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Nitrogen cycling in a changing world: Response of a California grassland to multiple global change factors

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Nitrogen cycling in a changing world: Response of a California grassland to multiple global change factors

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

In

Environmental Systems

by

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True teachers use themselves as bridges over which they invite their students to cross; then, having facilitated their crossing, joyfully collapse, encouraging them to create bridges of their own. – Nikos Kazantzakis

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Abstract of the Dissertation

Nitrogen cycling in a changing world: Response of a California grassland to multiple global change factors

by

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Doctor of Philosophy, Environmental Systems Program
University of California, Merced, 2014
Dr. Stephen C. Hart, Chair

The cycling of nutrients, such as nitrogen (N), is arguably one of the most critical ecosystem services provided by soil. Nitrogen is the limiting nutrient for plant growth in many terrestrial ecosystems and can consequently regulate net primary production, plant diversity, and community composition. Transformations of available N, which are catalyzed by soil microorganisms, can also affect air and water quality, with possible implications for climate change and human health. In an era of global environmental change, it is paramount to gain a mechanistic understanding of how soil N is affected by anthropogenically derived perturbations such as exotic plant invasion and elevated nutrient deposition. Using a multifactor global change experiment, I assessed how three principal global change factors – exotic plant invasion, N deposition (simulated by N fertilization), and aboveground vegetation removal (to simulate cattle grazing or mowing) – affected soil N cycling, namely \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) availability and potential rates of nitrification and denitrification, in a California grassland. In order to increase understanding of how soil microbial communities regulate changes in N cycling, I concurrently measured broad-scale community structure of bacteria and archaea and the abundances of ammonia-oxidizing bacteria and archaea. I found that two invasive plants, \textit{Aegilops triuncialis} and \textit{Elymus caput-medusae}, reduced soil N availability and nitrification and denitrification potentials compared to perennial-dominated native communities but not naturalized exotic communities. Aboveground vegetation removal, which is often used as a tool to manage invasive plant populations (through cattle grazing or mowing), tended to exacerbate the effects of invasion by further reducing nitrification potential and soil \( \text{NO}_3^- \) availability. Fertilization with \( \text{NH}_4\text{NO}_3 \) consistently increased nitrification potential and soil \( \text{NO}_3^- \) availability, yet \( \text{NH}_4^+ \) remained unaffected and
denitrification potential was reduced. When combined, defoliation and N fertilization always produced additive effects. Finally, despite the sometimes dramatic shifts in N availability and potential rates that were observed, microbial community composition remained unaffected by changes in plant composition, N fertilization and defoliation. Overall, these findings provide evidence that N cycling is uniquely affected by each individual global change factor, and that the interactive effects of N fertilization and defoliation can be predicted based on combining single factor studies. These results also suggest that microbial communities composition is insensitive to global change in this system, and that microbial activity – as measured by rates of N cycling – is decoupled from community composition.
1 Introduction

Soils are complex systems comprised of pore space, minerals, and organic matter. A diverse group of macro- and micro-organisms reside in soil, where nutrients are transformed, gases are produced and consumed, and water is transported. In this way, soil supports valuable ecosystem services - e.g., plant growth, water and air quality, and habitat for soil-dwelling organisms (Daily et al., 1997). The cycling of nutrients, such as nitrogen (N), is arguably one of the most critical functions affecting the provision of ecosystem services by soil. Nitrogen is the limiting nutrient for plant growth in most terrestrial systems (Vitousek & Howarth, 1991); as such, soil N availability regulates net primary production (NPP; LeBauer & Treseder, 2008), plant diversity (Bobbink et al., 2010), and community composition (Tilman, 1987; Hueneke et al., 1990; McClean et al., 2011). Moreover, transformations of available N via nitrification (NH$_4^+$ to NO$_3^-$) and denitrification (NO$_3^-$ to N$_2$) produce nitrous oxide (N$_2$O; Bateman & Baggs, 2005; Parton et al., 2012), a critical greenhouse gas, and nitric oxide (NO; Bollmann & Conrad, 1998), which is quickly transformed into the air pollutant nitrogen dioxide (NO$_2$). Water quality is also affected by soil N cycling; nitrate, and to a lesser extent NH$_4^+$, can leach into groundwater (MacDonald et al., 2002; Rowe et al., 2006; Jahangir et al., 2012), create eutrophication of adjacent aquatic ecosystems (Smith et al., 1999), and lead to unsafe drinking water conditions (Ward et al., 2005). The regulation of N cycling thus has potentially large consequences for maintenance of soil-derived ecosystem services.

Soil microorganisms are responsible for catalyzing key processes in the N cycle (Figure 1-1). For instance, heterotrophic bacteria and fungi excrete extracellular enzymes to access C contained in organic matter (Sinsabaugh, 1994); as a “byproduct” of this search for energy, organically bound N is released into the soil solution as a microbial and plant available form (NH$_4^+$; McGill & Cole, 1981; Zumft, 1997; Schimel & Bennett, 2004). A small group of microorganisms (ammonia oxidizing bacteria and archaea) can then utilize the NH$_4^+$ that is released as a source of electrons to produce adenosine triphosphate (ATP; Hofman & Lees, 1953; Stahl & la Torre, 2012), a key energy molecule. The byproduct, NO$_2^-$, is utilized in the same way by nitrite oxidizers (Lees & Simpson, 1957), and together, these two steps make up nitrification. A phylogenetically broader group of microorganisms (Shoun et al., 1992; Philippot, 2002), including bacteria, archaea, and fungi, utilize NO$_3^-$ as a terminal electron acceptor during anaerobic respiration, thereby reducing NO$_3^-$ to NO, N$_2$O and finally N$_2$ (i.e., denitrification), which is released into the
atmosphere. Clearly, these processes are inextricably linked to the microorganisms that catalyze them, suggesting that the composition, relative abundances, and richness (together = ‘structure’) of the microbial community should be of importance for soil N cycling and availability. Results from an increasing amount of studies, however, demonstrate that the relationship between N cycling and microbial community structure is not straightforward (Swift et al., 1998; Cavigelli & Robertson, 2000; Griffiths et al., 2000; Setälä & McLean, 2004; Salles, 2012; Weedon et al., 2012) even for specialized processes such as nitrification (Prosser & Nicol, 2008; Hallin et al., 2009; Di et al., 2009; Yao et al., 2011), highlighting the need for continued research.

