined in the prior step. All samples were filtered and soluble Ca was analyzed as above. Total calcium was determined on whole rock digests via lithium metaborate/tetraborate fusion and dilute nitric digestion, with analysis by inductively coupled plasma spectroscopy, at Acme Labs, Vancouver BC. There was no replication in this portion of the experiment.

Data analysis

In tests of Ca-ox dissolution in salt solutions without soil, we used two-way analysis of variance (ANOVA) to determine the effect of extractant and Ca-ox addition level on the percent dissolution of Ca-ox spikes and on equilibrium concentration products. Comparisons were made between solutions across Ca-ox addition levels, between Ca-ox addition levels within a solution, and between solutions within a Ca-ox addition level using LSMEANS. For the spike-recovery experiment, separate one-way ANOVAs were used for the low-Ca and high-Ca soils to determine differences in soluble Ca among treatments. Comparisons between each treatment and the control were performed using LSMEANS to determine whether the treatment would dissolve Ca-ox. The low-Ca and high-Ca soils were known to differ in exchangeable Ca concentrations (Perakis et al. in prep) and so were not directly compared to one another. All models were fit to the data using PROC MIXED in SAS v9.2 (SAS Institute Inc., Cary, NC).

Results and Discussion

Solubility experiments

Ca-ox has been considered inert in soil chemical analyses that occur at neutral pH (Cromack et al. 1979; Bailey et al. 2003). However, we found that Ca-ox partially dissolves in

several common pH-neutral salt solutions used in the determination of soil exchangeable-Ca. Crystals dissolve until a chemical equilibrium is reached with the salt solution, which may result in complete dissolution even in low ionic strength solutions if the amount of Ca-ox is small. For example, 1 mg of Ca-ox completely dissolved in 20 ml of 0.1 M $BaCl_2$ (Table 5.2). All of the typical salt solutions used for exchangeable cation determination dissolved Ca-ox to a greater extent than did water (two-way ANOVA, $P<0.001$, Table 5.2). The maximum observed dissolved Ca_{ox} from salt solutions was 36.1 mg Ca_{ox} L^{-1} in 0.1 M $BaCl_2$, which was 10-fold higher than water. Ionic concentrations of exchangeable cation extractants (I= 0.1 to 2, Table 5.3) are several orders of magnitude higher than native soil solution (e.g., 0.006 M, (Gillman and Bell, 1978). This disparity in ionic strength of soil versus extraction solutions and the resultant effects on Ca-ox solubility highlights the dramatic chemical shift that the addition of the highly concentrated extractants may have on soil. In this way, common salt extractants may release some Ca_{ox} into solution as free Ca in excess of what is typically available for immediate plant uptake and/or leaching loss.

In our solubility experiment with water and strong acids Ca-ox crystal dissolution behaved as expected, with low dissolution in water and near complete dissolution in strong acids. The solubility products in H_2O were calculated for each level of Ca-ox addition, though solubility products estimated from lower levels of Ca-ox addition were closest to standard values (Table 5.3). Our estimation of K_{sp} in H_2O, 2.6 (±0.2 SE) x 10^{-9}, at lower levels of Ca-ox addition matched the K_{sp} reported in literature of 2.57 x 10^{-9} at an ionic strength (I) of zero and at room temperature (Ringbom 1963). Ca-ox dissolved completely in acid solutions (i.e., HCl, HNO_3), probably via both dissolution and dissociation from acidic reactions; the highest level of Ca-ox addition (50 mg) was 97% (±0.4 SE) and 94% (±1.3 SE) dissolved in 20 mL of 1N HNO_3 and 0.5 *N* HCl, respectively (Table 5.2).

A change in solubility of a solute in a salt solution that has no ion in common with the solute is considered a neutral salt effect, where an increase in solution entropy increases dissolution of the solute (Mendham et al. 2000). The neutral salt effect likely explains our observations of Ca-ox dissolution in salt solution where the ionic strength of the salt influenced the amount of Ca-ox dissolution. As expected, when the concentration of the

solution increased in molarity, the dissolution of Ca-ox increased. Ca-ox dissolution increased with higher concentrations of NH_4OAc so that dissolution in a 2 M solution was no different from a weak acid of the same molarity, 2 *N* HOAc, where Ca-ox dissociation may also occur. Between 0.1 M and 1 M NH_4OAc, dissolution of Ca-ox increased (two-way ANOVA, $P<0.01$, Table 5.2), although not as much as proportional differences in solution molarity might suggest; the 10-fold more concentrated solution dissolved only 2-fold more Ca_{ox} in the three highest levels of Ca-ox addition (1, 10, 50 mg). When NH_4OAc concentration increased 2-fold between 1 M and 2 M NH_4OAc, there was a 1.4-fold increase in Ca_{ox} that dissolved in the highest Ca-ox level, though differences were not statistically significant between the two extraction solutions. This non-1:1 relationship is likely due to thermodynamics of dissociation that have equilibrium constants that are much lower than chemical reactions which cause complete dissolution, such as strong acids like HCl and HNO_3.

