Response of nitrogen cycling to atmospheric nitrogen deposition in Japanese forests

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Abstract

Elevated atmospheric nitrogen (N) deposition by human activities has changed N cycling including N leaching. Understanding the N leaching from forested ecosystems and its susceptibility to elevated atmospheric N deposition is essential for assessing the impact of atmospheric N deposition on forested ecosystems. However, the factors that cause nitrate (NO_3^-) leaching from the forest ecosystem and its response to increased atmospheric N deposition are not fully understood in Japan.

The aim of this thesis is to improve our understanding of NO_3^- leaching from forested areas and its susceptibility to elevated atmospheric N deposition and to identify the possible reasons causing NO_3^- leaching. We evaluated the NO_3^- leaching in a stand-scale from plantations of Japanese cedar, the main plantation species in Japan, and Japanese oak, the dominant deciduous species across Japan. In this study, we first analysed the soil solution below the rooting zone and its possible reasons including N uptake and nitrification, and second conducted the N manipulation (50 kg N ha^{-1} yr^{-1} as ammonium nitrate) experiments for three-year experimental at each site to evaluate the susceptibility of NO_3^- leaching to elevated atmospheric N deposition.

The first part of the thesis explored NO_3^- leaching from different tree species and its susceptibility in response to elevated nitrogen deposition, in addition with its possible reasons for NO_3^- leaching. The results showed that there was high N leaching and higher susceptibility from the Japanese cedar plantations, and a linear regression model indicates that the relatively low N uptake by Japanese cedars could be an important reason for the high N leaching. (Chapter 2)

The second part of the thesis explored the site variability in NO_3^- leaching from Japanese oak forests and its susceptibility to elevated atmospheric N deposition from five sites (Shibecha, Ashoro, Nakagawa, Shiiba, Fukuoka) in broadleaved forests grown on Andosols and Cambisols from northern to southern Japan. The results showed that NO_3^- leaching was variable among the five sites and it was the highest in the location (Shibecha) where soil net nitrification tended to be higher than elsewhere. In model selection for NO_3^- leaching, the soil parent material was not selected but soil net nitrification was

selected, indicating that soil net nitrification could explain the variability of NO_3^- leaching. In addition, the susceptibility of NO_3^- leaching is high in locations (Shibecha) where N leaching was originally high. Furthermore, we also could conclude that Shibecha is N saturated because significantly higher NO_3^- leaching than Ashoro and Nakagawa and a significant increase of NO_3^- leaching to N fertilization over three years were observed in Shibecha. In contrast, both Ashoro and Nakagawa are N limited, because lower NO_3^- leaching than Shibecha and no significant increases in NO_3^- leaching to N fertilization over three years. (Chapter 3)

In the third part of the thesis, the importance of understory vegetation in the response of the forest N cycle to increased atmospheric N deposition was evaluated by assessing the response of understory sasa as well as overstory Japanese oak forests in three areas of Japanese oak forests (Shibecha, Ashoro, and Nakagawa). The results indicated that the fertilization increased N uptake by Sasa in the N limited site (Ashoro and Nakagawa) but not in the N saturated site (Shibecha). Therefore, we concluded that understory vegetation should be included in addition to overstory vegetation when assessing the response of forest ecosystems to elevated atmospheric N deposition. (Chapter 4)

In summary, this study evaluated the factors that cause the variability in NO_3^- leaching from forested areas due to increased atmospheric N deposition and the response of the N cycle over a wide area, which have been poorly understood in the forested areas of Japan. As a result, it was clarified that N uptake by vegetation and nitrification in soil, which are important processes in the N cycle in forest areas, are important factors affecting NO_3^- leaching.

Contents

List of Tables

List of Figures

Chapter 1

General introduction

1.1 Background

Planet's environment has maintained a stable state over the past 10,000 years (Rioual et al., 2001), recognized the period of stability by geologists as the Holocene. However, Industrial Revolution marked a significant departure from this stability, with human activities such as the use of fossil fuels and industrialized agriculture, causing substantial global environmental changes (Steffen et al., 2007).

In response to the challenges posed by these changes, Rockström et al., (2009) proposed a framework based on planetary boundaries. This framework identifies planetary boundaries into nine interconnected processes critical to Earth's stability: climate change; rate of biodiversity loss (terrestrial and marine); interference with the nitrogen and phosphorus cycles; stratospheric ozone depletion; ocean acidification; global freshwater use; change in land use; chemical pollution; and atmospheric aerosol loading (Figure,1 Rockström et al., 2009). Among the nine interlinked planetary boundaries, three of them have been found to be overstepped by Rockström et al., (2009); however, the transgressed planetary boundaries framework was updated to be six (Figure 2, Richardson et al., 2023), which indicated that Earth is now well outside of the safe operating space for humanity (Richardson et al., 2023).

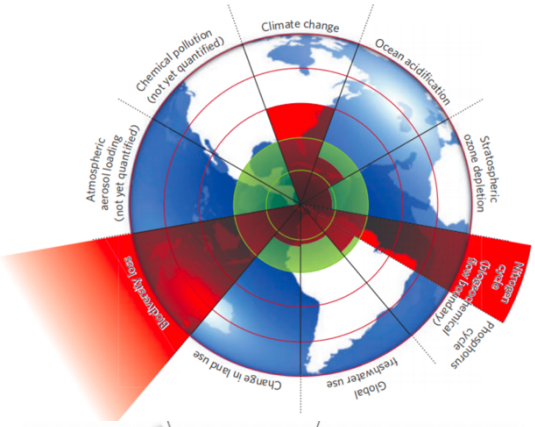

Figure 1.1. Beyond the boundary. The inner green shading represents the proposed safe operating space for nine planetary systems. The red wedges represent an estimate of the current position for each variable. The boundaries in three systems (rate of biodiversity loss, climate change and human interference with the nitrogen cycle), have already been exceeded (Rockström et al., 2009)

Fig. 1.2. Current status of control variables for all nine planetary boundaries. Six of the nine boundaries are transgressed. The green zone is the safe operating space (below the boundary). Yellow to red represents the zone of increasing risk. Purple indicates the high-risk zone where interglacial Earth system conditions are transgressed with high confidence (Richardson et al., 2023).

One of these planetary boundaries, the nitrogen cycle, has garnered attention due to the potentially disastrous consequences for the environment and humanity when certain biophysical thresholds are surpassed (Rockström et al., 2009; Richardson et al., 2023). Human processes removed the N_2 from the atmosphere into reactive forms per year from 0 (in pre-industrial) to approximately 120 million tonnes, which the value was higher than the planetary boundary (35 million tonnes per year) greatly (Rockström et al., 2009). However, the value of the total anthropogenically fixed N applied to the agricultural system was updated to be ~190 Tg year^{-1} according to Food and Agriculture Organization (2022), which the planetary boundary for N is the application rate of intentionally fixed N to the agricultural system of 62 Tg of N year^{-1}, indicating the boundary is globally transgressed (Richardson et al., 2023).

Human activities, particularly the release of reactive nitrogen to land and oceans, have profound effects on ecosystem composition and long-term stability. Global N cycle have been amplified by 10000%, due to intensified human activities (Elser et al., 2007). Especially, the elevated N levels contribute to water pollution, coastal zone degradation, which therefore, evaluating cycle including N leaching and its response to elevated available N is essential.

1.2 Nitrogen cycle in forests

Nitrogen (N) stands as a fundamental element within Earth's ecosystems, constituting 78% of the atmosphere but remaining inaccessible to plants in its atmospheric form. Therefore, the undisturbed forest ecosystems traditionally derive relatively little N annually from biological N fixation or atmospheric inputs (Melillo, 1981). The essential conversion of atmospheric N into inorganic forms, specifically, ammonium (NH_4^+) and nitrate (NO_3^-) is crucial for plant utilization.

In forest ecosystems, the N cycle operates as a biogeochemical phenomenon, systematically converting N through various forms in both internal and external cycles. Within forest ecosystems, processes such as mineralization, nitrification, and immobilization play pivotal roles. For instance, the organic N present in plant biomass, supplied to the soil surface as leaf litter, undergoes mineralization, converting organic N into inorganic forms, predominantly NH_4^+. Subsequently, by the process of nitrification NH_4^+ was transformed into NO_3^-. Notably, NH_4^+ is positively charged, making it easily

absorbable into the soil, while NO_3^-, being negatively charged, is prone to leaching from the soil. This generated inorganic N is absorbed and assimilated by plants, completing the fundamental decomposition-recycling process recognized as the elementary internal circulation (Fukushima, 2015).

In contrast, the external nitrogen cycle involves intricate processes, including dry and wet deposition, N uptake from the atmosphere, N release from the soil to the atmosphere, and N leaching from the soil to aquatic systems (Fukushima, 2015).

1.3 Effect of human activity on the nitrogen cycle in forests

Persistent high levels of N deposition exert a profound influence on N cycling in forests ecosystem, leading to increased foliar N concentration (Tietema and Beier 1995; Du 2017; Tian et al., 2018), increased N uptake (Ashton et al., 2010), and soil mineralization (Matson et al., 2014; Sun et al., 2016; Kooch et al., 2018). When plant capacity to assimilate N is surpassed, it may contribute to N saturation in forest ecosystems (Aber et al., 1989; Gundersen 1991) and high NO_3^- leaching (Aber et al., 1998; Chen et al., 2000; Corre et al., 2010; Stevens 2019), thereby exacerbating downstream eutrophication and water pollution (Chiwa et al., 2012; Shinozuka et al., 2017), polluting waterways and the coastal zone slowly erodes the resilience of important Earth subsystems (Rockström et al., 2009; Richardson et al., 2023).

1.4 Variability in N leaching and the factors affecting N leaching

Dise and Wright (1997) demonstrated variability in the response of N leaching among forested watersheds across Europe, where atmospheric N deposition ranging from 10 to 25 kg N ha^{-1} yr^{-1}. Japan, with approximately two-thirds of its land covered by forests consisting of diverse types including coniferous and broadleaf, and different soil compositions such as Andosols and Cambisols, which make it possible to present the variability in atmospheric N deposition and N leaching across Japanese forested watersheds. However, our understanding of the variability in N leaching within Japan's forest ecosystem remain incomplete.

To address this gap, we conduct an N manipulation experiment at multiple sites to provide insight into the susceptibility of N leaching to elevated N deposition in Japanese

forests. While similar studies investigating the response of N leaching to simulated N addition have previously been conducted in North America (Aber 1992; Aber et al. 1998), Europe (Tietema 1998; Corre et al. 2007), and China (Song et al. 2019). However, no study has conducted an N manipulation experiment at multiple sites in Japan, where Andosols are broadly distributed throughout forest soils.

Multiple factors, including successional age (Fang et al., 2008; Fukushima & Tokuchi, 2009; Goodale et al., 2000), vegetation and soil characteristics (Gundersen et al., 2006; Mitchell 2011; Takagi 2015), and topography (Creed & Band, 1998), may contribute to the heterogeneous response of NO_3^- leaching in forested watersheds to atmospheric N deposition (Campbell et al., 2014; Christopher et al., 2016; Lovett et al., 2000). Understanding these factors is imperative for effective water quality management in headwater streams. Although studies have addressed these factors globally, the specific factors influencing N leaching in the forest ecosystem of Japan remain incompletely understood.

Nitrification emerges as a crucial factor explaining the variability in N leaching from forested ecosystems across Japan. The higher N leaching due to elevated rates of nitrification (Gundersen et al., 1998). Considering nitrification is one of the important processes in N cycle, in the forest site with high soil nitrification, it is high possibility with high NO_3^- leaching.

Generally, N uptake by trees is an important factor regulating N leaching (Vitousek and Reiners, 1975; Chiwa et al., 2015). Lovett and Goodale (2011) showed that N added to a forest ecosystem can flow simultaneously to four possible “fates”: incorporation into vegetation, incorporation into the detritus and soil organic matter, leaching loss, or gaseous loss, depending on the strength of the sinks. Magill et al (2004) showed that N uptake by vegetation is one of the factors affecting N leaching. In addition, it has also been suggested that N uptake by coniferous species is higher than in broad-leaved species in Harvard Forest in the United States (Magill et al., 2004). However, N uptake by tree within Japan’s forest ecosystem remain poorly understood.

Significant N leaching has been suggested in the main planting areas for Japanese cedars, which constitute a substantial portion of Japan's plantation area and forested land. However, our understanding of N leaching from Japanese cedar plantations remains incomplete. While previous studies have indirectly evaluated N leaching from Japanese cedar plantations through statistical analyses involving synoptic sampling of stream water

within forested watersheds, stand-scale investigations directly assessing N leaching from specific vegetation types are limited, particularly in suburban forests in eastern Japan (Oyanagi et al., 2002).

Soil parent material, characterized by soil groups, influences the balance between nitrogen retention and loss (Anderson, 1988). Andosols, derived from volcanic sediments and widespread in Japanese forest soils, cover a significant portion of the land. N leaching from andosols is not fully underestood in Japan. This is because andosols have two opposite characteristics: in the one hand, the soils, with an anion exchange capacity under neutral to acidic conditions, retard NO_3^- transport due to anion adsorption (Miki et al., 2009). However, on the one hand, Andosols, which are rich in soil organic matter, may exhibit high soil nitrification rates (Urakawa et al., 2016), potentially leading to increased N leaching.