Understanding how microbial community structure regulates soil N cycling is becoming even more crucial with accelerated global change. Microorganisms differ in their sensitivity to biotic (e.g., changes in plant composition) and abiotic (e.g., temperature and precipitation) variables; as such, global change could favor certain groups of microorganisms over others (Fierer et al., 2007; Verhamme et al., 2011; Ramirez et al., 2012), leading to shifts in microbial composition, diversity, and subsequent N cycling. To understand if this is the case, numerous studies have manipulated one or more global change factor(s) and monitored the response of either soil N cycling (e.g., Barnard et al., 2006; Liao et al., 2008; Niboyet et al., 2010; Dijkstra et al., 2010; Brown et al., 2011; Ochoa-Hueso et al., 2013) or the microbial community (e.g., Horz et al., 2004; Zhang et al., 2005; Batten et al., 2006; Treseder, 2008; Castro et al., 2010; He et al., 2010; Shen et al., 2011; Ramirez et al., 2012; Gutknecht et al., 2012); however, relatively few have monitored soil N cycling and microbial community composition from the same treatments, especially in multifactor global change experiments. This is a large research need, given that multiple global change factors often simultaneously influence ecosystems and likely lead to unanticipated interactive effects.

The overall aim of my dissertation was to quantify the single and combined effects of three principle global change factors affecting Mediterranean grasslands today: exotic plant invasion, nitrogen (N) deposition, and aboveground vegetation removal (one component of cattle grazing). In California especially, these factors are of particular importance, given their high occurrences on the landscape. Indeed, California is home to over 200 invasive plants (Cal-IPC) and 5 million cattle (USDA NASS 2012), and experiences some of the highest N deposition rates in North America (≥ 90 kg N ha\(^{-1}\) y\(^{-1}\) in some areas; Fenn et al., 2010). Understanding how factors, such as these, interact will help provide a more realistic approximation of ecosystem dynamics in the future. This understanding may increase management success by helping land managers identify scenarios where the mitigation of one global change driver may require the concurrent mitigation of another global change driver acting simultaneously.

In order to increase understanding of how soil microbial communities drive ecosystem response to global change, I measured the response of both N cycling processes and soil microbial community structure to exotic plant invasion, N deposition, and aboveground vegetation removal. Additionally, because Mediterranean-type ecosystems experience
strong seasonality in soil temperature, soil water content, and plant phenology, I evaluated if and how the relative effects of these global change factors varied throughout the year. All data was collected from a well-replicated multifactor global change field experiment located in Davis, CA.

1.1 Organization of the Dissertation

The research from this dissertation is divided into three self-contained chapters (2, 3, and 4), which are written in manuscript format. In Chapter two, “Exotic plant invasion slows nitrogen cycling in a California grassland,” I investigated how soil N cycling differed among native grassland communities, naturalized communities (i.e., communities of exotic species that are now so ubiquitous that they are considered part of the native flora), and communities comprised of two aggressive invasive species, *Aegilops triuncialis* and *Elymus caput-medusae*. This chapter will be submitted to the journal, Ecology. In the third chapter, “Nitrogen fertilization and defoliation influence soil nitrogen cycling but not the abundance of ammonia oxidizing microorganisms in an invaded grassland community,” I first compared N cycling of native communities to invaded mixtures (native, naturalized, and invasive species), then determined how N fertilization, aboveground vegetation removal, and their interaction affected soil N cycling and the abundances of key microorganisms (ammonia oxidizing bacteria and archaea) in invaded grasslands. This chapter will be submitted to Global Change Biology. In the fourth chapter, “Microbial community composition is insensitive to multiple global changes in experimental grasslands,” I used next-generation sequencing techniques to determine if bacterial and archaeal community structures were affected by exotic plant invasion, N fertilization, aboveground vegetation removal, or the interaction of N fertilization and aboveground vegetation removal. This chapter will be submitted to the ISME Journal. All chapters were written using the pronoun “we”, which refers to co-authors of the manuscript and myself.

1.2 References


1.3 Figures

Figure 1-1 Simplified diagram of the nitrogen (N) cycle. 1) N fixation, 2) N mineralization/ammonification, 3) microbial immobilization or plant uptake of NH$_4^+$, 4) microbial immobilization or plant uptake of NO$_3^-$, 5) nitrification, 6) denitrification, 7) leaching of NH$_4^+$, 8) leaching of NO$_3^-$. Steps 1-6 are mediated by microorganisms, which are denoted by the small open circles. Plant uptake of N (steps 3 and 4) can be, but is not necessarily, facilitated by microorganisms.
2 Exotic plant invasion slows nitrogen cycling in a California grassland

2.1 Abstract

Invasive species constitute a significant component of global environmental change. In California grasslands, annual exotic grasses have been replacing native species since European colonization, many of which are now so ubiquitous that they are considered part of the regional flora (‘naturalized’). New invasive plants, such as *Aegilops triuncialis* (Barb goatgrass) and *Elymus caput-medusae* (Medusahead), continue to spread throughout the state today. It is critical to understand how exotic plant invasion affects nitrogen (N) cycling, given that changes in N availability can have consequences for vegetation dynamics, restoration efforts, and air and water quality. This study compared soil N cycling and microbial biomass of experimental grasslands comprised of (1) invasives *A. triuncialis* and *E. caput-medusae*, (2) exotic annual species that are now naturalized, and (3) native annual and perennial species. We found that *A. triuncialis* and *E. caput-medusae* significantly reduced N availability and nitrification and denitrification potentials compared to native communities. These differences were associated with lower microbial biomass and volumetric soil moisture in surface soil. In contrast, soil N cycling was similar between invaded and naturalized communities, suggesting that invasion of naturalized grasslands by *A. triuncialis* and *E. caput-medusae* may not induce changes in soil N cycling.