The molarity of extractant solutions affected dissolution similarly regardless of the type of counter-anion involved. For example, the dissolution of Ca-ox crystals was similar in 1 M NH_4OAc and 1 M NH_4Cl at the two highest levels of Ca-ox addition (Table 5.2). However, the type of cation in extractant solutions affected Ca-ox dissolution. For example, 0.1 M $BaCl_2$ dissolved nearly 4-times more Ca-ox on average than 0.1 M NH_4OAc (0.1 M). The divalent charge of barium (Ba^{2+}) and lower ionic radius (Ba^{2+} = 1.35 Å) as compared to monovalent ammonium (NH_4^+) with a higher ionic radius (NH_4^+= 1.43 Å) yields a 4-fold higher ionic potential (2.96 in Ba^{2+} versus 0.70 in NH_4^+), which allows Ba^{2+} to more strongly attract oxalate anions. Additionally, the ionic strength of the $BaCl_2$ solution was 4-fold higher than the NH_4OAc solution (0.4 in $BaCl_2$ versus 0.1 in NH_4OAc) due to its divalent charge (Table 5.3).

Although we tested only monohydrate Ca-ox in this experiment, crystal solubility is also influenced by crystal phase properties, suggesting that di-hydrate Ca-ox would likely have different dissolution behaviors. However, monohydrate Ca-ox (or whewelitte) is more common and more stable than di-hydrate Ca-ox (weddellite) and is much more prevalent in natural systems (Dana, 1997; Baran and Monje, 2008). Therefore, dissolution information

provided in this study may be a conservative assessment of calcium oxalate behavior overall, with wide applicability to acid forest soils.

Ca-ox spike/recovery in soil exchangeable-Ca analysis

We have thus far discussed the dissolution of calcium oxalate in pure solutions of water, acids, and extraction salts used in standard soil analyses. To better evaluate the amount of Ca-ox that could dissolve in actual soil extractions for exchangeable cations, we compared Ca_{ox} concentrations in soil extractions to the estimated amount of dissolved Ca_{ox} at equilibrium from solution experiments, i.e., the point at which no additional Ca-ox could go into solution. We used the Ca_{ox} concentration at the highest level of Ca-ox addition as an approximation of the amount of dissolved Ca_{ox} at equilibrium. For example, we found that 1 M NH_4OAc has the potential to dissolve up to 14.6 mg Ca_{ox} L^{-1} in aqueous solutions. We compared this Ca_{ox} concentration to soil extractions, which used *two* sequential 25 mL solutions to extract 10 g of soil (Sparks, 1996). If Ca-ox dissolution was the same in both procedures, we would predict a maximum dissolution potential of 0.37 mg Ca_{ox} *x 2 extractions*, or 0.74 mg Ca_{ox} total, for 10 g of soil. Below we evaluate how this dissolution potential may influence estimates of exchangeable-Ca in low- and high-Ca soils.

In 1 M NH_4OAc extraction of low-Ca soil, we extracted 0.98 mg Ca_{ox} from a Ca-ox spike containing a total of 3.2 mg Ca_{ox}, (31% dissolution), which is slightly more Ca than the predicted dissolution of 0.74 mg Ca_{ox} determined in aqueous 1 M NH_4OAc (Figure 5.1A). The increased dissolution of Ca-ox in the experiment containing soil could be due to lower pH and/or higher ionic strength of the soil extraction. Therefore, if Ca-ox were present in the low-Ca soil, a substantial portion of it would be indistinguishable from "exchangeable-Ca" using standard exchangeable-Ca methods. The dissolvable amount of Ca-ox in 1 M NH_4OAc (0.10 mg Ca g^{-1}) has the potential to increase the measured amount of exchangeable Ca in these low-Ca soils (0.19 mg Ca g^{-1}) by 52%. Some portion of the Ca-ox spikes were dissolved in soil extracted with 1 M NH_4OAc in all levels of Ca-ox addition, as evidenced by significantly greater Ca measured in spiked compared to the non-spiked control in low-Ca soils (compared *within* 1 M NH_4OAc controls, one-way ANOVA, $P>0.001$, Figure 5.1A). A portion of the Ca-ox spike was even detected in a 10-times less concentrated extractant (0.1 M NH_4OAc),

indicating the susceptibility of crystal to dissolution in this type of procedure (compared *within* 0.1 M NH_4OAc controls, one-way ANOVA, $P<0.001$, Figure 5.1A). This suggests that molarity adjustments to conventional soil extraction methods are unlikely to entirely eliminate dissolution of Ca-ox in the determination of soil exchangeable-Ca.