Less N uptake by vegetation (overstory and understory vegetation), which is part of the N cycling process, is likely to result in more N leaching. The importance of understory functional has been concluded by Landuyt et al. (2019), however, response of vegetation in overstory vegetation and understory vegetation to elevated atmospheric N deposition in forest ecosystem has little been assessed simultaneously. Sasa, the dominant understory vegetation in northern Japan, has been reported to mitigate N leaching (Fukuzawa et al., 2006) due to its fast annual increase in biomass (Watanabe et al., 2016). Therefore, it seems necessary to include the understory vegetation in addition to the overstory vegetation when assessing the response of forest ecosystems to increased atmospheric N deposition.

1.5 Objectives this study

The overall goal of this thesis is to improve our understanding of NO_3^- leaching from forested areas, its susceptibility to elevated atmospheric N deposition, and the potential factors driving NO_3^- leaching. To achieve this, we conducted a two-fold approach: first, analyzing soil solutions below the rooting zone and their interplay with soil parent material and net nitrification; and second, conducting N manipulation experiments at five broadleaved forest sites, involving three years of experimental N fertilization (50 kg N ha^{-1} yr^{-1} as ammonium nitrate) at each site to evaluate the susceptibility of NO_3^- leaching to elevated atmospheric N deposition.

The specific objectives of this study are as follows:

1. To valuate stand-scale N leaching in different tree species, especially Japanese cedar plantations in suburban forests in western Japan, and explore factors influencing N leaching in Japanese cedar plantations (Chapter 2).
2. To investigate site variability in NO_3^- leaching and factors influencing NO_3^- leaching from forested areas across Japan, including those with Andosols, and evaluate its susceptibility to elevated atmospheric N deposition (Chapter 3).
3. To evaluate the importance of the understory vegetation when assessing the response of forest ecosystems to elevated atmospheric N deposition (Chapter 4).

Chapter 2

Nitrate leaching and its susceptibility in response to elevated nitrogen deposition in different tree species

2.1 Introduction

Human activities, such as fuel combustion and agricultural activities, have increased anthropogenic nitrogen (N) deposition, especially since 1960(Cunningham et al., 2015). Chronic high N deposition has strongly influenced N cycling and may potentially cause N saturation in forested ecosystems, leading to high levels of N leaching from these ecosystems (Aber et al., 1998). Extensive studies in Europe (Dise and Wright,1995), China (Fang et al., 2009), North America (Aber et al., 2003), Japan (Chiwa et al., 2019; Ohrui and Mitchell, 1997) have shown the major role of atmospheric deposition on elevated nitrate (NO_3^-) concentrations in stream water from forest ecosystems, contributing to downstream eutrophication (Chiwa et al., 2012; Shinozuka et al., 2017)

Although the effects of elevated atmospheric N deposition on N leaching from forested ecosystems are widely recognized, there are various responses of N leaching to elevated atmospheric N deposition among forested watersheds. Dise and Wright (1995) showed that the response of N leaching is variable among forested watersheds with atmospheric N deposition from 10 to 25 kg N ha^{-1} $year^{-1}$ throughout Europe. This is because there are many factors that affect the response of NO_3^- leaching from forested watersheds to atmospheric N deposition (Campbell et al., 2004; Christopher et al., 2006; Lovett et al., 2000), including successional age (Fang et al., 2008; Fukushima and Tokuchi, 2009; Goodale et al., 2000; Tokuchi and Fukushima, 2009; Vitousek and Reiners, 1975) vegetation and soil characteristics (Christopher et al., 2006; Crowley and Lovett, 2017; Gundersen et al., 2006; Mitchell, 2011; Takagi 2015) and topography (Creed and Band, 1998). Understanding the factors affecting N leaching from forested watersheds is critical for managing the water quality in headwater streams.

In Japan, several studies have investigated the factors affecting the responses of N

leaching to elevated N deposition. Many studies (Campbell et al., 2004; Chiwa et al., 2004, 2015; Nishina et al., 2017; Watanabe et al, 2018) have shown that high NO_3^- concentrations in stream waters are observed near urban or suburban forested watersheds, where atmospheric N deposition is high. It has also been suggested that there is significant N leaching from the main planting areas for Japanese cedars (Chiwa et al., 2015; Nishina et al., 2017; Watanabe et al, 2018). Because Japanese cedar plantations account for approximately 44% of the plantation area and 18% of the total forested area in Japan, N leaching from Japanese cedar plantations should be intensively evaluated. However, N leaching from Japanese cedar plantations is not completely understood.

Many studies have evaluated N leaching from Japanese cedar plantations indirectly by statistical analysis in which synoptic sampling of stream water was conducted within a forested watershed containing various overstory species (Nishina et al., 2017; Watanabe et al, 2018). Stand-scale investigations can directly evaluate N leaching from specific vegetation types (Bahr et al., 2018; Gundersen et al., 1998; Vestgarden et al., 2001). However, studies evaluating N leaching from Japanese cedar plantations at the stand scale are limited to studies in suburban forests in eastern Japan by Oyanagi et al., (2002).

The presence of arbuscular mycorrhizae (AM) (Yamato et al., 2002) in Japanese cedar forests could lead to high NO_3^- leaching from plantations of this species. The high chemical quality of needle litter in AM-dominated stands results in rapid C mineralization, therefore contributing to a relatively low soil C/N ratio in AM-associated forests (Phillips et al., 2013). Phillips et al. (2013) revealed that there is an inorganic nutrient economy in AM-dominated forests, owing to the lower soil C/N ratio, which increases the risk of NO_3^- leaching because of the high rate of nitrification (Gundersen et al., 1998). Midgley and Phillips (2014) indicated that forests with AM leach more NO_3^- in response to

elevated atmospheric N deposition.

In addition, high NO_3^- leaching should be evaluated from a forestry point of view. In Japan, Japanese cedar was extensively planted in the 1950s and 1960s, and these trees are now reaching maturity (> 50 years). Fukushima et al. (2011) revealed that the amount of N uptake by Japanese cedars decreases as the trees age. Therefore, N retention, defined as biological utilization (including tree N uptake), might also increase the likelihood of N leaching from mature Japanese cedar plantations in response to elevated N deposition. Topography also has a large influence on stream N leaching. NO_3^- leaching largely occurs in relatively steep catchments (Watanabe et al., 2018; Fujimaki et al., 2008). Because many Japanese cedars are planted on steep slopes in mountainous areas in Japan (Yamamoto et al., 2017), steep topography might also be a reason for the high NO_3^- leaching in plantations of this species.

The objectives of this study are (1) to evaluate stand-scale N leaching and retention in Japanese cedar plantations in suburban forests in western Japan and (2) to explore possible factors affecting N retention in a Japanese cedar plantation.

2.2 Materials and methods

2.2.1 Study sites

This study was conducted in two forest stands, a Japanese cedar (*Cryptomeria japonica*) plantation and a neighboring Japanese oak (*Quercus crispula*) plantation, both located in the Kasuya Research Forest (KRF; 33° 38′ N, 130° 33′ E) of Kyushu University, situated 15 km west of the Fukuoka metropolitan area (Supplementary Fig. S2.1). In this region,

high levels of NO_3^- leaching from headwater streams are observed (Chiwa et al., 2012; Shinozuka et al., 2017). The mean annual temperature and precipitation are 15.9 °C and 1769 mm over the last 10 years, respectively (Chiwa, 2020). The atmospheric N deposition via bulk and throughfall and stemflow deposition in the Kasuya Research Forest are 11.3 and 15.5 kg N ha^{-1} $year^{-1}$, respectively (Chiwa, 2020). We compared plantations of Japanese cedar, the main plantation species in Japan, and Japanese oak, the dominant deciduous species across Japan, which served as a reference for evaluating the impact of tree species on N leaching. The distance between the two stands is approximately 3 km. Previously, these sites had been under natural forest and were planted for Japanese cedar in 1949–1954 and Japanese oak in 1994. Topography, tree age, diameter at breast height (DBH), and tree density in the Japanese cedar and oak plantations are shown in Supplementary Table S2.2.

In the Japanese cedar plantation, five 10 m × 10 m and five 15 m × 15 m plots with 2.5 m treated buffers on all sides (10 plots in total) were established as unfertilized and fertilized plots, respectively. In the Japanese oak forest plantation, one 10 m × 10 m plot and one 15 m × 15 m plot with 2.5 m treated buffers on all sides (in total 2 plots) were established as a unfertilized and a fertilized plot, respectively. In the fertilized plots, N fertilization (50 kg N ha^{-1} $year^{-1}$ as ammonium nitrate) was conducted at the beginning of April 2018. The purpose of the fertilization treatment in this study was to evaluate the short-term response of N leaching to an elevated inorganic N pool in the surface soil in Japanese cedar and oak plantations. N fertilizer was added directly to the forest floor.

2.2.2 Sample collection

Soil solutions were collected monthly from April to November 2018 using a tension

lysimeter with a porous cup to evaluate N leaching. Each lysimeter was installed at a depth of 50 cm below the predominant rooting zone so that the fraction of soil solution that represents potential leachate could be obtained. Above the surface, we used an injection syringe to collect the soil solution; the syringe was set up on the first day, and we collected the soil solution the next day. After collection, the soil solution samples were transported to a laboratory within approximately 4 h and immediately filtered by passing through a prewashed 0.45-μm membrane filter (Pall, Ekicrodisc syringe filter). Then, the concentration of NO_3^- was analyzed by ion chromatography (Aquion, Dionex, Japan). Because the NH_4^+ content in the soil solution was negligible, the data are not shown in this paper.

The soil net N mineralization and nitrification in the surface mineral soil (0–5 cm) in the unfertilized plots of the Japanese cedar and oak plantations were measured using in situ incubation of buried bags following the methods detailed in Eno, 1960. The collected soils were divided into two subsamples. The first was used to determine the initial mineral N content and the second was buried in the field at the same time and same depth for the incubation (0–5 cm). Soil incubation was conducted from 31st March to 30th June 2018 and from 30th June to 31st October 2018. The soils were sieved through a 4 mm screen before incubation, and roots and coarse organic matter were discarded. The soils were extracted at the beginning and end of the incubation period using 2 N KCl. The extracted solution was analyzed for NH_4^+ by the indophenol blue method, and NO_3^- was determined spectrophotometrically after cadmium reduction. The net mineralization and nitrification rates were calculated as the difference in the NH_4^+ and NO_3^- pools before and after in situ incubation, respectively, by quantifying the changes in NH_4^+ and NO_3^- over the same time period.

The soil samples used for the soil C and N analyses were also collected in the surface mineral soil (0–5 cm). They were passed through a 2 mm sieve and air-dried, and the sieved dry soil samples were gently ground with a mortar and pestle. The C and N concentrations were measured by the combustion method (MT-700, Yanaco, Kyoto, Japan).

To evaluate the uptake of N and its effect on N retention by plants, litterfall was collected from the Japanese cedar plots and oak control plots from April 2018 to March 2019. N uptake was evaluated as the N amount contained in the leaf fall. The N amount in the leaf fall was calculated by multiplying the leaf fall weight by the N concentration in the leaf fall. One and 5 litter traps (0.5 m^2 in size) per plot were established in the Japanese cedar and oak stands, respectively. Each litter trap was constructed from a steel hoop (80 cm aperture) and a woven nylon bag (80 cm depth) and was positioned 1 m above the ground with plastic stakes. Samples were dried for 48 h at 80 °C, sorted into leaf fall and other material, and weighed. The N concentrations were measured by the combustion method (CN Corder MT-700, Yanaco, Kyoto, Japan).

The woody and leaf N biomass were calculated to evaluate the biomass N pool. Woody N biomass was calculated by multiplying the wood mass by the trunk N concentration. The wood mass (kg) was determined using the following allometric equation (Komiyama et al., 2022):

$W = 0.1853\ p\ DBH^{2.491}$,

where DBH is the diameter at breast height (cm) and ρ is the specific weight (kg m^{-3}) for cedar (0.352) and oak (0.523) (Komiyama et al., 2011). The trunk N concentration used for the calculation was 0.12% for Japanese cedar (Shimono, 2020) and 0.39% for

Japanese oak (Takagi et al., 2010) The leaf N biomass was calculated by multiplying the leaf mass by the leaf N concentration. The leaf mass was determined from the leaf lifetime and weight of leaf fall collected from the Japanese cedar and Japanese oak plots from April 2018 to March 2019. The leaf N concentration used for the calculations was 1.06% for Japanese cedar (Shimono, 2020) and 1.80% for Japanese oak, as determined in this study. Surface (0–5 cm) soil N storage was calculated by multiplying total N concentration by soil density (0.33 g cm^{-3} for Japanese cedar and 0.55 g cm^{-3} for Japanese oak).

2.2.3. Statistics

The effects of tree species (Japanese cedar and Japanese oak), fertilization, and their interactions on NO_3^- leaching were analyzed using two-way repeated measures ANOVA. A linear regression model that can evaluate the relationship between a scalar response and multiple explanatory variables was used to evaluate the multiple factors (nitrification, N uptake, and slope) influencing NO_3^- leaching. The coefficient of determination for the regression model was 0.874 at a significance level of $p < 0.001$.

$$NO_3^- \ leaching = a_0 + a_1 Nit + a_2 Nup + a_3 Sl$$

where *Nit*, *Nup*, and *Sl* are soil net nitrification (mg N kg^{-1} day^{-1}), tree N uptake (kg N ha^{-1} $year^{-1}$), and slope (°), respectively. Comparisons of soil chemical properties and N uptake between Japanese cedar and oak plantations were performed using *t*-tests. All the statistical analyses were performed using SPSS 22.0J (SPSS Japan Inc, Tokyo, Japan).