2.2 Introduction

Invasive species occupy many ecosystems worldwide and are considered an important component of global environmental change (Vitousek et al., 1997; Wilcove et al., 1998; Sala et al., 2000; Tylianakis et al., 2008). With over 2,100 established invasive vascular plants in the United States (Vitousek et al., 1997), an annual management cost of $27 billion (Pimentel et al., 2005), and the potential for significant increases in invasive species establishment (Sala et al., 2000), many studies have focused on understanding the mechanisms by which invasive species establish, persist, and spread (e.g., Mack et al., 2000; Davidson et al., 2011; Bradley et al., 2012; Drenovsky et al., 2012). Additionally, a growing number of studies have sought to understand the effects of invasive plants on...
key ecosystem processes, such as nutrient cycling and net primary production (Vitousek et al., 1987; Ehrenfeld, 2003; Liao et al., 2008; Vilà et al., 2011). These changes in ecosystem function have the potential to greatly alter ecosystem services (e.g., air and water purification) on which humans rely (Charles & Dukes, 2007; Eviner et al., 2012).

Understanding how invasive plants influence soil nitrogen (N) availability is of particular importance. Nitrogen is a limiting nutrient for plant growth in most terrestrial ecosystems (Vitousek & Howarth, 1991; LeBauer & Treseder, 2008) and modifications to N availability may facilitate persistent invasive populations (Kulmatiski, 2006) and potentially inhibit successful re-vegetation of desired species (D’Antonio & Meyerson, 2002; Suding et al., 2004b; Corbin & D'Antonio, 2012). Recent reviews by Ehrenfeld, (2003), Liao et al., (2008), and Vilà et al., (2011) suggest that invasive plants vary in their effects on N pools and processes, with some studies demonstrating increases and others showing decreases with invasion. For instance, Hawkes et al., (2005) found that two exotic species, *Avena barbata* and *Bromus hordeaceous*, increased gross nitrification rates compared native species (*Nassella pulchra* and *Lupinus bicolor*), and Holly et al., (2009) showed that litter of the exotic species, *Imperata cylindrica*, decomposed more rapidly than that of a native grass species (*Andropogon glomeratus*). In contrast, Evans et al., (2001) reported decreases in pools of inorganic soil N and potential rates of net N mineralization and denitrification associated with *Bromus tectorum* invasion, and Dassonville et al., (2011) demonstrated reduced nitrification and denitrification potentials associated with the invasive species, *Fallopia japonica* and *Fallopia xbohemica*. Moreover, there are examples where the same invasive species (e.g., *Bromus tectorum*) has been shown to differentially affect soil N dynamics across studies (Bolton et al., 1990; Evans et al., 2001; Svejcar & Sheley, 2001; Sperry et al., 2006; Schaeffer et al., 2012). Variability among results, which may be due to differences in pre-invaded site dynamics (Suding et al., 2004a; Kulmatiski et al., 2006), exotic and native plant traits (Drenovsky et al., 2012; Castro-Diez et al., 2014), and time since establishment (Strayer et al., 2006), highlight the complexity of ecosystem responses to invasion and demonstrate the need for continued research in this area.

Our study aimed to elucidate how two species invading California grasslands, *Aegilops triuncialis* (Barb goatgrass) and *Elymus caput-medusae* (Meduseahead), transform soil N cycling and availability. *A. triuncialis* is a winter annual native to Asia and Mediterranean Europe. It was introduced to the U.S. in the early 1900s (first sighting in 1914; Peters et al., 1996), and continues to spread throughout California, Oregon, and Nevada (D'Antonio & Malmstrom, 2007). *E. caput-medusae* is also a winter annual native to Mediterranean Europe (Bossard et al., 2000) that was introduced in the late 1800s to Oregon, and which quickly spread across the western U.S. (D'Antonio & Malmstrom, 2007). It continues to increase its range by 12% (in area) every year (Duncan et al., 2005). Because *A. triuncialis* and *E. caput-medusae* are proving to be aggressive invaders (rated as ‘high threats’ by the California Invasive Plant Council), it is critical to understand the mechanisms by which they invade and the resulting effects on ecosystem structure and function.
Previous work has shown *A. triuncialis* and *E. caput-medusae* have traits that could affect soil N availability and rates of cycling compared to other species. For instance, reduced rates of litter decomposition have been observed with *A. triuncialis* (Eviner, 2004; Drenovsky & Batten, 2007) and *E. caput-medusae* (Bovey et al., 1961) invasion, which may be due to high litter carbon to nitrogen (C:N; Eviner, 2004; Drenovsky & Batten, 2007) and lignin to N mass ratios (Drenovsky & Batten, 2007) in the case of *A. triuncialis*, and high silica content in the case of *E. caput-medusae* (Bovey et al., 1961; Swenson et al., 1964). Litter decomposition is an important mechanism by which available N (NH$_4^+$) is released into the soil (Chapman et al., 2006; Parton et al., 2007); consequently, species-specific alterations in litter quality and decomposition rates may translate into changes in N availability and rates of N cycling (Facelli & Pickett, 1991; Stump & Binkley, 1993).

In order to understand how *A. triuncialis* and *E. caput-medusae* affect soil N cycling, we examined N dynamics in three experimental grassland communities comprised of native perennials and annuals, naturalized exotic annuals, and *A. triuncialis* and *E. caput-medusae*. Naturalized exotics comprise a large portion of California grasslands, and native perennials are often the target community in restoration efforts; as such, these are two ecologically relevant plant communities by which to compare the effects of *A. triuncialis* and *E. caput-medusae* invasion. We hypothesized that *A. triuncialis* and *E. caput-medusae* would slow soil N cycling compared to native communities (H1) and naturalized communities (H2). To test this, we employed a combination of in-situ measurements and laboratory potential assays multiple times throughout the year. In-situ measurements (e.g., ion-exchange resin bags) provide insight into actual rates of N availability at a given time, while potential assays approximate the size of a specific microbial group (e.g., nitrifiers or denitrifiers). For nitrification (an obligate process), a potential assay approximates the likely rates experienced over longer periods of time (Hart et al., 1994). We also measured the soil microclimate, total microbial biomass, and abundances of protozoa, in order to provide a mechanistic insight into the effects of *A. triuncialis* and *E. caput-medusae* on soil N cycling.