In the high-Ca soil, we could not detect additional Ca from the dissolution of Ca-ox spikes due to the high background exchangeable-Ca concentration of 2 mg g^{-1} soil (i.e., 10-fold higher than the low-Ca site, Figure 5.1B). The predicted 0.74 mg of dissolved Ca_{ox} in two 25 ml extractions with 1M NH_4OAc was only 3.5% of the amount of exchangeable-Ca present in the high-Ca control soil (20 mg exchangable-Ca in 10 g of soil), which was below the average range of experimental variability in replicated extraction measurements at the high-Ca site (± 10%). Only the $CaCl_2$ in 1 M NH_4OAc, and the high Ca-ox spikes (i.e., 500 mg and 1000 mg Ca-ox in 1 M NH_4OAc) were higher than the 1 M NH_4OAc control (one-way ANOVA, $P<0.001$, Figure 5.1B), yielding mean spike recovery rates of 92% (100 mg Ca-ox), 2.5% (500 mg Ca-ox) and 2.6% (1000 mg Ca-ox) for each treatment, respectively. Forest soils are unlikely to have Ca-oxalate concentrations as high as the spikes used in this experiment. The highest reported Ca-ox literature value was 3.32 mg Ca_{ox} g^{-1} soil in ectomycorrhizal mats (Cromack et al. 1979) whereas our high spikes in the high-Ca soil represent 16 and 33 mg Ca_{ox} g^{-1} soil (spike tmt 7 and 8, Table 5.1). In comparison, the lower level spikes added to the low-Ca soil (0.3 mg Ca_{ox} g^{-1} soil, spike tmt 3, Table 5.1), which more closely resemble native soil Ca-ox concentrations, underwent 31% dissolution and increased total soil exchangeable-Ca levels by 52%. Therefore, unless Ca-ox soil concentrations are extremely high, our results suggest that high-Ca soils may be less susceptible than low-Ca soils to be strongly biased by Ca-ox dissolution in measurements of exchangeable-Ca. The wide variance in dissolution properties of Ca-ox in standard exchangeable cation methods may make cross-site comparisons difficult when background Ca levels also vary, and add complexity to calculating soil Ca pools for mass balance budgets.

The cold spiked treatment extracted at 5°C at the low-Ca site yielded less Ca-ox recovery than the same spike and extraction conducted at 23°C (one-way ANOVA, $P=0.03$, Figure 5.1A). Several factors may explain this difference. Temperature affects solubility by

changes in entropy, and as an endothermic reaction, solubility is predicted to increase with increasing temperature (Le Chatelier's Principle). In addition, the lower temperature extraction may have decreased oxalotrophic microbial activity, which is primarily enzymatic (Khammar et al. 2009; Svedruzic et al. 2005) and is therefore likely suppressed at lower temperatures. Soil microbes that utilize oxalate for energy, electron transfer and carbon are widespread (Verrecchia et al. 2006; Sahin, 2003), and although mechanisms by which carbon in crystalline Ca-ox is accessed by microbes is unstudied, oxalate originating as Ca-ox has been observed to be degraded by microbes at relatively rapid rates in petri cultures (Braissant et al. 2004; Jayasuriya, 1955). Additional work is needed to determine the actual mechanism(s) of temperature effects on Ca-ox dissolution, as such effects may also be relevant in field degradation of Ca-ox in soils.

Differences in pH between low-Ca (4.6) and high-Ca (5.5) soils may contribute to dissolution of additional Ca-ox in the low-Ca soil. Ca-ox solubility is highly pH-dependent (Gadd, 1999) and our spikes were completely dissolved in the highly acidic HCl and HNO_3 solutions we examined. However, despite the acidity of both soils, extractions occurred at a pH that is higher than the soil solution due to the > 7 pH of NH_4OAc solutions. In the high-Ca soil the final solution pH was 7.1 and 5.7 in 1 M NH_4OAc and 0.1 M NH_4OAc, respectively. In the low-Ca soil the final solution pH was 6.2 and 5.7 in 1 M NH_4OAc and 0.1 M NH_4OAc, respectively. The higher pH measured in 1M NH_4OAc extractions may be due to greater buffering by this more concentrated extractant after the addition of soil. Despite the consistently lower soil pH that resulted from extraction with the more dilute 0.1M NH_4OAc, this extraction nevertheless dissolved a smaller percentage of the Ca-ox spike than 1 M NH_4OAc, suggesting that differences of extraction pH in the range of 5 to 7 had less of an effect than the 10-fold difference in ionic concentrations of extractant.

Determining an alternative solution for exchangeable cation extraction that excludes Ca-ox proves to be a difficult problem. Our solubility experiments indicated Ca-ox dissolution in NH_4OAc (0.1 M) was the most similar to dissolution in H_2O (Table 5.2), which raises the possibility that 0.1 M NH_4OAc could be an alternative exchangeable-Ca extractant that does not dissolve a substantial amount of Ca-ox. In the soil experiment using low-Ca soil, 0.1 M

NH_4OAc dissolved less Ca-ox crystals (15% recovery of 10 mg Ca-ox) than 1 M NH_4OAc (31% recovery). However, the exchangeable-Ca concentration using 0.1 M NH_4OAc (control treatment) was less than 1 M NH_4OAc (one-way ANOVA, $P<0.001$) by 49% in the high-Ca soil and 61% in the low-Ca soil, suggesting that more dilute concentrations of extractants may not give comparable baseline exchangeable-Ca concentrations. Extracting at a higher pH (7) may have dissolved less Ca-ox in our low-Ca soil, and may provide another way to exclude Ca-ox from exchangeable-Ca extractions. However, extractions that occur at a high pH in buffered NH_4OAc are known to overestimate CEC and underestimate exchangeable-Ca as compared to ammonium salts (Borge, 1997; Skinner et al. 2001), because increasing the negative charges on the surface of soil results in stronger adsorption of Ca^{2+} ions. Where calcium oxalate is expected to contribute substantially to measurements of exchangeable Ca, it may be possible to independently measure Ca-ox by measuring both oxalate and Ca ions and using stoichiometric calculations to account for Ca_{ox} contributions to extracted Ca.