2.3 Results and Discussion

2.3.1 High N leaching and low N retention in a suburban Japanese cedar plantation

The NO_3^- concentration in the soil solution below the rooting zone (collected from a depth of 50 cm) in the unfertilized plots of the Japanese cedar plantation (607 ± 59 μmol L^{-1}) was much higher than that in the unfertilized plots of the Japanese oak plantation (8.3 ± 8.1 μmol L^{-1}) (Table 2.1). In addition, the difference between the two sites was consistent during the observation period from April to November 2018 (Fig. 2.1). Higher NO_3^- concentration in the Japanese cedar plantation than in the Japanese oak plantation indicates high levels of NO_3^- leaching from the Japanese cedar plantation. The high NO_3^- leaching from the Japanese cedar plantations in this study was consistent with the findings of a study by Oyanagi et al. (2002), which showed that the NO_3^- concentration in a soil solution from deeper soil (50 and 80 cm depths) was high (approximately 240 μmol L^{-1}) in a N-saturated Japanese cedar plantation in eastern Japan, where high levels of atmospheric N deposition (10.5 kg N ha^{-1} $year^{-1}$) and stream water N leaching (13.5 kg N ha^{-1} $year^{-1}$) have been observed (Ohrui and Mitchell, 1997).

Watanabe et al. (2018) reported that catchments with high coniferous coverage, including Japanese cedar plantations near urban areas, tend to have high NO_3^- concentrations in stream water. It has also been reported that there is significant N leaching from forested watersheds in which the main species is Japanese cedar (Chiwa et al., 2015; Nishina et al., 2017; Watanabe et al., 2018). However, N leaching from Japanese cedar plantations has been evaluated indirectly by statistical analysis in which synoptic sampling of stream water was conducted within a forested watershed containing various overstory species (Nishina et al., 2017; Watanabe et al., 2018). Our stand-scale

investigation confirmed the presence of high N leaching from Japanese cedar plantations in suburban areas.

Table 2.1. Average NO_3^- concentration in soil solution below rooting zone (50 cm depth), soil chemical properties of A layer (0-5 cm) in no-fertilization plots of Japanese cedar and oak plantations during the study period from April to November 2018.

	Japanese cedar	Japanese oak	*p* value
NO_3^- concentration in soil solution (μmol L^{-1})	617 ± 59[a]	8.7 ± 8.1	***
Total C (%)	6.37 ± 1.2	7.77 ± 1.22	ns[c]
Total N (%)	0.37 ± 0.06	0.46 ± 0.06	ns[c]
C/N ratio	17.0 ± 1.1[a]	16.2 ± 0.8	ns [c]
Inorganic N (mg N kg^{-1})	4.71 ± 1.73	4.35 ± 0.85	ns[c]
Net mineralization (mg N kg^{-1} day^{-1})	0.71 ± 0.38	0.71 ± 0.26	ns[c]
Net nitrification (mg N kg^{-1} day^{-1})	0.63 ± 0.36	0.67 ± 0.26	ns[c]
Relative net nitrification [b] (%)	90 ± 13	96 ± 4	ns[c]

[a] mean ± standard deviation (n = 5); [b] defined as the proportion of net nitrification to net N mineralization; [c] no significant difference (P > 0.05 for t-test); significant difference (P < 0.001 for t-test)

The NO_3^- concentration in the soil solution collected from a depth of 50 cm in the fertilized plots of the Japanese cedar plantation increased immediately after fertilization and then returned to the same NO_3^- concentrations found in the unfertilized plots (Fig. 2.1 a). In contrast, there was only a small increase in NO_3^- concentration in the fertilized plots of the Japanese oak plantation after fertilization (Fig. 2.1b). Another experiment also showed that the response of NO_3^- concentration in the soil solution from below the rooting zone is variable in response to N addition and that the response of N leaching is faster in coniferous forests than in deciduous forests in stand-scale fertilization experiments (Magill et al., 2004). The immediate NO_3^- leaching in the Japanese cedar plantation and the small increase in leaching in the Japanese oak plantation observed in this study after fertilization suggest that the response of N leaching to N addition is

variable.

The NO_3^- concentration in the soil solution in the Japanese cedar plantation increased approximately three-fold after the addition of ammonium nitrate at a rate of 50 kg N ha^{-1} (Fig. 2.1a). Considering the current atmospheric N deposition in this study area (Chiwa, 2020) (approximately 16 kg N ha^{-1} $year^{-1}$), it is likely that NO_3^- leaching in Japanese cedar plantations has increased in response to elevated atmospheric N deposition.

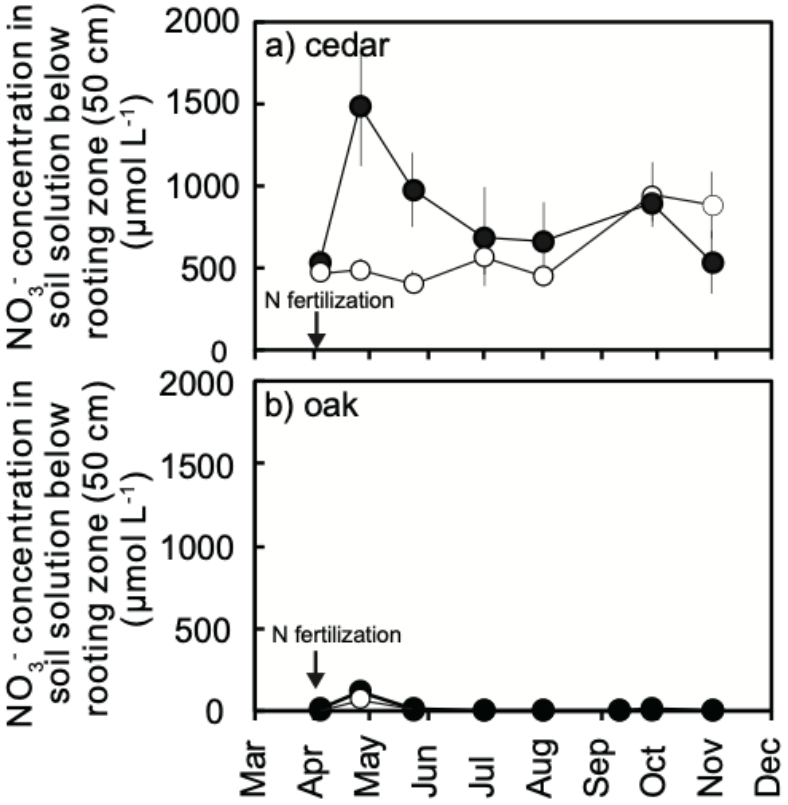

Fig. 2.1 Changes in the NO_3^- concentration in the soil solution below the rooting zone (50 cm depth) in the a) Japanese cedar and b) oak plantations during the study period from April to November 2018. The error bars indicate the standard errors (n = 5). The open circles refer to the no-fertilization plots; the closed circles refer to the fertilized plots. The arrows indicate the timing of the addition of N fertilizer (50 kg N ha^{-1} $year^{-1}$ as ammonium nitrate) to the surface of the forest floor.

2.3.2 Possible mechanisms for high N leaching and low N retention in Japanese cedar plantations

A high soil nitrification rate may increase the risk of NO_3^- leaching (Gundersen et al., 1998). Because Japanese cedar is an AM-associated species (Yamato et al., 2002) that is likely to leach more NO_3^- in response to N deposition (Midgley and Phillips, 2014), high N leaching from Japanese cedar plantations may be caused by high soil net nitrification rates. The soil net mineralization rates in the unfertilized Japanese cedar plots in this study (0.71 ± 0.38 mg N kg^{-1} day^{-1}; Table 2.1) were comparable to those (0.9 mg N ka^{-1} day^{-1}) in the neighboring forests, where high N leaching via stream water has been reported (Chiwa et al., 2010). The lower end of the range of soil nitrification rates (0.63 ± 0.36 mg N kg^{-1} day^{-1}; Table 2.1) were also similar to those in Japanese cedar plantations in southern Japan (0.29 mg N kg^{-1} day^{-1}) (Tateno et al., 2010) and central Japan (0.3 mg N kg^{-1} day^{-1}) (Fukushima et al., 2011). In addition, the relative net nitrification, defined as the proportion of net nitrification to net N mineralization, was 90% in the Japanese cedar plantation (Table 2.1). These results indicate that soil nitrification in the Japanese cedar plantation in this study was high enough to cause significant N leaching.

However, the soil net nitrification in the Japanese cedar plantation was not significantly higher than that in the Japanese oak plantation (Table 2.1). The lack of significant differences in the soil total C, total N, C/N ratio, and inorganic N between the Japanese cedar and oak plantations (Table 2.1) may explain the lack of significant differences in the soil net nitrification between the plantations, because soil N status generally affects soil nitrification rates (Gundersen et al., 1998). In addition, NO_3^- leaching was not correlated with soil net nitrification (Table 2.2). Therefore, soil nitrification could not

explain the difference in NO_3^- leaching from the Japanese cedar and oak plantations, although soil nitrification may be an important source of NO_3^- leaching in Japanese cedar plantations.

Table 2.2 Coefficient of determination and regression coefficients of multiple linear regression of NO_3^- leaching.

ç	NO_3^- leaching (μmol L^{-1})	*p*
R^2		< 0.001
Intercept	913	< 0.001
Nitrification (mg N kg^{-1} day^{-1})	–	0.700
N uptake (kg N ha^{-1} yr^{-1})	-22.9 (-0.935)	< 0.001
Slope (°)	–	0.686

Numbers in parentheses indicate standardized coefficients. Stepwise F <0.05

The finding that soil nitrification could not explain the difference in NO_3^- leaching between the Japanese cedar and oak plantations is consistent with the results of the N fertilization experiments in this study. N fertilization enhances the contents of inorganic N in surface soil. The inorganic N contents in surface (5 cm) soil after the N fertilization were calculated to be 303 and 190 mg N kg^{-1} for Japanese cedar plantation and Japanese oak plantation, respectively, which was higher than those before the N fertilization (Table 2.1). The smaller increases in NO_3^- leaching despite the elevated inorganic N contents caused by N fertilization in the Japanese oak plantations than in the Japanese cedar plantation in this study (Fig. 2.1) suggest that other factors that lower N retention and subsequently increase N leaching are also important for NO_3^- leaching.

Generally, N uptake by trees is an important factor regulating N retention (Vitousek and Reiners, 1975; Chiwa et al., 2015). N uptake was significantly lower in the Japanese cedar plantation than in the oak plantation (Table 2.3). The low N uptake in the Japanese cedar plantation compared with that in the Japanese oak plantation is supported by the lower N biomass pool and soil N storage in the Japanese cedar compared with the

Japanese oak (Supplementary Table S2.1), even though the Japanese cedar is older than the Japanese oak (Supplementary Table S2.2). In addition, NO_3^- leaching was significantly correlated with tree N uptake (Table 2.2), indicating that the lower N uptake by the Japanese cedars could be an important reason for the high N leaching in plantations of this species. The low N uptake in the Japanese cedar plantation compared to that in the Japanese oak plantation may also be supported by other studies showing that broadleaf forests have significantly higher N uptake than coniferous forests in China (Liu et al., 2001) and Europe (Kavvadias et al., 2001). In addition, it has been shown that Japanese cedar is characterized by lower N uptake than other species, including deciduous hardwood and Japanese cypress forests (Inagaki and Kohzu, 2005).

Table 2.3. Estimates of N uptake by Japanese cedar and Japanese oak plantations.

	N concentration of leaffall (A) (%)	weight of leaffall (B) (g m^{-2})	N uptake (A×B) (kg N ha^{-1} yr^{-1})
Japanese cedar	0.62 ± 0.04	217 ± 18	13 ± 1.1
Japanese oak	0.91 ± 0.03	433 ± 33	39 ± 3.0
	**	**	**

[a] mean ± standard deviation (n = 5); [b] significant difference ($P < 0.01$ for t-test)

In this study, N uptake, calculated by multiplying the leaf fall amount by the N concentration in the leaf fall, may be underestimated because the annual increase in the N stored in the woody biomass was not included. However, the finding that the Japanese cedar plantation had a lower N uptake than the Japanese oak plantation is reliable because the annual N increase in the woody biomass in the mature (64–69 year) Japanese cedar plantations used in this study would be smaller than that in the Japanese oak plantations because woody N storage is lower in the Japanese cedar plantation than in the Japanese oak plantation (Supplementary Table S2.1), even though the Japanese cedar is older than the Japanese oak at this study site (Supplementary Table S2.2).