2.3 Methods

2.3.1 Site Description & Experimental Design

To test our hypotheses, we collected soil samples from an experimental site located in Davis, California (38°32′45.52″N, 121°47′05.37″W). The site elevation is 18.6 m and the climate is Mediterranean, with hot dry summers and cool wet winters. Mean annual temperature (MAT) is 15.7 °C. In 2011, summer (June – September) and winter (December – February) mean daily maximum air temperatures were 32.2 °C and 13.8 °C, respectively. In 2012, mean daily maximum air temperatures in summer were 33.3 °C and winter temperatures averaged 15.3 °C. Mean annual precipitation is 485 mm, with 75-100% of that occurring between November and April. In 2011 and 2012, annual precipitation was 173 mm and 216 mm, respectively. The soils at this site are primarily
 (> 75% of the area) of the Reiff series (coarse-loamy, mixed, superactive, nonacid, thermic Mollic Xerofluvents); the other soil series present (< 25% of the area) is the Brentwood soil series (fine, smectitic, thermic Typic Haploxerepts) with a 0-2% slope (USDA Web Soil Survey, http://websoilsurvey.sc.egov.usda.gov).

In 2006, experimental grassland communities were established in an old agricultural field. Plots measuring 1.5 x 1.5 m each were arranged in a randomized complete block design and seeded as monocultures or mixtures of: (1) native species (Bromus carinatus, Elymus glaucus, Elymus triticoides, Ascispon americanus, Lupinus bicolor, Stipa pulchra, Poa secunda, and Festuca microstachys) (2) naturalized annual species (Avena fatua, Bromus hordeaceus, Festuca multiflorum, and Trifolium subterraneum) or (3) invasive winter annual species (Aegilops triuncialis and Elymus caput-medusae). Prior to seeding, the site was irrigated to stimulate germination of the existing seedbank within the soil; plants that germinated were sprayed with glyphosate. A total of 139 g of seed was added to each 1.5 x 1.5 m plot, with an equal proportion of each species in the mixture. Plant communities were allowed to diverge over time; however, non-planted species were removed periodically. For this study, we utilized three of the vegetation treatments: communities of native species, communities of naturalized species, and communities of invasive species. At the time of collection, N fixing species (Acmispon americanus, Lupinus bicolor, Trifolium subterraneum) were absent from all treatments and the native communities were dominated by perennial plants Elymus glaucus and Stipa pulchra.

2.3.2 Sample Collection

Mediterranean-type ecosystems experience strong seasonality in soil temperature, soil water content, and plant phenology, and thus the relative effects of plant community composition may vary considerably throughout the year. In order to account for this, we sampled in April 2011 and four times throughout 2011-12 during periods representative of the major seasons (October – warm and wet; January – cool and wet; April – warm and wet; July – hot and dry). Within each date, we sampled five soil cores (2.5 cm diameter x 15 cm deep) per plot and combined them into a single sample in order to account for within-plot spatial variability. For all soil analyses, there were 8 replicates per treatment (n = 8), except for April 2011 in which case there were 10 replicates (n = 10). Field moist soils were sieved (2 mm) and stored at 4 °C until analysis (the average time to analyze all variables was 1 week).

2.3.3 Soil Properties

Soil moisture was determined gravimetrically by drying the samples at 105°C until constant mass. For total carbon (C) and N analysis, we ground the soils to a fine powder and composited samples over the four sampling dates (October 2011 – July 2012) prior to analysis on an Elemental Combustion System (Costech Analytical Technologies Inc., Valencia, CA, USA). Samples were composited within each treatment because total C
Volumetric water content (VWC) was measured at each sampling date using a Time-Domain Reflectometer (TDR; MiniTrase, Soil Moisture Equipment Corp., Santa Barbara, CA, USA). Fifteen-cm long TDR probes were inserted vertically into the soil in October 2011 and 30-cm long probes were installed adjacent to the 15-cm probes in January 2012 (n = 6/depth). This design allowed measurement of VWC within 0-15 cm and 15-30 cm soil layers. Soil pH was measured using a pH electrode (Orion DUAL STAR meter, Thermo Scientific, Waltham, MA, USA) after allowing 15 g fresh weight soil to equilibrate with 30 mL 0.01 CaCl$_2$ for 30 minutes (the soil slurry was mixed continuously during measurement). Soil temperature was measured at every sampling date using a traceable metal thermometer (Control Company, Friendswood, TX, USA) at 7.5 cm depth.

### 2.3.4 Nitrogen Availability

Soil available N was measured using two indices. First, static (instantaneous) inorganic (NH$_4^+$ and NO$_3^-$) pool sizes were measured four times (October, January, April, July) by extracting 15 g field-moist soil with 100 mL of 2 M KCl. Samples were shaken on a mechanical shaker for 1 hour, filtered using pre-leached Whatman No. 1 filter paper, and stored at -20 °C until analysis on a Lachat AE Flow Injection Autoanalyzer (Lachat Instruments, Inc., Loveland, CO, USA). In addition, ion-exchange (IER) resin bags were used to measure the accumulation of in-situ NH$_4^+$ and NO$_3^-$ over time, expressed on a per g dry resin per day basis (Binkley & Matson, 1983; Binkley, 1984). Bags were prepared by measuring 15 mL (approximately 8 g wet weight and 4 g oven-dry equivalent) of cation and anion resin beads (J.T. Baker Mixed Bed Exchange Resin, IONAC NM-60 H$^+$/OH$^-$/Form, Type 1, 16-50 Mesh) into undyed nylon stockings that were subsequently tied shut. One bag per plot was buried at a depth of 7.5 cm and incubated between 5 and 7 weeks. After the end of the allotted incubation time, each resin bag was removed and exchanged with a new one (using the same hole at 7.5 cm depth). The first bags were buried on October 18, 2011 and the last bags were removed on August 31, 2012, totaling eight incubations. Some of the removal and insertion dates corresponded with soil sampling dates. Once removed from the field, resin bags were immediately air-dried, weighed, and placed into 125 mL flasks. To each flask, 50 mL of 2M KCl was added. Samples were then shaken uncovered for 1 hour on an orbital shaker, filtered, and stored at -20°C until analysis of filtrate on a Lachat AE Flow Injection Autoanalyzer.