Soil extractions with dilute HCl may be effective in quantifying Ca-ox independently from exchangeable-Ca by measuring both Ca ions and oxalate ions in solution (Lilieholm et al. 1992). It is likely that freed oxalate measured in these acid extracts was once bound to Ca, as Ca-ox is by far the most abundant and least soluble metal oxalate (Baran and Monje, 2008; Echigo and Kimata, 2011), although measurements of oxalate would confirm this assumption. We compared soil extractions with dilute HCl to extractions with NH_4OAc in a smaller subset of unreplicated samples from the low-Ca site. In a 0.5 M HCl acid-extraction we recovered 83% of the Ca from a Ca-ox spike in the low-Ca soil (0.22 mg Ca g^{-1} in control unspiked soil versus 0.46 mg Ca g^{-1} in spiked soil), suggesting that the dilute acid was effective in extracting the majority of Ca-ox crystals although this was not confirmed with measurements of oxalate ions. In comparison, the 0.1 M NH_4OAc extraction recovered only 18% of the Ca from a Ca-ox spike in the low-Ca soil (0.22 mg Ca g^{-1} in control unspiked soil versus 0.46 mg Ca g^{-1} in spiked soil), similar to the larger study at the low-Ca site, which recovered 15% of the spike. However, interestingly, the 0.5 M HCl acid extraction yielded 4-fold more Ca than soil exchangeable-Ca (0.22 mg Ca g^{-1} in HCl versus 0.06 mg Ca g^{-1} in NH_4OAc). This suggests a sizeable pool of Ca-ox in mineral soil that is not detected with exchangeable-Ca analysis, and

which has unknown dynamics as a source of Ca for plant uptake, leaching loss, and other ecosystem processes.

Given the emphasis placed on Ca returns from litter as an important source of plant nutrition (Likens et al. 1998; Blum et al. 2008), and the high organic matter content of 0-10 cm mineral soils this site (low-Ca site = 9.22 %C, Perakis and Sinkhorn, 2011) we expected that combustion of the soil before 0.5 M HCl extraction would release additional Ca bound in organic matter. Instead we found little difference in Ca concentration of acid extractions between combusted and non-combusted soil (<0.01 mg Ca g^{-1}) in both spiked and non-spiked soil. This suggests that either Ca in organic matter is a small pool of soil Ca compared to Ca-ox in this soil, or that extraction with 0.5 N HCl could access Ca that is operationally indistinguishable between Ca-oxalate, mineral-Ca and organic-Ca. Additionally, the combusted sample was only 12% of the Ca in a whole-rock digest (1.93 mg Ca g^{-1} soil) suggesting that a large portion of Ca exists in inaccessible Ca that did not dissolve with 0.5 N HCl.

Addition of $CaCl_2$ spikes to the soil was intended to provide an easily dissociated form of Ca that could be used to estimate soil effects on extractable Ca recovery independent of effects of Ca-ox addition. $CaCl_2$ is expected to dissolve 745 mg $CaCl_2$ L^{-1} at 20°C and 1 atm, so we expected that 0.2 mg $CaCl_2$ L^{-1} (low-Ca soil) and 2.0 mg $CaCl_2$ L^{-1} (high-Ca soil) would be 100% dissolved in soil extraction with 1 M NH_4OAc. We measured 80% and 92% Ca recovery from $CaCl_2$ spikes in the low- and high-Ca soil (respectively), suggesting that there may be a small amount of interference in soil that complexes or adsorbs some free Ca^{2+} ions. This may have happened to a greater extent in the low-Ca soil due to higher clay content (39% versus 25% at the high-Ca site). Although, because the proportion of unrecoverable Ca from $CaCl_2$ was lower (8%) compared to recoverable Ca from Ca-ox (15 to 30%) at the low-Ca site, we consider it likely that interference did not occur to the extent that experiments containing soil were incomparable to solubility experiments.

How much does Ca-ox potentially contribute to measures of exchangeable Ca across forest soils?

Assuming a bulk density of 0.48 g cm^{-3} in 0-10 cm in low-Ca soil where this sample was taken (Perakis and Sinkhorn, 2011) and using data from our high-Ca spike (100 mg), the amount of Ca_{ox} dissolved during exchangeable-Ca measurements could be as high as 188 kg Ca_{ox} ha^{-1}. While the concentration of Ca-ox in soils is relatively unstudied (Dauer, Chapter 2), one estimate ranged from 95 to 1895 kg Ca_{ox} ha^{-1} (Cromack et al. 1979). Calcium oxalate can vary even more when other soil types are considered, yet generally mechanisms of calcium oxalate accretion and dissolution remain poorly characterized (Dauer, Chapter 2). Given that values of exchangeable-Ca reported in some temperate forest surface soils range from 160 and 3600 kg ha^{-1} (Cole and Rapp, 1981), even limited quantities of Ca-ox have the potential to be important for Ca dynamics, especially in low-Ca soils.

Conclusions

Standard methods for the measurement of exchangeable soil cations likely include some portion of the Ca-ox pool. The contribution of calcium oxalate to exchangeable pools may be especially large (as a fraction of total) in low-Ca soils. Measurements of exchangeable-Ca are often used mechanistically or to indicate soil function, such as quantifying the pool of cations available for plant uptake, leaching loss or other processes in the soil. Our results highlight potential limitations to application of operational techniques to quantify Ca pools in soil, and suggest that operationally defined methods may be more critically considered in a comparative sense, as an index of soil cation status. Analyses of cations in soil, especially Ca, may be improved upon to contain more functional information in order to more fully understand ecosystem Ca biogeochemical processes.