N uptake by trees is also affected by tree age (Ohrui and Mitchell, 1997). Fukushima et al. (2011) evaluated tree N uptake at different ages (5–89 years) and demonstrated that N uptake by Japanese cedar was higher in younger stands (16 years old: 53 kg N ha^{-1} $year^{-1}$) than in older stands (31 years old: 29 kg N ha^{-1} $year^{-1}$, 42 years old: 24 kg N ha^{-1} $year^{-1}$, and 89 years old: 34 kg N ha^{-1} $year^{-1}$). The Japanese cedar plantation in this study is 64–69 years old and has reached maturity, resulting in high susceptibility to NO_3^- leaching in response to elevated N deposition because of the reduction in tree N uptake with age. Therefore, low N uptake owing to forest maturity could be an important factor causing the low N retention in the Japanese cedar plantations.

The steep slope in the Japanese cedar plantation could be an additional reason for the low N retention. However, NO_3^- leaching was not correlated with slope in this study (Table 2.2). Generally, stream NO_3^- concentrations tend to be higher on steep slopes (Watanabe et al., 2018; Fujimaki et al., 2008). Steep slopes increase N flushing through water discharge (Creed and Band, 1998; Fujimaki et al., 2008), as NO_3^- leaching occurs from upper soils following stormflows. Because N leaching loss was significantly higher in Japanese cedar planted on steep slopes than in Japanese oak, a steep slope may also support high leaching losses in the cedar plantation. A greater range of slope conditions will be required in future research to examine the topographic effect on NO_3^- leaching. It is possible that other factors, including soil microbial immobilization, lowered N retention. Soil microbial NO_3^- immobilization is reportedly lower in Japanese cedar plantations than in broadleaf and Japanese cypress forests (Komiyama et al., 2011). Further investigations are required to explore the mechanisms lowering N retention in Japanese cedar plantations.

2.4 Conclusion

This study conducted a stand-scale investigation of N leaching from a specific species. The results confirmed high NO_3^- leaching from a Japanese cedar plantation. N fertilization experiments indicate that the high NO_3^- leaching observed in the Japanese cedar plantation could have been caused by low N retention. Although soil nitrification in Japanese cedar plantations could be an important source of NO_3^- leaching, soil nitrification was not correlated with NO_3^- leaching. The relatively low N uptake by Japanese cedar planted on the steep slopes could be an important contributor to the high N leaching. Because Japanese cedar is the main plantation species, accounting for approximately 44% of the plantation area in Japan, N leaching from these plantations may be an important source of N in headwater streams. Further investigations are required to explore the reasons for low N retention in Japanese cedar plantations.

Chapter 3

Nitrate leaching and its susceptibility in response to elevated nitrogen deposition in Japanese forests

3.1 Introduction

Understanding the variability in nitrogen (N) leaching from forested ecosystems and its susceptibility to elevated atmospheric N deposition is essential for assessing the impact of N pollution on forested ecosystems. Atmospheric N deposition has dramatically increased, especially since the 1960s because of the increase in reactive N produced by industrial processes and the extensive use of chemical N fertilizers for agriculture (Galloway et al. 2008). Chronic high atmospheric N deposition has strongly influenced N cycling in forested ecosystems, leading to high levels of N leaching (Chiwa et al. 2019). Although it is generally accepted that elevated atmospheric N deposition can cause high levels of N leaching, Dise and Wright (1995) showed that N leaching is variable among forested areas with moderately high levels of atmospheric N deposition throughout Europe.

Soil parent material, as identified by soil groups, influences the balance between N retention and loss (Anderson 1988). Williard et al. (2005) found that the characteristics of the soil substrate explained most of the variation in stream nitrate (NO_3^-) from the mid-Appalachian region, USA. Cambisols are widely distributed in temperate and boreal forests. Andosols (IUSS Working Group WRB, 2014; Soil Survey Staff, 2014), which are generated from volcanic sediments, are also broadly distributed throughout forest soils in Japan (Nanko et al. 2014), covering one-sixth of Japanese land surface (Nanzyo 2007). These soils are typically characterized by an anion exchange capacity under neutral to acidic conditions, which causes the retardation of NO_3^- transport because of anion adsorption (Miki et al. 2009). In contrast, Andosols have large amounts of soil organic matter and therefore may potentially have high soil nitrification rates (Urakawa et al. 2016), which may lead to high N leaching.

Nitrification could be an important factor explaining the variability of N leaching from forested ecosystems across Japan. The Carbon: Nitrogen ratio (C:N ratio) of forest floor has been used as an index to explain N leaching from forested ecosystems across Europe (Gundersen et al. 1998; Gundersen et al. 2006). Higher N leaching with lower C:N ratios has been observed because lower C:N ratios generally cause higher rates of nitrification (Gundersen et al. 1998). However, no correlation between the organic layer C:N ratio and soil N transformation, including soil net nitrification, was found in Japan when both Cambisols and Andosols were studied (Urakawa et al. 2016). Andosols showed high soil nitrification rates (Urakawa et al. 2016), which could be an important factor in explaining N leaching from Japanese forested ecosystems.

Here, we conduct an N manipulation experiment at multiple sites to provide insight into the susceptibility of N leaching to elevated N deposition in Japanese forests. Studies investigating the response of N leaching to simulated N addition have previously been conducted in North America (Aber 1992; Aber et al. 1998), Europe (Tietema 1998; Corre et al. 2007), and China (Song et al. 2019). However, no study has conducted an N manipulation experiment at multiple sites in Japan, where Andosols are broadly distributed throughout forest soils.

In Europe and America, it has been reported that the response of N leaching to elevated N deposition is related to its original N leaching status. Negligible responses of N leaching to N addition were found where N leaching was originally low (Magill et al. 2004). In addition, in an N fertilization experiment at multiple sites across Europe, Gundersen et al. (1998) showed that the susceptibility of NO_3^- leaching to N addition increased in the forests in which N leaching was originally high. Therefore, we hypothesize that the susceptibility of NO_3^- leaching in response to elevated N deposition

in Japanese forests also depends on its original N leaching status.

This study explored the site variability in NO_3^- leaching and the factors affecting NO_3^- leaching from forested areas across Japan, including those with Andosols, and evaluated its susceptibility to elevated atmospheric N deposition. To achieve this goal, we first analyzed the soil solution below the rooting zone and its relationship with soil parent material and net nitrification, and second conducted the N manipulation experiments at five broadleaved forest sites grown on Andosols and Cambisols.

3.2 Materials and methods

3.2.1 Study site

This study was conducted in five Japanese oak (*Quercus crispula*) forests, located in Shibecha, Ashoro, Nakagawa, Shiiba, and Kasuya, together stretching from the north to the south of Japan (Figure S3.1). All sites are registered as either core or associate sites of JaLTER (the Japan Long-Term Ecological Research Network; http://www.jalter.org/en/researchsites/). Their locations, soil groups, mean annual precipitation, mean annual temperature, and atmospheric N deposition rates are presented in Table 3.1. In Kasuya, the forest floor is covered by several species of herbaceous plants. In Shiiba, there is almost no understory vegetation because of deer overpopulation. In the three sites on Hokkaido (Shibecha, Ashoro, and Nakagawa), the forest floor is covered by dense stands of Sasa dwarf bamboo; *Sasa nipponica* in Shibecha (at 0.8–1.0 m in height, Tateno et al. 2019), *Sasa nipponica* in Ashoro (0.5 m in height, Chiwa and Nakamura 2020), and *Sasa senanensis* and *Sasa kurilensis* in Nakagawa (1.5−2.0 m in

height, Shibata et al., 2005). According to the classification system of the IUSS Working Group WRB (2014), soil groups were Cambisols (two sites) and Andosols (three sites) (Table 1).

We established pair plots of unfertilized and fertilized plots (15 m × 15 m) at each site (Table 1). In Kasuya, one 15 × 15 m pair plot was established because of the limited extent of Japanese oak here. In the fertilized plots, N fertilization (50 kg N ha^{-1} yr^{-1} in the form of granular solid ammonium nitrate) was carried out during the period of April–May 2018, 2019, and 2020, before the growing season (at early-April in 2018 and in 2019, at mid-May in 2020 in Kasuya, ; at mid-April in Shiiba; at mid-May in Shibecha, from late-April to mid-May in Ashoro; from early-May to late-May in Nakagawa). N fertilizer was added directly to the forest floor. We divided a plot (15 m × 15 m) into five subplots (15 m × 3 m) to spread the N fertilizer homogenously over the survey plot.

3.2.2. Sample collection

Soil solutions were collected monthly from April to November 2018 in unfertilized plots, and in both unfertilized and fertilized plots in 2020 (after the 3-year fertilization experiments), using a tension lysimeter with a porous cup (Daiki, Japan) to measure N leaching. In each plot, a lysimeter (five in Kasuya) was installed in mineral soil at a depth of 50 cm below the predominant rooting zone. Above the ground, we use an injection syringe to collect the soil solutions by forming vacuums on the first day, to let the soil

solution flow up to the injection syringe according to the actual pressure difference. The soil solution was then collected the next day and the volume of soil solution obtained in one injection syringe was approximately 10–50 ml. After collection, the soil solution samples were transported to our laboratory and filtered by passing them through a pre-washed 0.45 μm membrane filter (Pall, Ekicrodisc syringe filter, Japan). The soil solutions were refrigerated at 4 ºC until chemical analysis. The concentration of NO_3^- was analyzed by ion chromatography (Aquion, Thermo Fisher Scientific, Japan). The concentrations in soil solution were measured separately and an average was calculated in each site.

Soil net N mineralization and nitrification rates in the surface mineral soil (0–5 cm) were measured using *in situ* incubation of buried bags following the methods detailed in Eno (1960). In each subplot, one soil sample (five in Kasuya) was collected. The collected soils were divided into two subsamples. The first was used to determine the initial inorganic N content, and the second was buried in the field at the same time and at same depth for the incubation (0–5 cm). *In situ* soil incubation was conducted from July to October, once in 2018 and again in 2020. The soils were sieved through a 4 mm screen before incubation to remove coarse roots, gravel, and organic matter. The soils were extracted at the beginning and end of the incubation period using 2 mol L^{-1} KCl. Concentrations of NO_3^- and NH_4^+ in the extracted solution were analyzed using an auto analyzer (AACS-4, BL-TEC Inc., Japan). The net N mineralization and nitrification rates

per unit dry soil weight were calculated as the difference in the NH_4^+ and NO_3^- pools, and NO_3^- pools before and after *in situ* incubation divided by the incubation days. Gravimetric water content was measured by drying at 105 °C for 24 h.

The soil samples used for analyzing the soil pH and total C and N were also collected in the surface mineral soil (0–5 cm). They were air-dried and passed through a 2 mm sieve, and the sieved dry soil samples were then gently ground with a mortar and pestle prior to soil C and N measurement. Soil pH and the CN ratio were measured approximately once a year. C and N concentrations were measured by the combustion method (MT–700, Yanaco, Kyoto, Japan). pH (H_2O) was measured by glass electrode after water extraction (air-dried soil:deionized water = 1:2.5).

3.2.3. Statistical analysis

Soil net N mineralization, net nitrification in the A layer (0–5 cm), and soil solution NO_3^- concentration in the unfertilized plots were compared across the five sites using the least significant difference (LSD) test. The above statistical analyses were performed using SPSS 22.0J (SPSS Japan Inc, Tokyo, Japan). Comparison of soil pH (H_2O), C:N ratio, net N mineralization, and net nitrification in the A layer (0–5 cm), and NO_3^- concentration in soil solutions between unfertilized and fertilized plots were analyzed using the *t*-test. The differences were considered significant at the $P < 0.05$ level. We used soil solution NO_3^- concentration as the dependent variable, and soil parent material

(S), nitrification (N), and their interaction ([S] × [N]) as the independent variables after checking the independence between [S, soil parent material] and [N, nitrification] (P = 0.3). The relationship between soil solution NO_3^- concentration and multiple explanatory variables (soil parent material, nitrification, and their interaction) were analyzed using a generalized linear model (GLM). The model selection was based on Akaike's Information Criterion (AIC). For each model, the AIC was computed and the model with the lowest AIC value was selected as the best model. The above statistical analyses were performed using R version 3.5.0 for Windows (R Core Team, 2018).

3.3 Results

3.3.1 Site variability of nitrate leaching among five sites.

Average of soil solution NO_3^- concentration below the rooting zone (collected from a depth of 50 cm below the ground surface) in the unfertilized plots varied greatly among five sites, ranging from 8.71 ± 8.05 μmol L^{-1} in Kasuya to 259 ± 78.1 μmol L^{-1} in Shibecha in 2018 (Figure 3.1a), and from 0.39 ± 0.20 μmol L^{-1} in Kasuya to 70.8 ± 30.6 μmol L^{-1} in Shibecha in 2020 (Figure 3.1b). Soil solution NO_3^- concentrations in Shibecha were significantly higher than those at the other four sites (Ashoro, Nakagawa, Shiiba, and Kasuya) in 2018 (Figure 3.1a), and at three sites (Nakagawa, Shiiba, and

Kasuya) in 2020 (Figure 3.1b). No clear and common seasonal changes in the five sites were observed at each site (Figure S3.2).