### 2.3.5 Potential Assays and Microbial Biomass

Potential rates of nitrification were measured using a shaken soil-slurry method as described by (Hart et al., 1994). By creating optimal conditions for nitrification, this 24-h incubation provides an approximation of the population size of nitrifying microorganisms in the soil. Briefly, 100 mL of a solution containing 1.5 mM of NH$_4^+$ and 1 mM of PO$_4^{3-}$ (pH = 7.2) was added to 15 g field-moist soil in a 250 mL flask. Flasks were capped with a rubber stopper containing a hole and placed on an orbital shaker at 180 rpm for 24
hours. Sampling occurred at 2, 4, 22, and 24 hours by removing a 10 mL aliquot of the suspension from each flask and centrifuging at 8,000 x g for 8 minutes. Five mL of supernatant was then placed into a disposable polypropylene tube, capped, and stored at -20°C until analysis for NO$_3^-$ on a Lachat AE Flow Injection Autoanalyzer. Concentrations of NH$_4^+$ were also analyzed from these samples in order to check that nitrification never became limited by NH$_4^+$.

Similar to nitrification potential, denitrification potential is an index of the size of the denitrifying microbial community and the pool of denitrifying enzymes. We measured denitrification potential by modifying a protocol developed by (Smith & Tiedje, 1979). Briefly, non-limiting conditions were created by amending 50 g field-moist soil with NO$_3^-$ and labile C (0.1 mg N-NO$_3^-$ g$^{-1}$ soil, 1 mg C-glucose g$^{-1}$ soil, and 1 mg C-glutamic acid g soil$^{-1}$) in a 250 mL flask. The flasks were sealed tight with a rubber stopper and septum and 20 mL of acetylene was added in order to inhibit the reduction of N$_2$O to N$_2$. The soils were incubated anaerobically on an orbital shaker (180 rpm) for 90 minutes; 15 mL of the headspace was sampled at 30 and 90 minutes. Gas samples were stored in evacuated Exetainer® until analysis for N$_2$O production on a Shimadzu GC-2014 electron capture detector (Shimadzu Corporation, Columbia, MD, USA). Values of N$_2$O produced were used to estimate the overall size of the denitrifying microbial community.

Microbial biomass was estimated using three methods that provide complementary information: substrate induced respiration (SIR; measured at all 4 time points from October 2011 – July 2012), chloroform fumigation-extraction (CFE; measured in January 2012), and microscopic enumeration (measured in April 2011; n = 10). By measuring CO$_2$ production, substrate induced respiration estimates those microorganisms that are active and glucose-responsive (Wardle & Ghani, 1995). The chloroform fumigation extraction method more closely captures total microbial biomass and provides information on biomass N (Martikainen & Palojärvi, 1990; Bailey et al., 2002), while direct enumeration allows for the separate quantification of bacteria and fungi (Paul et al., 1999).

Substrate induced respiration was modified from (West & Sparling, 1986). Fifteen g of field-moist soil was weighed out into 250 mL flasks and 30 mL of a glucose solution (30 mg glucose ml$^{-1}$ H$_2$O) was added to each. The flasks were sealed tight with a rubber stopper and septum and placed on a shaker (180 rpm) for 2.5 hours. At 0.5, 1.5, and 2.5 hours, 15 mL of the headspace was sampled for CO$_2$ and stored in evacuated Exetainer® until analysis on a Shimadzu GC-2014 thermal conductivity detector. Changes in [CO$_2$] over time were used to calculate microbial biomass (West & Sparling, 1986). The chloroform fumigation-extraction method was used to estimate microbial biomass carbon (C) and N (Haubensak et al., 2002). Microbial C and N were calculated by dividing the fumigation C-flush and N-flush by a k$_{EC}$ factor of 0.49 (Joergensen, 1996) and 0.68 (Brookes et al., 1985), respectively. Direct enumeration of total bacteria and fungi was conducted using microscopy. Samples (10 g) were shipped overnight on dry ice to Soil Food Web, Inc. (Corvallis OR, USA) where total bacteria was estimated by direct
counting using a fluorescein isothiocyanate (FITC) method (Babiuk & Paul, 1970) and fungal biomass was calculated by measuring the diameter and length of hyphae (Lodge & Ingham, 1991). The Soil Food Web Inc. also estimated abundance of protozoa in each sample. Protozoa can be categorized into three subgroups depending on size: flagellates, and amoeba. Subgroups were differentiated and enumerated by direct counting of serial dilutions using microscopy. The direct counts were then used to estimate total protozoa population sizes using the most probable number approach (Darbyshire et al., 1974).

2.3.6 Statistical Analysis

Repeated measures analysis of variance (RM ANOVA) was used to assess significant effects of treatment (“plant community type”) and date on instantaneous and accumulated (IER bag) inorganic N pools, nitrification and denitrification potential, microbial biomass as measured by SIR, soil water content, and temperature. A one-way ANOVA was used to assess significant differences in protozoan biomass and microbial biomass as determined by CFE and direct enumeration because they were sampled only once. Soil NO$_3^-$ and NH$_4^+$ pools, IER-NO$_3^-$ and NH$_4^+$, and volumetric water content (0-15 cm) were log transformed in order to fulfill the assumptions of an ANOVA. In addition, two IER-NO$_3^-$ outliers (values > 3 SD of mean) were removed. A post-hoc Tukey’s test ($\alpha = 0.05$) was used to make comparisons between treatment means when the ANOVA model was significant. Potential nitrification and denitrification were correlated using Pearson’s product moment correlation coefficient, as were volumetric and gravimetric water content.

We used bidirectional stepwise multiple regression in order to explore potential relationships among potential rates and biotic (e.g., microbial biomass) and abiotic (e.g., inorganic N, soil moisture) variables (Table 3). When necessary, data was transformed to meet the assumption of normality. All statistical analyses were performed in ‘R’ statistical software (www.r-project.org).