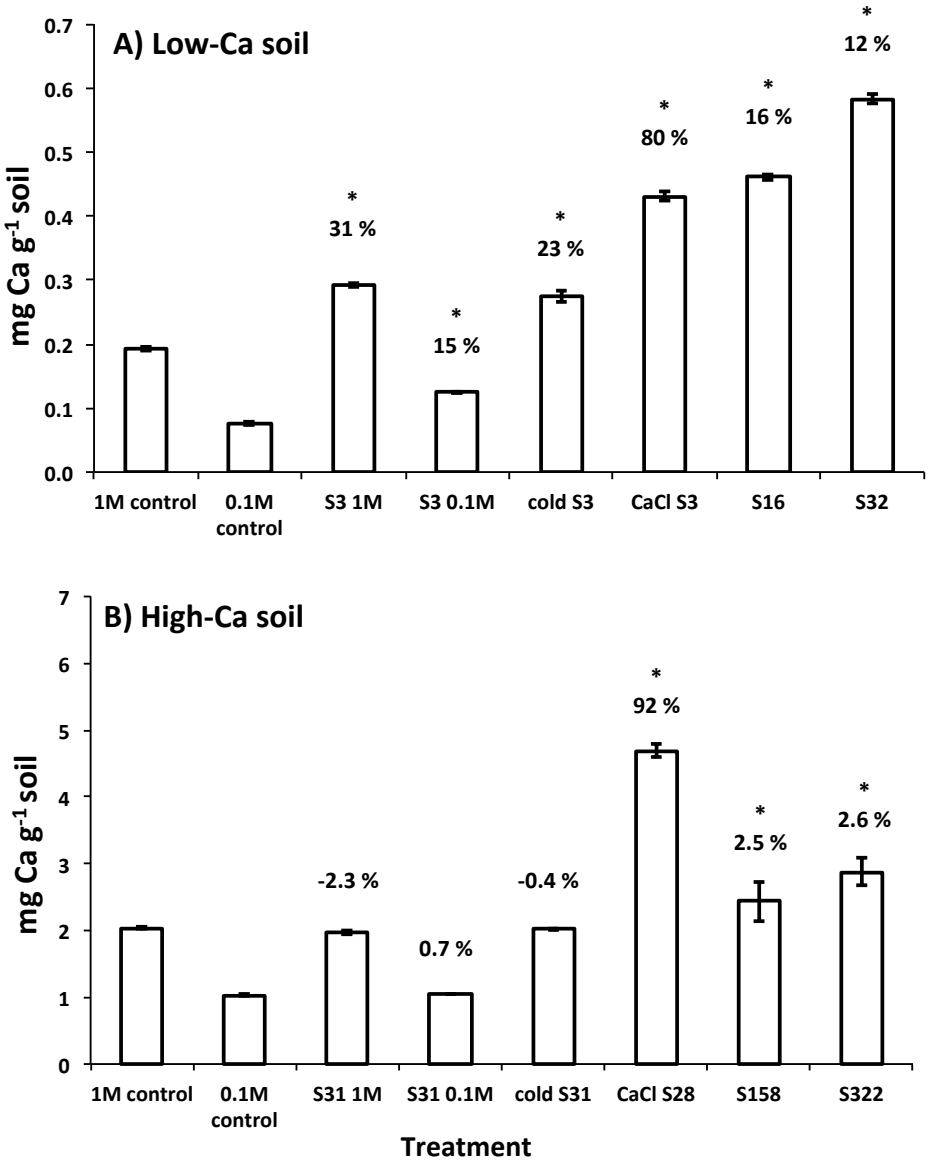

Figure 5.1. A) Low-Ca soil and B) high-Ca soil Ca concentrations (mg Ca g^{-1} soil) spiked with Ca-ox (S10=3 mg Ca_{ox}; S50=16 mg Ca_{ox}; S100=32 mg Ca_{ox}; S500= 158 mg Ca_{ox}; S1000= 322 mg Ca_{ox}) or $CaCl_2$ ($CaCl_2$ S10=3 mg Ca from $CaCl_2$; S100= 28 mg Ca from $CaCl_2$) and extracted with either 1 M NH_4OAc (1M) or 0.1 M NH_4OAc (0.1M). The percent recovery of the spike is indicated above each bar and was calculated by comparing the concentration of the spiked sample to the concentration of the control. Bars with (*) indicate significantly different from their respective 1 or 0.1 M NH_4OAc control (one-way ANOVA, $P<0.05$).

Table 5.1. Treatments in soil exchangeable analysis of Ca-ox spike-recovery for both the high and low Ca-soils. Concentration of extractants and amount of spikes (mg Ca-ox) are given. The cold Ca-ox spike was extracted at 4°C and all other samples were extracted at 23°C. Soil weight was approximately 10 mg dry weight, and extraction occurred twice with 25 ml each time.