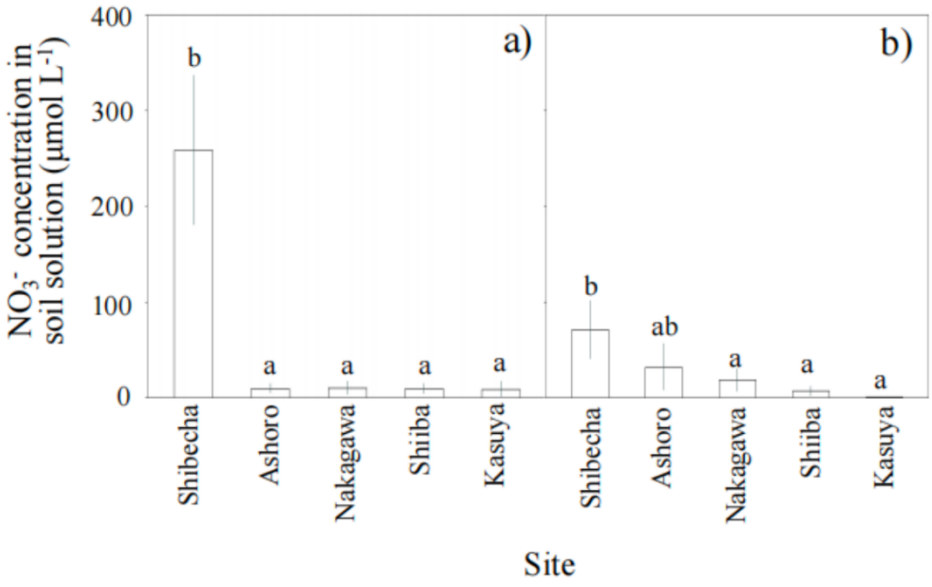

Figure 3.1 Average of soil solution NO_3^- concentration below the rooting zone (50 cm depth below the ground surface) in unfertilized plots among five Japanese oak forests in a) 2018 and b) 2020. The error bar indicates the standard error.

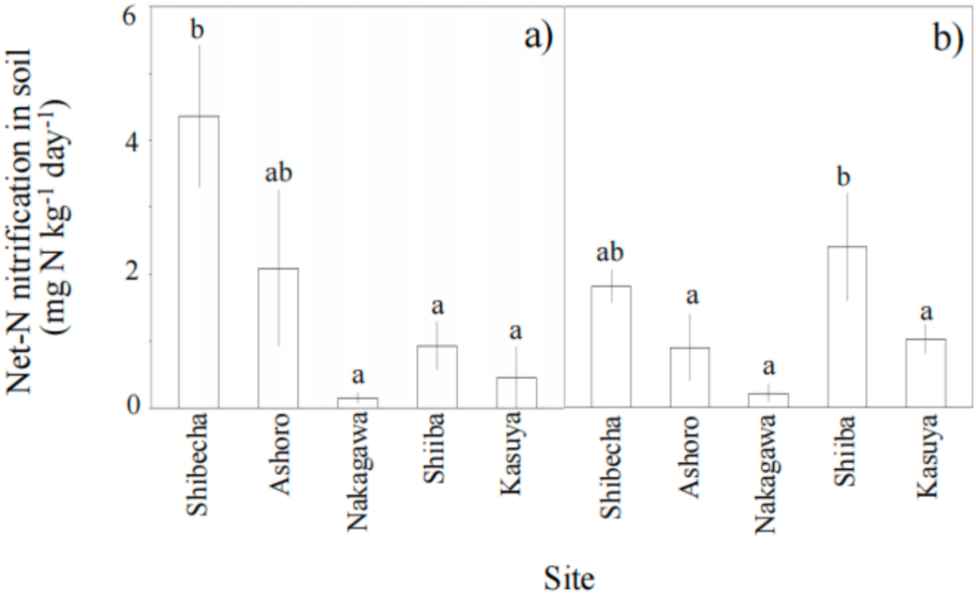

Figure 3.2. *In situ* net nitrification in unfertilized plots of five Japanese oak forests in a) 2018 and b) 2020. The error bar indicates the standard error.

3.3.2. Soil parent material, soil net nitrification and its relationship with N leaching among five sites

Soil net nitrification varied greatly between the five sites, ranging from 0.15 ± 0.09 mg N kg^{-1} day^{-1} in Nakagawa to 4.36 ± 1.06 mg N kg^{-1} day^{-1} in Shibecha (Figure 2). Soil net nitrification in Shibecha was significantly higher than at three other sites (Nakagawa, Shiiba, and Kasuya) in 2018 (Figure 3.2a), and tended to be higher than at three sites (Ashoro, Nakagawa, and Kasuya) in 2020 (Figure 3.2b).

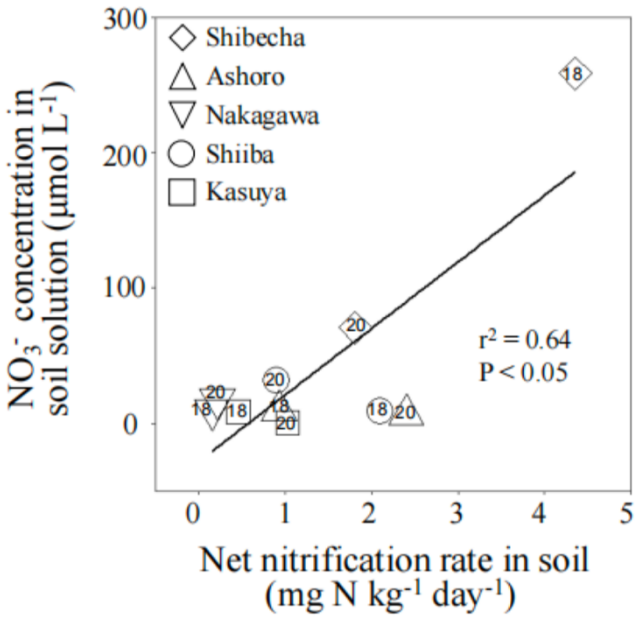

Figure 3.3 The correlation between soil net nitrification and average NO_3^- concentration in the soil solution below the rooting zone (50 cm depth) in unfertilized plots among five Japanese oak forests in 2018 and 2020. The labels "18" and "20" represent "in 2018" and "in 2020."

In selecting a model to predict soil solution NO_3^- concentration, soil parent material was not included as a predictor, but nitrification was (Table S3.1). Soil solution NO_3^- concentration was positively correlated with soil net nitrification (Figure 3.3). There was also a significant relationship between net nitrification and nitrate concentration in the soil solution using all the replicates (Figure S3.3). At each individual study site, the NO_3^-

concentration does not increase even if the nitrification rate increases in Ashoro and Kasuya. In contrast, the NO_3^- concentration seems to increase with the nitrification rate in Nakagawa, which indicated the different sensitivity of NO_3^- concentration in response to the increases in nitrification (Figure S3.3). The soil C:N ratio was not correlated with soil solution NO_3^- concentration ($P > 0.05$, Figure S3.4).

3.3.3. Responses of N leaching to elevated N deposition among the sites

In Shibecha, average NO_3^- concentrations in the soil solutions below the rooting zones (collected from a depth of 50 cm below the ground surface) in fertilized plots were significantly higher than in unfertilized plots after the 3-year fertilization experiment (Figure 3.4). In contrast, there was no significant difference in the NO_3^- concentrations between unfertilized and fertilized plots at the other sites (Ashoro, Nakagawa, Shiiba, and Kasuya). Neither were there any significant differences in soil net nitrification between unfertilized and fertilized plots at any of the five sites (Table 3.2).

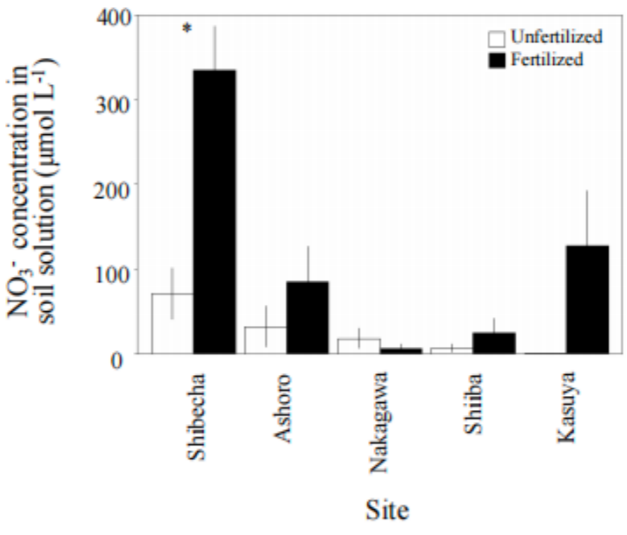

Figure 3.4 Average of soil solution NO_3^- concentration below the rooting zone (50 cm depth below the ground surface) in unfertilized plots and fertilized plots of five Japanese oak forests in 2020. Error bar indicates standard error. * $P < 0.05$.

3.4 Discussion

3.4.1. Site variability of NO_3^- leaching and its relationship with soil parent material and soil net nitrification

Significantly higher levels of soil solution NO_3^- concentration below the rooting zones at natural forests at Shibecha compared with other sites (Figure 3.1ab) suggest that there is inter-site variability in NO_3^- leaching, and that this can be high even in certain natural Japanese forests. We made an approximate evaluation of the amount of leaching (Table S3.2). The amount of N leaching from Shibecha was higher than from other sites, which also supports the result of higher leaching from Shibecha based on concentration. In the USA, it has been reported that soil solution NO_3^- concentrations under root zone in natural hardwoods forests were low, even near detection limits, where atmospheric N deposition was low (Magill et al. 2004). The reported levels of soil solution NO_3^- concentration were similar to those measured in this study in Nakagawa, Shiiba, and Kasuya. However, soil solution NO_3^- concentration at Shibecha in 2018 (Figure 3.1a) was almost at the same level as the N leaching from Japanese cedar plantations in central Japan (240 μmol L^{-1}; Oyanagi et al. 2002) and from an adjacent Japanese cedar plantation near the Kasuya site in this study (600 μmol L^{-1}). Japanese cedar plantations are known to undergo higher levels of NO_3^- leaching (Chiwa et al. 2015b; Nishina et al. 2017; Yang and Chiwa 2021), because mature trees are characterized by lower N uptake（Yang and Chiwa 2021).

The inter-site variability of NO_3^- concentrations in soil solutions observed in this study cannot be explained by differences in atmospheric N deposition alone. It has been suggested that N leaching among forested areas was related to atmospheric N deposition throughout Europe, where N leaching is low or even could not be detected when atmospheric N deposition was less than 10 kg N $ha^{-1}yr^{-1}$; N leaching is variable when atmospheric N deposition is from 10 to 25 kg N $ha^{-1}yr^{-1}$, and N leaching is high when atmospheric is more than 25 kg N $ha^{-1}yr^{-1}$ (Dise and Wright 1995). The low NO_3^- concentrations in soil solution at Ashoro, Nakagawa, and Shiiba could be explained by Dise and Wright (1995) because atmospheric N deposition is less than 10 kg N $ha^{-1}yr^{-1}$ at these three sites. However, a significantly higher NO_3^- concentration of soil solution in Shibecha was observed (Figure 1, Nagano et al., 2021) despite the low atmospheric N deposition (Table 1). Although atmospheric N deposition, especially dry deposition, is dominated by NH_4^+ in Shibecha because the forest is surrounded by dairyland (Nagano et al., 2021; Katata et al., 2023), the elevated levels of N deposition were moderate (< 10 kg N ha^{-1} yr^{-1}) (Nagano et al., 2021). In addition, although atmospheric N deposition is higher in Kasuya (15.5 kg N $ha^{-1}yr^{-1}$), there were no significant differences in NO_3^- concentrations of soil solutions among the sites except for at Shibecha.

Annual precipitation is also unable to explain the site variability of soil solution NO_3^- concentration in this study. Usually, NO_3^- concentration in soil solution may be more diluted at the sites with higher precipitation. The annual precipitation in Shiiba is

more than 3000 mm, which is substantially higher than that at the other four sites (Table 3. 1). However, there were no significant differences between soil solution NO_3^- concentrations measured at Ashoro, Nakagawa, Shiiba, and Kasuya.

It is possible that the inter-site variability of soil solution NO_3^- concentration reported in this study may be explained by the parent material (Anderson 1988). Soil parent material, as identified by soil groups, influences the balance between nitrogen loss and retention. Williard et al. (2005) found that the characteristics of the soil substrate could explain most of the variation in stream nitrate levels from the mid-Appalachian region, USA. Andosols, which are characterized by an anion exchange capacity under neutral to acidic conditions, cause the retardation of nitrate transport because of anion adsorption (Miki et al. 2009). In addition, it has been reported that nitrate adsorption of Andosols, which are rich in amorphous aluminum (Kinjo and Pratt 1971; Tani et al. 2004), was much higher than those reported in Ultisols and Oxisols, which are rich in iron oxides (Cahn et al. 1992; Eick et al. 1999). Therefore, it is possible that nitrate leaching in Andosols is lower than in other soil types. However, soil parent material was not selected for N leaching in model selection, indicating that soil parent material could not explain the site variability of soil solution NO_3^- concentrations in this study. This may be because of the contradictory characteristic of Andosols; they have large amounts of soil organic matter, and may therefore be associated with high soil nitrification rates (Urakawa et al. 2016), increasing N leaching.