2.4 Results

2.4.1 Nitrogen Availability

Pools of soil NH$_4^+$ varied by date but not plant community type (RM ANOVA; plant community type, $P = 0.22$; date, $P < 0.001$; community x date, $P = 0.54$). Soil NO$_3^-$ pools were also affected by date and were smaller in naturalized treatments compared to native treatments, although this effect was only marginally significant (RM ANOVA; plant community type, $P = 0.06$; date, $P < 0.001$; community x date, $P = 0.5$). Total inorganic N (TIN; NH$_4^+$ and NO$_3^-$) was affected by plant community type and date (RM ANOVA; plant community type, $P = 0.04$; date, $P < 0.001$; community x date, $P = 0.82$); however, when analyzed using a post hoc analysis, naturalized ($P = 0.08$) and invaded ($0.06$) treatments were only marginally reduced compared to native treatments.
Ion-exchange resin bags are likely a more sensitive measure of N availability than pools of soil N, given that the buried bags capture N accumulated in soil over time and pools of N are instantaneous measurements. Indeed, soil IER-$\text{NH}_4^+$ was significantly affected by plant community type and date (Figure 2-2, RM ANOVA; plant community type, $P = 0.03$; date, $P < 0.001$; community x date, $P = 0.61$). Specifically, invaded treatments had lower IER-$\text{NH}_4^+$ values than native treatments ($P = 0.02$). Plant community type and date had significant interactive effects on IER-$\text{NO}_3^-$ (Figure 2-2, RM ANOVA; plant community type, $P < 0.001$; date, $P < 0.001$; community x date, $P = 0.02$), with invaded treatments exhibiting lower IER-$\text{NO}_3^-$ than native treatments in April ($P = 0.04$). Total available IER-N ($\text{NH}_4^+$ and $\text{NO}_3^-$) was affected by plant community type, independent of date (RM ANOVA; plant community type, $P < 0.001$; date, $P < 0.001$; community x date, $P = 0.11$). Here, native treatments had higher values of IER-N than invaded treatments ($P < 0.001$) and naturalized communities had intermediate values (Naturalized – Invasive, $P = 0.04$; Naturalized – Native, $P = 0.04$).

### 2.4.2 Potential Rates of Nitrification, Denitrification, and Microbial Biomass

Plant community type and date had significant independent effects on potential nitrification rates (Figure 2-3, RM ANOVA; plant community type, $P < 0.001$; date, $P < 0.001$; community x date, $P = 0.31$). Potential nitrification was significantly lower in naturalized ($P < 0.001$) and invaded ($P < 0.001$) treatments than native treatments. Community effects on potential nitrification were correlated with soil moisture and microbial biomass for all dates except April 2011 and 2012 (Table 2-4), in which case potential nitrification was not explained by any of the variables included in the model (Table 2-3).

Potential denitrification was affected independently by plant community type and date (Figure 2-3, RM ANOVA; plant community type, $P < 0.001$; date, $P < 0.001$; community x date, $P = 0.41$), following a similar pattern to potential nitrification over time (lowest values in October and April, highest values in January and July; $R = 0.68$, $P = 0.03$). Rates of potential denitrification were significantly reduced in invaded ($P < 0.001$) and naturalized plots ($P = 0.004$) compared to native plots. Changes in potential denitrification were closely associated with microbial biomass during the growing season (October – April) and were correlated with both microbial biomass and soil moisture during the summer (Table 2-4).

Microbial biomass as measured by SIR was significantly affected by plant community composition (Figure 2-3, RM ANOVA; plant community type, $P < 0.001$; date, $P < 0.001$; community x date, $P = 0.46$). Values were significantly reduced in invaded plots compared to native plots and naturalized communities had intermediate values. SIR varied significantly over time, with the highest values occurring during July.
Total fungi and bacteria, measured using direct enumeration in April 2011, did not show significant differences among plant community type (Table 2-2, ANOVA; total bacteria, P = 0.84; total fungi, P = 0.50). However, microbial C, which was measured by CFE, was significantly reduced in naturalized (Table 2-2, ANOVA; P = 0.002) and invaded communities (P = 0.04) compared to native. Microbial N was also affected by plant community type (ANOVA; P = 0.05). Naturalized communities had significantly lower microbial N than native communities (P = 0.04); invaded plots did not differ from either native (P = 0.22) or naturalized (P = 0.60) plots.

Soils from April 2011 were analyzed for biomass within each subgroup of protozoa (Figure 2-4). Across all treatments, amoeba dominated the soils (mean = 17.5 organisms kg⁻¹ soil), followed by flagellates (mean = 7.8 organisms kg⁻¹ soil), and finally ciliates (mean = 0.4 organisms kg⁻¹ soil). Plant community type had no significant effect on the abundance of flagellates (ANOVA; P = 0.25) and ciliates (P = 0.61); naturalized plots had higher amounts of amoeba per kg of soil than invaded treatments, although this difference was only marginally significant (P = 0.06)

2.4.3 Soil Properties

Plant community type and date had significant, independent effects on volumetric soil water content at 0-15 cm depth (Figure 2-1, RM ANOVA model; plant community type, P < 0.001; date, P < 0.001; community x date, P = 0.16) and 15-30 cm depth (RM ANOVA model; plant community type, P <0.001; date, P< 0.001; community x date = 0.81). Volumetric water content was also affected by soil depth (P < 0.001); on average, shallower soils (0-15 cm) had less soil moisture than deeper soils (15-30 cm), although differences by depth were dependent on time (P = 0.006). Gravimetric and volumetric water content within the top 15 cm of the soil profile were positively correlated (r = 0.76, P < 0.001). Despite this, gravimetric soil water content (0-15 cm depth) was statistically similar among plant community type, indicating that it was a less sensitive measure of community effects. Gravimetric water content did, however, differ by date (RM ANOVA model; plant community type, P = 0.14; date, P < 0.001; community x date, P = 0.40). Soil pH and temperature were significantly affected by date but not by treatment (Table 2-1, RM ANOVA; pH: plant community type, P = 0.95; date, P < 0.001; community x date, P = 0.80; temperature: plant community type, P = 0.91; date, P < 0.001, community x date, P = 0.32). There were no significant effects of plant community type on soil total C, total N, or C/N ratio (Table 2-1, ANOVA; P = 0.251, P = 0.184, P = 0.843, respectively).