Treatment	Extractant	Ca-ox Spike (mg Ca-ox)		Ca-ox Spike (mg Ca_{ox})	
		Low Ca site	High Ca site	Low Ca site	High Ca site
1) Control 1	1 M NH_4OAc	0	0	0	0
2) Control 0.1	0.1 M NH_4OAc	0	0	0	0
3) Ca-ox spike 1	1 M NH_4OAc	10	100	3	31
4) Ca-ox spike 0.1	0.1 M NH_4OAc	10	100	3	31
5) Cold Ca-ox spike	1 M NH_4OAc	10	100	3	31
6) $CaCl_2$ spike	1 M NH_4OAc	10	100	3	28
7) 5-x Ca-ox spike	1 M NH_4OAc	50	500	16	158
8) 10-x Ca-ox spike	1 M NH_4OAc	100	1000	32	322

Table 5.2. Percent dissolution of Ca in a range of Ca-ox additions measured as dissolved Ca_{ox} (mg L^{-1}) in seven solutions after 20 hours at 25°C at each approximate amount of Ca-ox (mg) added. There were significant interactive and main effects of solution type and amount of Ca-ox added to solution (two-way ANOVA, P<0.001). Standard error in parenthesis (n=3). Lower-case letters indicate significant differences between percent dissolution within a solution (P<0.02), numbers without letters are non significantly different. Upper-case letters indicate significant differences between percent dissolution between solutions (P<0.001). The maximum amount of Ca_{ox} observed to dissolve in a given volume of extractant given as the mg Ca L^{-1} that dissolved in the highest level of Ca addition (50 mg).

Ca-oxalate (mg) added	**H_2O**	**0.1 M NH_4OAc**	**1 M NH_4OAc**	**2 M NH_4OAc**	**1 M NH_4Cl**	**2 *N* HOAc**	**0.1 M $BaCl_2$**	**0.5 *N* HCl**	**1 *N* HNO_3**
	A	**B**	**C**	**C**	**D**	**C**	**E**	**F**	**G**
0.5	28 (2.5) a	76 (1.2) a	98 (1.0) a	99 (0.5) a	100 (1.3) a	97 (2.4) a	104 (1.2)a	99 (5.3)	120 (5.8) a
1	14 (0.7) b	46 (1.3) b	98 (1.9) a	98 (0.8) a	81 (1.0) b	101 (0.9)a	98 (1.7) b	96 (0.4)	108 (0.9) b
10	2 (0.1) c	5 (0.2) c	9 (0.5) b	13 (0.1) b	10 (0.3) c	13 (0.6) b	27 (2.8) c	94 (0.1)	98 (0.3) c
50	0 (0.0) c	1 (0.0) c	2 (0.1) c	3 (0.0) c	2 (0.0) d	3 (0.1) c	5 (0.7) d	94 (1.3)	97 (0.4) c
Max. Ca_{ox} dissolution mg Ca L^{-1}	3.2	7.2	14.6	20.6	14.6	18.8	36.1	633.2	706.1

Table 5.3. The mean equilibrium concentration products $[Ca^{2+}]$ $[C_2O_4^{2-}]$ of each solution calculated at each approximate amount of Ca-ox (mg) added. For water where ionic strength= 0 and activity coefficients = 1, Ksp = (activity coefficient) $2[Ca^{2+}][C_2O_4^{2-}]$ was estimated. There were significant interactive and main effects of solution type and amount of Ca-ox added to solution (two-way ANOVA, P<0.001). Lower-case letters indicate significant differences between percent dissolution within a solution (P<0.001), numbers without letters are non significantly different. Upper-case letters indicate significant differences between percent dissolution between solutions (P<0.001). The mean solution pH after extraction, except for 0.1 M NH4OAc and 0.1 M BaCl2 which were estimated from pre-extraction values. Ionic strength (I) calculated based on oxidation states and concentration of solution.

Ca-oxalate (mg) added	**H_2O**	**0.1 M NH_4OAc**	**1 M NH_4OAc**	**2 M NH_4OAc**	**1 M NH_4Cl**	**2 *N* HOAc**	**0.1 M $BaCl_2$**	**0.5 *N* HCl**	**1 *N* HNO_3**
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
0.5	2.6×10^{-9}	2.3×10^{-8}	4.2×10^{-8}	4.3×10^{-8}	4.7×10^{-8}	5.0×10^{-8}	5.2×10^{-8}	3.7×10^{-8} a	5.1×10^{-8} a
1	2.6×10^{-9}	2.7×10^{-8}	1.3×10^{-7}	1.5×10^{-7}	1.1×10^{-7}	1.3×10^{-7}	1.5×10^{-7}	1.2×10^{-7} a	1.6×10^{-7} a
10	3.4×10^{-9}	2.7×10^{-8}	1.3×10^{-7}	2.4×10^{-7}	1.2×10^{-7}	2.1×10^{-7}	9.8×10^{-7}	1.4×10^{-5} b	1.3×10^{-5} b
50	6.7×10^{-9}	3.2×10^{-8}	1.3×10^{-7}	2.7×10^{-7}	1.3×10^{-7}	2.2×10^{-7}	8.4×10^{-7}	2.5×10^{-4} c	3.1×10^{-4} c
pH of extraction	**6.00**	**7.42**	**7.32**	**7.23**	**5.89**	**2.29**	**5.53**	**0.98**	**0.11**
I	**0**	**0.1**	**1**	**2**	**1**	**2**	**0.4**	**0.5**	**1**

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CHAPTER 6 – CONCLUSIONS

The objectives of this dissertation were to investigate: 1) amount, distribution and degradation rates of Ca-oxalate (Ca-ox), 2) the use of Ca-ox for foliar diagnosis to response to fertilization, 3) influence of Ca-ox on Ca/Sr discrimination and Ca-isotope ($^{44}Ca/^{40}Ca$) fractionation in biological systems and, 4) Ca-oxalate measurement in soil and separation from other chemical forms of Ca in soil. I used contrasting Ca-status sites to represent the range of soil calcium (Ca) in the Oregon Coast Range, and in order to contrast models of cation cycling in natural systems. Ca-oxalate is an important component of ecosystem Ca cycling with dynamics that differ from other chemical Ca fractions in terms of physiological and biogeochemical processes. This dissertation illustrated some ways that Ca-ox is important to ecosystem Ca cycling however much work remains on this topic.