A tendency for higher soil nitrification in Shibecha (Figure 3.2ab), where the highest NO_3^- concentration in soil solution was observed (Figure 3.1ab), alongside a significant relationship between NO_3^- leaching and soil net nitrification (Figure 3.3), indicates that soil net nitrification is an important factor for high N leaching from forested ecosystems across Japan. Our model selection for N leaching (Table S3.1) also suggests that soil net nitrification is an important factor explaining variability in N leaching. Our results are supported by previous studies that reported that NO_3^- export was positively related to nitrification rates (Ross et al. 2012) and high NO_3^- leaching occurs in locations where nitrification is high (Christ et al. 2002).

The association between lower C:N ratio and higher NO_3^- leaching has been observed by Phillips et al. (2013) and Midgley and Phillips (2014), and arises lower C:N ratios usually cause higher rates of soil nitrification (Gundersen et al. 1998). Andersson et al. (2002) also concluded that the C:N ratio was an important factor for N leaching across Europe. However, Urakawa et al. (2016) reported that the effect of organic layer C:N on soil net nitrification was not significant when Andosol sites were included, which supports the absence of any significant relationship between soil C:N ratio and NO_3^- concentrations observed in this study (Figure S3.4).

It is possible that the site variability in NO_3^- leaching in this study is explained by vegetation N uptake. This is an important factor regulating N retention (Vitousek et al., 1975; Fukuzawa et al., 2006; Yang and Chiwa, 2021). In unfertilized plots in Shibecha,

Ashoro, Nakagawa, Shiiba, and Kasuya, N uptake by plants – including overstory and understory vegetation – is estimated to be 49.5, 31.2, 28.9, 22.7, and 47.0 kg N ha^{-1} yr^{-1}, respectively (Yang et al., unpublished). This is much higher than the atmospheric N deposition in all five sites. Therefore, N uptake by plants was high enough to affect the retention of nitrogen inputs. However, N uptake by vegetation alone could not explain the site variability in NO_3^- leaching in this study because N uptake by vegetation in Shibecha was not lower than in other four sites. Further study of the plant response to elevated atmospheric N deposition will be needed to confirm this.

3.4.2. Susceptibility of NO_3^- leaching to elevated N deposition

Significant increases in NO_3^- concentration in soil solution after the 3-year N fertilization experiment in Shibecha (Figure 3.4), where the soil solution NO_3^- concentration in unfertilized plots was the highest (Figure 3.1ab), indicates that the significant increase in N leaching in response to N addition occurs at sites where N leaching was originally high. The N manipulation experiments at multiple sites across Europe also showed that the response of N leaching depends on its original N leaching status: low susceptibility of N leaching was reported at sites where N leaching was originally low (Gundersen et al. 1998). In contrast, significant increases in N leaching occurred at sites where N leaching was originally high (Gundersen et al. 1998). Similar

responses of high N leaching to N additions were also found in Chinese forests (Fang et al. 2009).

The amount of NO_3^- leaching in the fertilized plot of Shibecha was calculated to be 24 kg N ha^{-1} yr^{-1} (Table S3.2), approximately which was half of added nitrogen, indicating a lowering of N retention efficiency. In addition, when soil mineralization was considered as an input for calculating N retention efficiency, the value was 0.82. This is still lower than values reported from hardwood forests in Harvard Forest and Bear Brook (USA), where N retention efficiency was reported to be 0.93–0.99 (Aber et al., 1998). N retention efficiency in Ashoro, Nakagawa, and Shiiba (Table S2) was comparable to that in the Harvard Forest and Bear Brook sites (Aber et al., 1998).

It is also possible, but probably less likely, that the significant response of N leaching in Shibecha is explained by the increase in soil net nitrification rates caused by elevated N input. No significant difference in soil net nitrification between unfertilized and fertilized plots was found in this study (Table 3.2). Previous studies reported that responses of soil N transformation to elevated N deposition depend on several factors, including soil C:N ratio and pH (Urakawa et al. 2016), the abundance of ammonifiers and nitrifiers (Isobe et al. 2020) affecting soil microbial community structure (Wan et al. 2015), and microbial activities (Lupon et al. 2016). Although the reason why there was no significant increase in soil net nitrification remains elusive, it may simply be that lack of significant differences in chemical properties (including soil C:N ratio), and soil pH

(except for pH in Ashoro) between unfertilized and fertilized plots (Table 3.2) may lead to no significant difference in soil net nitrification between unfertilized and fertilized plots.

The lower soil NO_3^- immobilization in Shibecha reported by Urakawa et al. (2016) could be also the reason for a significant increase in N leaching in response to N addition in Shibecha that was observed (Figure 3.4). Urakawa et al. (2015) reported that soil NO_3^- immobilization in Shibecha (0.27 mg N kg^{-1} day^{-1}) was a lower level among 20 sites across Japan, with the average of 0.67 mg N kg^{-1} day^{-1} ranging from 0.05 to 3.5 mg N kg^{-1} day^{-1}. NO_3^- is consumed by the process of immobilization (Zhu and Wang 2011; Backer et al. 2017). In sites where N immobilization is low, large amounts of inorganic N result from the process of nitrification and an elevated pool NO_3^- in the surface soil (e.g. elevated N deposition and NO_3^- fertilization) can be leached into deeper soil from surface soil.

3.5 Conclusion

Our investigation evaluated the site variability of N leaching across five Japanese broadleaved forests. Soil parent material was not selected as a predictor for NO_3^- leaching, but soil net nitrification was. This indicates that soil parent material could not explain the variability of NO_3^- leaching, and that soil net nitrification could explain the N leaching from Japanese forests. We also found that the response of N leaching to elevated N deposition was more pronounced in sites where N leaching was originally high.

Chapter 4

Importance of understory vegetation and the response of overstory and understory to elevated N deposition

4.1 Introduction

Atmospheric nitrogen (N) deposition has increased by human activity (Galloway et al. 2008; Law et al., 2013; Engardt et al., 2017; Yu et sl., 2019; Gao et al., 2020). Chronic high atmospheric N deposition has strongly influenced N cycling in temperate forests that is generally limited by available N (Aber et al., 1998; LeBauer and Treseder, 2008). The increases in available N in soil due to elevated atmospheric N deposition can perturb the N cycling in forested ecosystems resulting in enhanced foliar N concentration (Tietema and Beier 1995; Du 2017; Tian et al., 2018), N uptake (Ashton et al., 2010), soil mineralization (Matson et al., 2014; Sun et al., 2016; Kooch et al., 2018), and nitrate (NO_3^-) leaching (Aber et al., 1998; Chen et al., 2000; Corre et al., 2010; Stevens 2019).

Sasa dwarf bamboo is a common understory vegetation that is dominated in Hokkaido, northen Japan (Toyooka et al., 1983; Watanabe et al., 2016). Litterfall of Sasa account for 30% of the total litterfall in a natural forest (Watanabe et al., 2016). In addition, it has been reported that Sasa dwarf bamboo mitigates N leaching because of the rapid annual increment of its biomass (Fukuzawa et al., 2006; Watanabe et al., 2016). Thus, assessing understory vegetation in addition to overstory vegetation is critical when assessing the response of forest ecosystems to elevated atmospheric N deposition. The importance of understory functional has been concluded by Landuyt et al. (2019), he also indicated that a simultaneous investigation of both overstory and understory functional responses to global change will be crucial, however, response of vegetation in overstory vegetation

and understory vegetation to elevated atmospheric N deposition in forest ecosystem has little been assessed simultaneously.

It can be hypothesized that the responses of understory vegetation to elevated N deposition including leaf N concentration and N uptake are more sensitive than those of overstory vegetation. Nitrogen isotope experiment indicated that understory vegetation had a higher N uptake than overstory vegetation (Buchmann et al., 1996). In addition, as vernal-dam hypothesis (Muller and Bormann, 1976) indicated that understory vegetation starts to absorb nutrients including nitrogen earlier in the growing season than deciduous canopy develops. Together, Makoto et al., (2021) demonstrate that increased available soil N will benefit understory vegetation without a commensurate change in overstory vegetation. Furthermore, it has also been reported that Sasa dwarf bamboo started growing faster than overstory vegetation in Shibecha, Northen Japan (Tateno et al., 2019), indicating faster acquisition of elevated available N in soil by understory vegetation than overstory vegetation. Therefore, it is possible that response of understory vegetation is faster than overstory vegetation to global climate change, especially elevated N deposition.

In this study, we evaluated N status including leaf N concentration and N uptake for both overstory and understory in three forested sites with different stages of N saturation, northern Japan, in conjunction with N fertilization experiment. Specific objectives of this study are 1) to investigate N status of overstory and understory

vegetation in N saturated and limited sites, 2) to evaluate the response of N status of overstory and understory vegetation in each site to elevated available N, and finally 3) to discuss the importance of understory vegetation to assess the response of forest ecosystems to elevated N deposition.

4.2. Materials and methods

4.2.1. Study site

The study was conducted in three sites, located in Shibecha, Ashoro, Nakagawa, northern Japan. All sites are registered as core or associate site of JaLTER (Japan Long-Term Ecological Research Network, http://www.jalter.org/en/researchsites/). The forests in three sites consists of overstory vegetation and understory vegetation. Overstory vegetation was Japanese oak (Quercus crispula) forests, understory vegetation was evergreen dwarf bamboo (Sasa nipponica in Shibecha and Ashoro, Sasa *senanensis & kurilensis* in Nakagawa). Detailed information at each site were shown in Table 1. According to Yang et al. (2023), Shibecha is N saturated because significantly higher NO_3^- leaching than Ashoro and Nakagawa and a significant increase of NO_3^- leaching to N fertilization (50 kg N ha^{-1} yr^{-1}) over three years (as described later in more details) were observed in Shibecha. In contrast, both Ashoro and Nakagawa are N limited, because

lower NO_3^- leaching than Shibecha and no significant increases in NO_3^- leaching to N fertilization (50 kg N ha^{-1} yr^{-1}) over three years (Yang et al., 2023)

Table 1. Annual precipitation, annual mean temperature, atmospheric N deposition, and number of pair plots; Species and height of understory vegetation; Diameter at breast height (DBH), density and Base area (BA) of overstory vegetation in the study sites

	Shibecha	**Ashoro**	**Nakagawa**
Annual precipitation (mm yr^{-1})	1066	951	1262
Annual mean temperature (ºC)	5.6	6.6	6.4
Atmospheric N deposition flux (kg N $ha^{-1}yr^{-1}$)	< 5[1)]	4.1[2)]	6.9[3)]
Number of pair plot	4	4	5
Understory vegetation (Sasa)			
Species	*Sasa nipponica*	*Sasa nipponica*	*Sasa senanensi &kurilensis*
Height (m)	0.8 –1.0	0.5	1.5−2.0
Overstory vegetation (Oak)			
DBH (cm) (Unfertilized)	30	32	47
DBH (cm) (Fertilized)	34	34	49
Density (trees ha^{-1})	450	500	300
(Unfertilized)	378	388	125
Density (trees ha^{-1}) (Fertilized)	42	41	54
BA (m^2 ha^{-1}) (Unfertilized)	62	33	23
BA (m^2 ha^{-1}) (Fertilized)			

1) Watanabe et al. (2019), 2) Chiwa et al. (2015a), 3) Shibata and Fukuzawa (2010)

We conducted the fertilization treatment to evaluate the response of vegetational N status including leaf N concentration and N uptake by overstory and understory vegetation to an elevated inorganic N pool in the surface soil. We established pair plots of unfertilized and fertilized plots (15 m × 15 m) in each site (Table 1). In the fertilized plots, N fertilization (50 kg N ha^{-1} yr^{-1} as granular solid ammonium nitrate) was

conducted during from late–April to late–May 2018, 2019, and 2020, before the growing season. N fertilizer was added directly to the forest floor. We divided a plot (15 m × 15 m) into 5 subplots (15m × 3m) to spread the N fertilizer homogenously over the survey plot.

4.2.2.1 Overstory trees

To assess leaf N concentration of overstory vegetation, we collected foliage of Japanese oak that is dominated overstory vegetation during growing season at each site (August 2020). At each site, three foliage samples from a branch exposed to sunlight was collected using a 15-m branch cutter. Collected leaf samples were dried for 72 hours at 70 °C. The N concentrations were measured by the combustion method (CN Corder MT-700, Yanaco, Kyoto, Japan). N resorption efficiency was calculated as:

N resorption efficiency = **(**N concentration in leaf - N concentration in leaffall)/ N concentration in leaf*100

To evaluate N uptake by overstory vegetation (Japanese oak), litterfall was collected from the Japanese oak (control and fertilization) from April 2018 to March 2020. The N uptake was calculated by multiplying the annual leaf fall weight by the N concentration in the leaf fall. To collect litterfall, one litter traps (0.25 m^2 in size) per plot were established in Shibecha, one litter traps (0.5 m^2 in size) per plot were established in Nakagawa and in Ashoro. Each litter trap was constructed from a steel hoop (80 cm

aperture) and a woven nylon bag (80 cm depth) and was positioned 1 m above the ground with plastic stakes. Samples were collected monthly and dried for 72 hours at 70°C, sorted into leaf fall and other material (did not use in this study), and weighed. The N concentrations were measured by the combustion method (CN Corder MT-700, Yanaco, Kyoto, Japan).