2.5 Discussion

Our study demonstrated that *A. triuncialis* and *E. caput-medusae* communities had significantly lower rates of N cycling than native communities (H1). These findings are similar to some studies comparing native perennials and exotic annuals (Evans *et al.*, 2001; Corbin & D'Antonio, 2011), but contrast with others that demonstrated an increase
in N cycling associated with exotic annual invasion (Hawkes et al., 2005; Parker & Schimel, 2010) or no differences between the two community types (Bolton et al., 1990; Svejcar & Sheley, 2001). In our study, reduced inorganic N availability and potential rates of nitrification and denitrification associated with invasion may be due, in part, to lower decomposition rates. Prior work at our site showed that native plants had significantly higher litter N concentrations than A. triuncialis and E. caput-medusae (Baty, 2012), which could result in higher rates of decomposition and N mineralization in native communities. Lower litter quality and reduced decomposition rates of A. triuncialis and E. caput-medusae have been demonstrated elsewhere (Bovey et al., 1961; Swenson et al., 1964; Eviner, 2004; Drenovsky & Batten, 2007) supporting this as a possible mechanism by which N cycling is altered in this system.

While lower N availability was observed in invaded compared to native communities (as measured by IER bags), neither changes in NH$_4^+$ nor NO$_3^-$ availability could account for reductions in nitrification and denitrification potentials (based on a multiple stepwise regression; Table 2-4). This finding is similar to Hawkes et al., (2005) where NH$_4^+$ concentrations failed to predict increases in gross nitrification with invasion. The lack of relationship between substrate availability and potential rates is surprising, given that actual rates of denitrification and nitrification should be strongly regulated by NO$_3^-$ and NH$_4^+$ supply (Booth et al., 2005). However, Wallenstein et al., (2006) argue that NO$_3^-$ availability has little effect on denitrifying microbial community structure and abundance, and thus potential rates, even though availability can affect actual in-situ rates of denitrification. Moreover, Horz et al., (2004) note that significant changes in NH$_4^+$ may be required to stimulate growth of ammonia oxidizing microorganisms; thus, it is possible that nitrifying microbial abundance and potential rates are insensitive to changes in NH$_4^+$ produced by alterations in plant community composition, and are instead regulated by other factors such as soil moisture availability.

Indeed, changes in potential nitrification and denitrification with invasion were best explained by reductions in soil moisture and total microbial biomass (SIR) at 0-15 cm depth (based on a multiple stepwise regression; Table 2-4). Specifically, nitrification potential was explained by soil moisture and total microbial biomass on all dates except April, in which case soil moisture and microbial biomass ceased to explain any variation. In addition, changes in denitrification potential with exotic plant invasion were influenced by shifts in total microbial biomass. Soil moisture was also a significant explanatory variable in July, with potential denitrification showing a positive response to increases in soil moisture. These results demonstrate that A. triuncialis and E. caput-medusae altered soil moisture availability and total microbial biomass compared to native communities, likely because annuals have shallower roots than perennials (Holmes & Rice, 1996). As a result, invaded communities had lower nitrification and denitrification potentials than native communities.

The disparity between N cycling in native and invaded soils may have implications for restoration. Native perennials are often used to re-vegetate sites after the removal of
invasive species (Stromberg et al., 2007); if legacies of altered N cycling persist after the removal of *A. triuncialis* or *E. caput-medusae*, as has been shown with other invasive plants (Corbin & D'Antonio, 2004; 2012), the competitive edge may be shifted away from natives and successful reestablishment may be hindered (Gorman & Suding, 2010). While *A. triuncialis* can reduce the success of native species by altering the soil microbial community (Batten et al., 2008), additional studies are needed in order to determine whether the same is true for alterations in soil N cycling by both *A. triuncialis* and *E. caput-medusae*.

In contrast to our second hypothesis, N cycling was not lower in invaded relative to naturalized plant communities (H2). This may be because the effect of an invasive plant is likely linked to how functionally distinct it is from the resident species (Corbin & D'Antonio, 2011). Thus, differences in important morphological or physiological characteristics between these two groups might not be large enough to induce changes in N cycling. For example, *A. triuncialis* and *E. caput-medusae* have similar litter C:N ratios as *Bromus hordeaceus* (Eviner, 2004) which dominated naturalized plots at the time of sampling. Additionally, invasive and naturalized exotics utilized in this study are all annuals and may consequently share similar rooting depths (as suggested by similarities in volumetric water content at 0-15 and 15-30 cm depths; Figure 2-1), and exploit N in similar ways. Additional studies explicitly looking at rooting depths and N use between these two communities are needed to confirm this hypothesis.

The similarity in N cycling between invaded and naturalized soils has at least two implications. First, alterations to N availability may not be a mechanism by which *A. triuncialis* and *E. caput-medusae* establish persistent populations in naturalized grasslands of California. Instead, changes to N cycling may be a “byproduct” of other mechanisms responsible for the success of these species. For instance, dense layers of *E. caput-medusae* litter have been shown to favor the emergence of *E. caput-medusae* seedlings while inhibiting the germination of other species, such as *Convolvulus arvensis* (Evans & Young, 1970). Second, the invasion of naturalized grasslands by *A. triuncialis* and *E. caput-medusae* may not result in altered soil N dynamics.