For the first objective, I found that Ca-ox comprised 4% to 18% of total ecosystem Ca in high- and low-Ca sites in the Coast Range of Oregon, which varied by ecosystem compartment. Sites examined in this study had up to 34% of tree biomass Ca in Ca-ox, and up to 20% of available Ca in a Ca-ox in 0 - 10 cm depth mineral soil. I found that Ca-ox was the dominant form of Ca returned from plants to soil, but disappeared rapidly from decomposing litter at both sites suggesting an important pathway for Ca recycling. This was surprising based on the insolubility of Ca-oxalate, and previous paradigms of Ca-ox as a non-labile storage-form of Ca in soils. In mineral soil, Ca-ox was a larger portion of total available Ca in the low-Ca site but had lower Ca-ox concentration overall, therefore Ca-ox has limited potential to buffer against Ca depletion in forests where Ca is in shortest supply. Tree species may vary greatly in their creation of Ca-ox crystals, as plant crystal formation is highly regulated with specific shapes and sizes associated with plant species (Franceschi and Nataka, 2005), and it is likely that the importance of Ca-ox varies greatly by species. Additionally, Ca-oxalate accumulation in pools and compartments of other ecosystems may be highly variable as it could depend not only on soil Ca supply status, but also plant species, soil fungi and soil texture. In particular, Ca-ox can accumulate to high levels in association with fungal mats

(Graustein et al., 1977), which likely contributes to heterogeneity in soil Ca-ox where such mats occur. Ca-ox appears to accumulate associated with organic soil and rotting woody debris (Hintikka, 1790; Jellison et al., 1997) and a more mechanistic understanding of the role of fungi in the creation of the crystals may be useful for predicting ecosystems where Ca-ox in organic matter plays an important role for Ca returns to soil. Evidence suggests that Ca uptake in trees is primarily recycled Ca released from organic horizons (Blum et al., 2008) so the differing dynamics and quick release of Ca from Ca-ox crystals of decomposing litter highlight the potential importance of this chemical Ca fraction, especially in areas where Ca is deficient and tightly cycled in biomass.

For the second objective, I investigated foliar chemistry as a method for diagnosis of nutrient deficiencies in sites with high and low-Ca supply status which often vary inversely with soil nitrogen (N) supply in the Oregon Coast Range. Both sites were fertilized with urea, lime and calcium chloride for three years. The high-Ca site displayed 20-60 times higher concentrations of Ca-ox than the low-Ca site. However, Ca-ox accumulation was unaffected by 3-year fertilization, implying that there is either a long time-lag in foliar response to fertilization, or that Ca-ox accumulation is related to more complex factors than Ca supply. Response to fertilization examined with foliar vector diagrams suggested N limitation at the low-N site and N sufficiency at the high-N site after 3-years fertilization. Soil nitrification responded to both N and lime fertilization at both sites, suggesting that fertilization with N may not result in sustained increases in soil N availability, but may stimulate nitrification that could accelerate soil Ca loss. Low Ca supply resulting from sustained Ca loss likely has a negative impact on tree growth at these sites (Mainwaring et al., 2011). The role of Ca-ox and tree function and health has rarely been studied, especially in a forest ecosystem context. Ca-ox may be involved in resistance to herbivory, permanent or impermanent sequestration of Ca in tissues, deposition of waste products from carbon metabolism or other physiological processes. On these sites, Swiss needle cast heavily affects low-Ca sites, and there may be interactive effects with Ca-ox. Low-Ca, high-N sites that are heavily infected with Swiss needle have dampened ability to photosynthesize (Manter et al., 2005), may inhibit the tree's carbon metabolism and therefore ability to create Ca-ox crystals.

For the third objective, I investigated how Ca-ox may influence cation tracers such as Ca and strontium (Ca/Sr) ratios and Ca-isotopes ($^{44}Ca/^{40}Ca$) as tools to identify sources and pathways of Ca cycling in ecosystem studies. I determined that crystal formation exhibited preference for Ca over Sr, and for ^{40}Ca over ^{44}Ca during synthesis of Ca-ox. Discrimination of Ca/Sr was detected in bulk plant tissues due to Ca-ox accumulation suggesting that Ca-ox accumulation related to tree Ca supply status can have a significant impact on bulk tree tissue Ca/Sr values. Fractionation of $^{44}Ca/^{40}Ca$ was related more to Ca movement within the plant between bole-wood and foliage than to Ca-ox accumulation. In order to have a mechanistic understanding of cation discrimination and isotope fractionation, more information about chemical fractions are needed, including more information about the role of crystalline Ca-ox. Ca-oxalate offers a foil to other biominerals such as Ca-carbonate that are subjects of much recent debate for mechanisms of Ca/Sr discrimination and $^{44}Ca/^{40}Ca$ fractionation (DePaolo et al., 2011; Nielsen et al., 2011), and especially inform questions about the role of discrimination and fractionation during dehydration of Ca existing in aquocomplexes.