4.2.2.2 Understory Sasa dwarf bamboo

We established one quadrat (one 1m × 1m, divided into four 50 cm × 50 cm) per plot to assess biomass N including leaf and culm N uptake by understory Sasa. We harvested aboveground living Sasa from the ground surface in each quadrat at the beginning of October in 2020, when Sasa biomass is almost at its peak. The collected Sasa biomass was divided into current- and previous-year leaves and culms. The collected samples were brought to the laboratory, dried for 72 hours at 70°C, and weighed. The N concentrations of current- and previous-year leaves and culms were measured by the combustion method (CN Corder MT-700, Yanaco, Kyoto, Japan). We calculated annual N uptake by Sasa (ANUS) as follows:

ANUS (kg N ha^{-1} yr^{-1}) = [current year leaf biomass (g m^{-2}) × current year leaf N concentration (%) + current year culm biomass (g m^{-2}) × current year culm N concentration (%)] ×10

4.2.3. Statistical analysis

Site comparison of leaf N concentration of overstory vegetation and of leaf and culm of understory vegetation were performed using *Tukey* tests. Comparisons of N concentration, weight, N uptake by understory vegetation and overstory vegetation between unfertilized and fertilized plots were performed using *t*-tests. The differences were considered significant at the $P < 0.05$ level. Above statistical analyses were performed using R version 3.5.0, for Windows (R Core Team, 2022).

4.3. Results

4.3.1. Overstory trees

There was no significant difference in leaf N concentration, N uptake, in overstory vegetation in unfertilized plots among three sites (Shibecha, Ashoro, and Nakagawa) (Table 2). Leaf N concentration did not differ significantly between unfertilized and fertilized plots in each three sites (Table 2). In addition, there was no significant difference of tree N uptake, N concentration in leaf fall, leaf fall biomass, N resorption efficiency (NRE) between unfertilized and fertilized plots at each three sites (Table 2).

Table 4.2. N concentration in leaves and leaffall, weight of leaf fall, N uptake, N resorption efficiency in trees.

	N concentration in leaf (A) (%)	N concentration in leaffall (B) (%)	weight of leaffall (C) ($g\ m^{-2}$)	N uptake ($kg\ N\ ha^{-1}$)	N resorption efficiency (A - B)/A*100 (%)
Shibecha					

Unfertilized	2.04 ± 0.08 a	0.99 ± 0.08 a	352 ± 7.59	34.6 ± 2.4 a	56 ± 5
Fertilized	2.30 ± 0.04	0.95 ± 0.18	333 ± 23.9	48.3 ± 14	59 ± 7
	ns	ns	ns	ns	ns
Ashoro					
Unfertilized	2.19 ± 0.17 a	0.73 ± 0.05 b	270 ± 24.1	22.3 ± 3.1 a	66 ± 5
Fertilized	2.33 ± 0.15	0.77 ± 0.07	289 ± 8.70	30.0 ± 9.0	67 ± 3
	ns	ns	ns	ns	ns
Nakagawa					
Unfertilized	2.10 ± 0.07 a	0.67 ± 0.04 b	140 ± 18.6	25.0 ± 3.7 a	68 ± 2
Fertilized	2.15 ± 0.06	0.70 ± 0.02	162 ± 15.3	20.3 ± 3.5	67 ± 1
	ns	ns	ns	ns	ns

All values show mean ± standard deviation (n = 5); ns shows not significant difference (P > 0.05 for t-test) between unfertilized and fertilized plot; N uptake was calculated as: weight of leaffall (g m^{-2}) × N concentration in leaffall (%)×10 / (base area of oak/total area×100)×10^3; Different capital letters mean significant differences among three unfertilized sites (P < 0.05, Tukey's HSD).

4.3.2. Understory Sasa

N concentration of Sasa was higher in N saturated site (Shibecha) of unfertilized plots for both leaf and culm than those in N limited sites (Ashoro and Nakagawa) (Table 3). In Shibecha, there were no significant differences in N uptake by Sasa, N concentration in culm, and Sasa biomass in leaf and culm between unfertilized and fertilized plots (Table 3). However, current leaf N concentration of Sasa was higher in the fertilized plot than in unfertilized plot (Table 3).

In Ashoro, one of the N limited sites, although there were no significant differences in N concentration and biomass in leaves and culm between the unfertilized plot and the fertilized plot, N uptake by Sasa was higher in the unfertilized plot than in the fertilized plot (Table 3).

In Nakagawa, which is also one of the N limited sites, N uptake by Sasa and biomass in leaves were higher in the fertilized than in unfertilized plots (Table 3). However, there

were no significant differences in N concentrations in current leaf and culm, and biomass in culm, between the unfertilized and fertilized plots (Table 3).

Table 4.3. N concentration in leaf and culm, weight of leaf and culm, N uptake in leaf and culm in sasa.

	N concentration (leaf) (A) N concentration (culm) (C) (%)	weight of leaf (B) weight of culm (D) (g m^{-2})	N uptake (A×B+C×D) (kg N ha^{-1})
Shibecha			
Unfertilized	2.5 ± 0.0(leaf) a	83.2 ± 6.5(leaf)	32.3 ± 3.9 a
Fertilized	2.7 ± 0.0(leaf)	79.1 ± 8.4(leaf)	33.8 ± 4.5
	*	ns	ns
Unfertilized	0.9 ± 0.0(culm)	130.4 ± 10.1(culm)	
Fertilized	1.05 ± 0.1(culm)	115.9 ± 13.3(culm)	
	ns	ns	
Ashoro			
Unfertilized	2.0 ± 0.1(leaf) b	54.4 ± 8.5(leaf)	14.3 ± 2.5 b
Fertilized	2.2 ± 0.1(leaf)	79.0 ± 10.6(leaf)	23.9 ± 3.4
	ns	ns	*
Unfertilized	0.57 ± 0.1(culm)	55.3 ± 10.1(culm)	
Fertilized	0.81 ± 0.1(culm)	83.2 ± 13.2(culm)	
	ns	ns	
Nakagawa			
Unfertilized	2.1 ± 0.1(leaf) b	70.7 ± 11.2(leaf)	19.7 ± 3.2 b
Fertilized	2.1 ± 0.1(leaf)	115.2 ± 16.2(leaf)	36.1 ± 6.2
	ns	*	*
Unfertilized	0.58 ± 0.1(culm)	87.4 ± 16.8(culm)	
Fertilized	0.63 ± 0.1(culm)	116.7 ±44.6(culm)	
	ns	ns	

All values show mean ± standard deviation (n = 5); ns shows not significant difference (P > 0.05 for t-test) between unfertilized and fertilized plot; significant difference (P < 0.05 for t-test) between unfertilized and fertilized plot; Different capital letters mean significant differences among three unfertilized sites (P < 0.05, Tukey's HSD).

4.4. Discussion

4.4.1. N status of overstory and understory vegetation in N saturated and limited sites

Leaf N concentration of overstory vegetation in unfertilized plots was not higher in the N saturated sites (Shibecha) than in the N limited sites (Ashoro and Nakagawa) (Table 2), which was not corresponding to the N status of the site judging from NO_3^- leaching

from soil (Yang et al., 2023). In general, foliar element concentrations reflect the nutritional status of trees (Garten 1993; Schmitz et al., 2019; Talkner et al., 2019). Our result was contrast with the study showing that foliar N concentration is enhanced in N saturated sites (Aber et al., 1998; Yu et al., 2018; Braun et al., 2020). The reason will be discussed in later section. However, foliar N levels have been found little difference along an N deposition gradient in the Asia, US and Europe (Gundersen et al., 1998; Magill et al., 2004; Huang et al., 2015), which indicated the seasonality of our result.

In contrast, N concentrations in current leaf and current culm of Sasa in unfertilized plots were significantly higher in the N saturated site (Shibecha) than those in N the limited sites (Ashoro and Nakagawa), corresponding to the N status of the sites (Table 2). Higher N uptake by Sasa in the N saturated site than in the N limited sites (Table 2) also support the corresponding relationship between vegetation and sites with respect to N status. Thus, the corresponding relationship between vegetation and sites with respect to N status differed between overstory and understory vegetation in this study.

4.4.2.1. Negligible response in overstory vegetation to N fertilization regardless of site N status

No significant increases in leaf N concentration and N uptake by overstory vegetation over three-year N fertilization (Table 2) indicate that the response of overstory vegetation to elevated N deposition is negligible even in the N limited sites (Ashoro and Nakagawa).

Previous studies have also indicated that there are no significant differences of leaf N concentration (Magill et al., 2000) and plant N uptake (Liu et al., 2023) after N addition in broad leaved forest. Oak was also found to have no foliar response to varied N availability (McNeil et al. 2007). No significant difference in NRE between unfertilized and fertilized plots (Table 2) further supports negligible response of N uptake to N status. These results suggests that response of Japanese oak to elevated N deposition can be negligible even in N limited areas. Thus, the fertilization experiments in this study confirm no corresponding relationship between Japanese oak and the site with respect to N status as discussed in the previous section.

4.4.2.2. Response in understory vegetation to N fertilization with dependence on site N status

No significant increase of N uptake by understory vegetation to N fertilization in the N saturated site (Shibecha) (Table 3) indicates that Sasa in Shibecha is N saturated. No significant increase in biomass in N fertilization plots despite significant increase in current leaf N concentration of Sasa in the N saturated site (Table 3) also supports the N saturation of Sasa.

The increases in N uptake by Sasa in the N-limited sites (Ashoro and Nakagawa) (Table 3) support a general idea that N uptake by vegetation increases in response to

elevated N deposition in N limited areas (Bobbink and Hettelingh, 2010; Templer et al., 2012). Interestingly, fertilization did not increase leaf N concentration of Sasa in the two sites unlike in Shibecha where leaf N concentration was significantly higher in the fertilized plots. Our results were consistent with Watanabe et al. (2016) showing that N fertilization (50 kg N ha^{-1} yr^{-1}) enhanced N uptake by Sasa without increase in leaf N concentration. Nordin et al. (1998) also indicated that N fertilization stimulated biomass and leave N concentration in understory vegetation after 50 kg N ha^{-1} yr^{-1} N addition, which is consistent to our two N limited sites (Ashoro and Nakagawa). These fertilization experiments in this study also confirm the corresponding relationship between Sasa and site with respect to N status as discussed before.

4.4.3. Importance of understory vegetation to assess the response of forest ecosystems to elevated N deposition

This study showed that the response to elevated atmospheric N deposition was different between overstory and understory, and the N fertilization experiment confirmed this. The reason why no corresponding relationship between overstory vegetation and sites with respect to N status (as discussed in section 4.1) and negligible response of Japanese oak to over three-year N fertilization remains elusive. However, three-year fertilization may be too short to enhance N concentration owing to lower sensitivity of Japanese oak to elevated N availability (Magill et al., 2004). Nagano et al. (2022) also

indicated that negligible Response of Leaf N contents to short-term nitrogen fertilization in Japanese oak forest.

Relatively high NRE in Japanese oak might be the reason for lower sensitivity of Japanese oak to N status. Higher N resorption efficiency (56%-68%; Table 2, the value is within the review papers reported by Zhang et al., 2018) indicates higher contribution of internal N cycling to N utilization for growth of overstory vegetation, which may cause lower sensitivity of Japanese oak to the changing N status. The results of N resorption efficiency were higher than reported data by Tateno et al. 2019, where N resorption efficiency was 50% in Shibecha. Aerts (1996) found a mean reabsorption of 50% for N during leaf senescence using a wide range of species. A recent report by Vergutz et al. (2012) suggests that N from leaves frequently may exceed 60%, therefore, a lower sensitivity of Japanese oak in this study is reasonable.

In contrast, increase in N uptake by understory vegetation in the N limited sites (Ashoro and Nakagawa) (Table 3) indicate that N uptake by Sasa is more sensitive than that by Japanese oak in response to elevated N availability. Muller (2003) pointed out that the herb layer is potentially the most sensitive to changes in nutrient availability. Nitrogen isotope experiment also indicated that understory vegetation had a higher N uptake than overstory vegetation (Buchmann et al., 1996). In addition, it has been shown that the response of nutrient cycling of understory vegetation to added N is more sensitive than overstory vegetation (Deng et al., 2016; Gilliam 2006; Zhang et al., 2016).

Higher sensitivity in Sasa may be caused by different phenology between Sasa and Japanese oak. Sasa grows faster than Japanese oak (Tateno et al., 2019), which result in faster N acquisition by Sasa from elevated N availability in soil caused by N fertilizer than by overstory vegetation. It is also generally known as the vernal-dam hypothesis that the understory vegetation in deciduous forests absorbs N in early spring before the forest canopy tree leaves develop (Muller and Bormann, 1976), which could explain higher sensitivity in understory vegetation than in overstory vegetation in this study. In addition, it has also been reported that root nitrate reductase activity tended to be lower in overstory trees than in the understory vegetation, contributing to lower NO_3^- uptake by overstory trees (Tateno et al., 2020).

A higher sensitivity in Sasa may be caused by different mycorrhizal fungi associations between Sasa and Japanese oak. Because Sasa is an AM-associated species (Tateno et al., 2020) in which the growth may be enhanced by N deposition (Thomas et al., 2010), whereas Japanese oak is ectomycorrhizal species (Tateno et al., 2020). Ectomycorrhizal species utilize the organic N whereas arbuscular mycorrhizal fungi usually utilize the inorganic N, contributing to a higher sensitivity to N fertilized addition (Thomas et al., 2010, Phillips et al., 2013). In addition, arbuscular mycorrhizal fungi producing low levels of proteolytic enzymes respond positively to increased nitrogen accumulation compared to ectomycorrhizal species (Thomas et al., 2010, Phillips et al., 2013).

4.5. Conclusion.

This study highlights the importance of understory vegetation when assessing the response of vegetation to elevated N deposition. To our knowledge, this is the first study conducting quantitative evaluation on the N status of overstory and understory vegetation simultaneously in forested sites with different N status. This study provides better understanding the effect of elevated N deposition on N cycling in forest ecosystems. Inclusion of understory vegetation to assess N status can explain N status of sites in this study. In the N limited sites (Ashoro and Nakagawa) where NO_3^- leaching is low (Yang et al., 2023), Sasa can mitigates NO_3^- leaching owing to high sensitivity to elevated N availability. In contrast, In the N saturated site (Shibecha) where NO_3^- leaching is high (Yang et al., 2023), Sasa has already reached at N saturated and cannot mitigate NO_3^- leaching. Therefore, understory vegetation should be included in addition to overstory vegetation when assessing the response of forest ecosystems to elevated atmospheric N deposition.

Chapter 5

Summary and conclusion

We investigated NO_3^- leaching from Japanese cedar plantation and Japanese oak forests and their susceptibility to elevated N addition by 3-year N fertilization experiment. We aimed to improve our understanding of nitrate (NO_3^-) leaching from forested areas and its susceptibility to elevated atmospheric N deposition and elucidate the possible reasons causing NO_3^- leaching. The results of our study divided into three main sections:

1) This study conducted a stand-scale investigation of N leaching from a specific species. The results confirmed high NO_3^- leaching from a Japanese cedar plantation. N fertilization experiments indicate that the high NO_3^- leaching observed in the Japanese cedar plantation could have been caused by low N retention. Although soil nitrification in Japanese cedar plantations could be an important source of NO_3^- leaching, soil nitrification was not correlated with NO_3^- leaching. Lower tree N uptake was a dominant factor contributing to the increase in N leaching. Because Japanese cedar is the main plantation species, accounting for approximately 44% of the plantation area in Japan, N leaching from these plantations may be an important source of N in headwater streams. Further investigations are required to explore the reasons for low N retention in Japanese cedar plantations (Chapter 2).

2) Our investigation evaluated the site variability of N leaching across five Japanese broadleaved forests. Soil parent material was not selected as a predictor for NO_3^- leaching, but soil net nitrification was. This indicates that soil parent material could not explain the variability of NO_3^- leaching, and that soil net nitrification could explain the N leaching from Japanese forests. We also found that the response of N leaching to elevated N deposition was more pronounced in sites where N leaching was originally high (Chapter 3).

3) This study is the first study conducting quantitative evaluation on the N status of overstory and understory vegetation simultaneously in forested sites with different N status. This study provides better understanding the effect of elevated N deposition on N cycling in forest ecosystems. Inclusion of understory vegetation to assess N status can explain N status of sites in this study. In the N limited sites (Ashoro and Nakagawa) where NO_3^- leaching is low, Sasa can mitigates NO_3^- leaching owing to high sensitivity to elevated N availability. In contrast, In the N saturated site (Shibecha) where NO_3^- leaching is high, Sasa has already reached at N saturated and cannot mitigate NO_3^- leaching. Therefore, understory vegetation should be included in addition to overstory vegetation when assessing the response of forest ecosystems to elevated atmospheric N deposition (Chapter 4).

In summary, this study evaluated the factors that cause the variability in NO_3^- leaching from forested areas due to increased atmospheric N deposition and the response of the N cycle over a wide area, which have been poorly understood in the forested areas of Japan. And we compared with Europe and the United States: 1) differences among tree species (Japanese cedar) are a unique factor influencing NO_3^- leaching in Japan (Chapter 2); 2) soil characteristics (nitrification rate, parent material) were consistent with those in Europe and the United States (Chapter 3); 3) the importance of understory vegetation was newly elucidated in this study by comparing the N uptake by overstory vegetation (Oak) and understory vegetation (Sasa), which was quantified (Chapter 4). As a result, it was clarified that N uptake by vegetation and nitrification in soil, which are important processes in the N cycle in forest areas, are important factors affecting NO_3^- leaching. Furthermore, in forests with low nitrification rate, low N uptake by overstory tree causes N leaching (Chapter 2); while in forests with high nitrification rate, low N uptake by overstory tree causes N leaching (Chapter 3, 4). Which means that in forests with a high

nitrification rate, N leaching occurs regardless of the amount of N uptake. Therefore, that means most of NO_3^- produced by nitrification was leached very soon, which cause high NO_3^- leaching even in the sites with the high N uptake. This study also highlights the importance of vegetation composition for managing the water quality in headwater streams from forest ecosystems disturbed by atmospheric N deposition.

Appendix

Table S2.1 Results (*P*-values) of ANOVA on tree species (S), fertilization (F) and their interactions on NO_3^- leaching.

Variable	4/4	4/26	5/24	7/1	8/1	9/28	10/30
Species (S)	< 0.001	< 0.001	< 0.001	< 0.001	0.01	< 0.001	0.001
Fertilization (F)	0.433	0.014	< 0.001	0.707	0.439	0.889	0.324
S × F	0.491	0.024	< 0.001	0.709	0.445	0.857	0.326

Bold values donate a significance at $P < 0.05$.

Table S2.2 Topographic slope, age of tree, diameter of breast height (DBH), density in Japanese cedar and Japanese oak plantations.

	Slope (°)	Age of tree (year)	DBH (cm)	Tree density (trees ha^{-1})
Japanese cedar	25 – 40	64 – 69	33.0 ± 0.9	1040
Japanese oak	3	24	22.8 ± 1.5	1600

[a] mean ± standard deviation ($n = 5$)

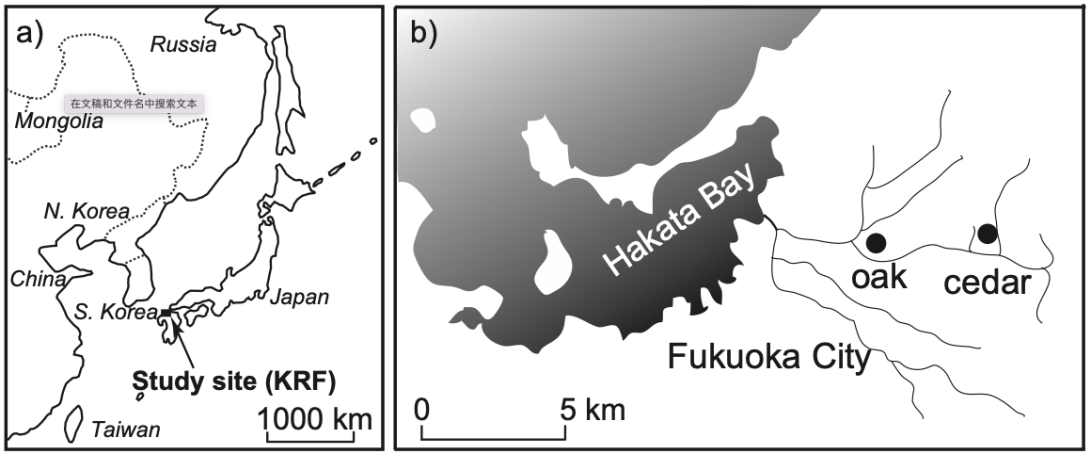

Fig. S2.1 Location of a) the study site (KRF; Kasuya Research Forest) and b) experimental stands in the Japanese cedar plantation and oak forests.

Table S3.1. General linear model (GLM) model selection for NO_3^- leaching. Full model is soil parent material (S) + nitrification (N) + the interaction ([S] × [N]).

Selected factor	AIC
1	118.62
Soil parent material (S)	119.22
Nitrification (N)	110.28
S + N	110.95
S + N + S × N	111.12

Table S3.2. N flux of N leaching in five sites in 2020 in unfertilized and fertilized plot.

Site	Runoff[a] (mm)	Amount of N leaching[b] (Kg N ha^{-1} $year^{-1}$)		N retention efficiency[c]	
		Unfertilized	Fertilized	Unfertilized	Fertilized
Shibecha	514	5.1	24	-0.02[d]	0.56
Ashoro	377	1.6	4.4	0.60	0.92
Nakagawa	685	1.7	0.6	0.75	0.99
Shiiba	2453	2.3	8.4	0.77	0.86
Kasuya	894	0.1	16	0.99	0.76

[a] Runoff was calculated by subtracting the annual evapotranspiration from the annual precipitation. The annual evapotranspiration was estimated using the model for Evapotranspiration as E (mm) = 31.4T (°C) + 376 (Komatsu et al., 2008), where T refers to the mean annual mean air temperature.

[b] Amount of N leaching was calculated by multiplying runoff (mm) by NO_3^- concentration in soil solution.

[c] N retention efficiency was calculated as the following equation: N retention efficiency = 1- amount of N leching/N input. N input includes atmospheric N deposition and N fertilizer (in fertilized plot).

[d]The negative value indicates a situation where the output of N exceeds its input.

Figure S3.1. Location of experiment sites

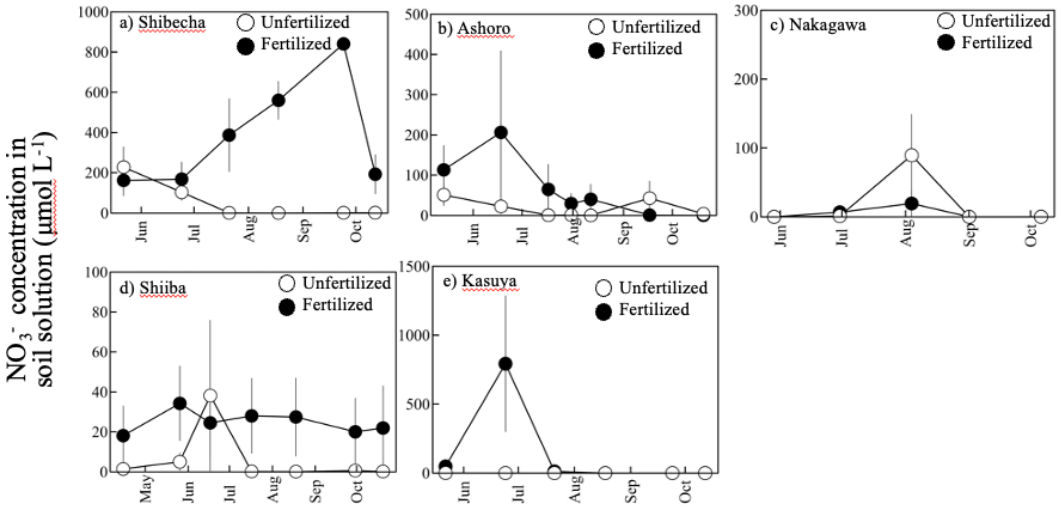

Figure S3.2. Changes in the NO_3^- concentration in the soil solution below the rooting zone (50 cm depth) in the (a) Shibecha (b) Ashoro (c) Nakagawa (d) Shiiba and (a) Kasuya during the study period in 2020. The error bars indicate the standard errors.

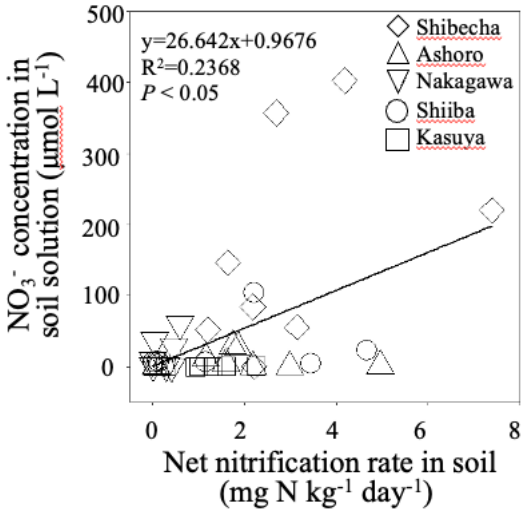

Figure S3.3. The correlation between soil net nitrification and average NO_3^- concentration in the soil solution below the rooting zone (50 cm depth) in unfertilized plots among replicates plots in five Japanese oak forests in 2018 and 2020.

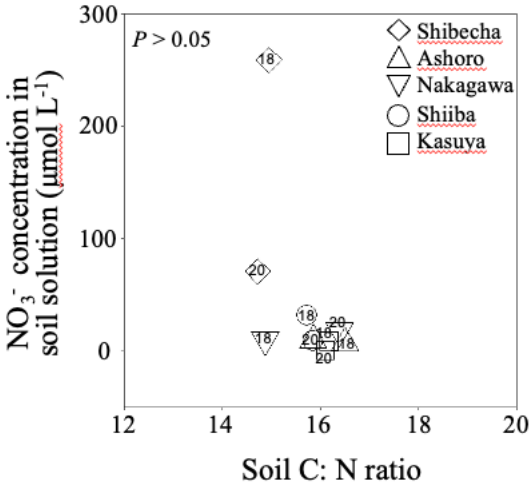

Figure S3.4. The correlation between soil CN ratio and average NO_3^- concentration in the soil solution below the rooting zone (50 cm depth) in unfertilized plots among five Japanese oak forests in 2018 and 2020. The labels "18" and "20" represent "in 2018" and "in 2020."

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