For the fourth objective I found that extraction-dissolved Ca-ox could potentially contribute an additional 52% to standard measurements of exchangeable-Ca pools in low-Ca sites. Standard methods for the determination of soil Ca often focus on the exchangeable pool as the most dynamic available soil Ca pool, but our data suggest that Ca-ox is a dynamic pool of Ca in soil that is not typically measured independently from exchangeable Ca. Much work remains in order to determine the best method for measuring Ca-ox as a distinct pool of soil Ca. However, it is likely that a dilute acid extraction that quantifies both Ca ions and oxalate ions may work in most soils, and spike-recovery analysis may be a way to determine if this method will be effective.

Ca-oxalate is a chemical form of Ca that is ubiquitous in plant and soil ecosystems, and its crystalline form likely results in differing dynamics in comparison to free Ca ions, Ca bound to pectin or lignin, or Ca associated with exchange sites in soil. With mechanistic information about this form of crystalline Ca, we can more fully understand single

physiological and biogeochemical processes involved in Ca cycling. A clearer understanding of these processes is useful in understanding ecosystem response to perturbations and strategies for sustainable nutritional management of forested ecosystems.

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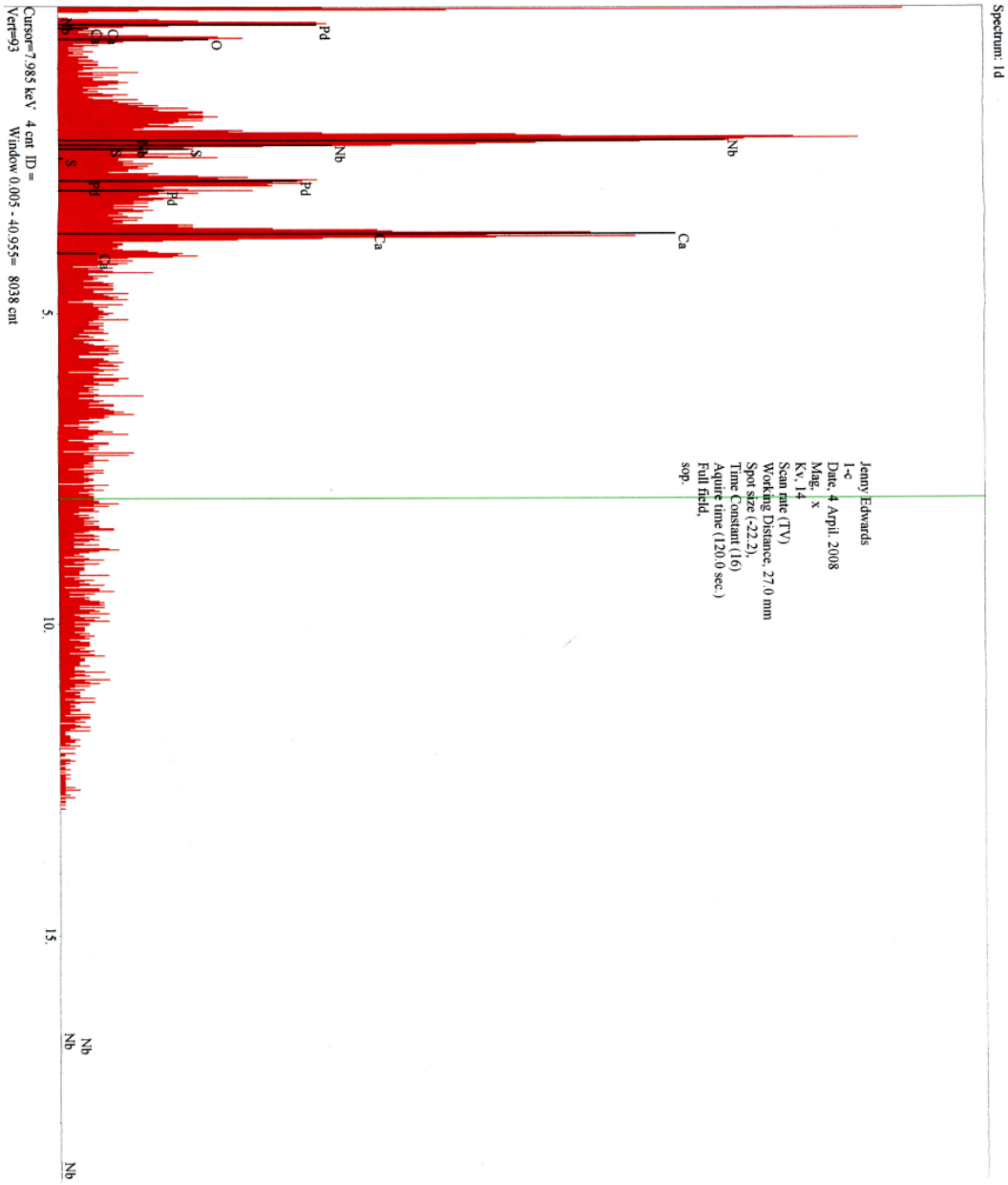
APPENDIX



Appendix 2.1. X-Ray Energy Dispersive Spectrophotometry Spectra indicating high calcium of crystal in Figure 2.4 A.