### 2.6 References


### 2.7 Tables

Table 2-1 Soil characteristics from 0-15 cm depth associated with each plant community type

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GWC (%)(^a)</th>
<th>pH(^b)</th>
<th>Temperature(*)</th>
<th>Total C (g kg(^{-1}) soil)</th>
<th>Total N (g kg(^{-1}) soil)</th>
<th>C:N(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>14.3 (1.1)</td>
<td>6.6 (0.03)</td>
<td>18.5 (1.3)</td>
<td>12.9 (0.1)</td>
<td>1.34 (0.01)</td>
<td>9.63 (0.02)</td>
</tr>
<tr>
<td>Naturalized</td>
<td>13.9 (1.1)</td>
<td>6.6 (0.02)</td>
<td>18.4 (1.3)</td>
<td>12.3 (0.1)</td>
<td>1.28 (0.01)</td>
<td>9.59 (0.03)</td>
</tr>
<tr>
<td>Invaded</td>
<td>14.4 (1.0)</td>
<td>6.6 (0.02)</td>
<td>18.1 (1.3)</td>
<td>12.1 (0.1)</td>
<td>1.27 (0.01)</td>
<td>9.55 (0.05)</td>
</tr>
</tbody>
</table>

\(^a\)Gravimetric water content, \(^b\)pH measured in 0.01 M CaCl\(2\) solution, \(*\)Temperature measured at 7.5 cm depth, \(^b\)Carbon to nitrogen mass ratio. Note: Values are means ± SE averaged over all four seasons (October 2011 – July 2012; \(n = 8\)). The absence of letters signifies that no significant differences occurred among treatments (\(P < 0.05\)).

Table 2-2 Comparison of direct count measurements and chloroform fumigation extraction as affected by plant community type

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Direct Counts: April 2011</th>
<th>CFE: January 2012</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Fungi</td>
<td>Total Bacteria</td>
</tr>
<tr>
<td></td>
<td>mg C kg(^{-1}) soil</td>
<td>mg C kg(^{-1}) soil</td>
</tr>
<tr>
<td>Native</td>
<td>181.85 (10.76)</td>
<td>344.40 (41.45)</td>
</tr>
<tr>
<td>Naturalized</td>
<td>163.25 (14.24)</td>
<td>350.35 (69.28)</td>
</tr>
<tr>
<td>Invaded</td>
<td>187.30 (20.15)</td>
<td>378.60 (34.52)</td>
</tr>
</tbody>
</table>

Note: Values are means ± SE (\(n = 8\)). Different letters denote significant differences among plant community type within a given microbial characteristic (determined by a Tukey post-hoc test [\(P < 0.05\)]). If letters are absent, no significant differences were present.
Table 2-3 Soil and microbial characteristics used in stepwise regression to account for plant community effects on nitrification and denitrification potentials

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Independent variable</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>April 2011</strong></td>
<td></td>
</tr>
<tr>
<td>Nitrification potential</td>
<td>Soil moisture</td>
</tr>
<tr>
<td></td>
<td>Fungi:bacteria ratio</td>
</tr>
<tr>
<td></td>
<td>Flagellates</td>
</tr>
<tr>
<td></td>
<td>Amoeba</td>
</tr>
<tr>
<td></td>
<td>Ciliates</td>
</tr>
<tr>
<td></td>
<td>C:N ratio</td>
</tr>
<tr>
<td><strong>October 2011 - July 2012</strong></td>
<td></td>
</tr>
<tr>
<td>Nitrification potential</td>
<td>Soil temperature</td>
</tr>
<tr>
<td></td>
<td>Soil moisture</td>
</tr>
<tr>
<td></td>
<td>Soil pH</td>
</tr>
<tr>
<td></td>
<td>Microbial biomass</td>
</tr>
<tr>
<td></td>
<td>NH$_4^+$ pool size</td>
</tr>
<tr>
<td></td>
<td>IER - NH$_4^+$</td>
</tr>
<tr>
<td>Denitrification potential</td>
<td>Soil temperature</td>
</tr>
<tr>
<td></td>
<td>Soil moisture</td>
</tr>
<tr>
<td></td>
<td>Soil pH</td>
</tr>
<tr>
<td></td>
<td>Microbial biomass</td>
</tr>
<tr>
<td></td>
<td>NO$_3^-$ pool size</td>
</tr>
<tr>
<td></td>
<td>IER - NO$_3^-$</td>
</tr>
</tbody>
</table>

Note: IER-NH$_4^+$/NO$_3^-$ are changes in NH$_4^+$ and NO$_3^-$ over time as captured by ion-exchange resin bags (buried at 7.5 cm depth).
Table 2-4 Stepwise multiple regression relating potential nitrification and denitrification to selected soil and microbial characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Regression coefficient</th>
<th>P</th>
</tr>
</thead>
</table>

**October 2011**  
*Nitrification potential: \( R^2 \) for full model = 0.33, \( P \) value = 0.02*  
- Soil moisture: 0.35, \( P \) value = 0.08  
- Microbial biomass: 0.01, \( P \) value = 0.02  
*Denitrification potential: \( R^2 \) for full model = 0.37, \( P \) value = 1.6\( \times \)10\(^{-3} \)*  
- Microbial biomass: 0.13, \( P \) value = 1.6\( \times \)10\(^{-3} \)

**January 2012**  
*Nitrification potential: \( R^2 \) for full model = 0.60, \( P \) value = 7.08\( \times \)10\(^{-5} \)*  
- Soil moisture: -0.06, \( P \) value = 7.7\( \times \)10\(^{-3} \)  
- Microbial biomass: 0.01, \( P \) value = 1.1\( \times \)10\(^{-4} \)  
*Denitrification potential: \( R^2 \) for full model = 0.27, \( P \) value = 0.01*  
- Microbial biomass: 0.01, \( P \) value = 0.01

**April 2012**  
*Denitrification potential: \( R^2 \) for full model = 0.27, \( P \) value = 9.4\( \times \)10\(^{-3} \)*  
- Microbial biomass: 0.10, \( P \) value = 9.4\( \times \)10\(^{-3} \)

**July 2012**  
*Nitrification potential: \( R^2 \) for full model = 0.47, \( P \) value = 1.3\( \times \)10\(^{-3} \)*  
- Soil moisture: 0.4, \( P \) value = 0.15  
- Microbial biomass: 0.03, \( P \) value = 5.9\( \times \)10\(^{-4} \)  
*Denitrification potential: \( R^2 \) for full model = 0.31, \( P \) value = 0.02*  
- Microbial biomass: 0.12, \( P \) value = 0.02  
- Soil moisture: 3.6, \( P \) value = 0.08

Note: Results are presented only for overall models found to be significant (\( P < 0.05 \)). Nitrification for October was power transformed. Potential denitrification and nitrification were log transformed for January.
2.8 Figures

Figure 2-1 Changes in mean soil volumetric water content over time as influenced by plant community type (native, naturalized, and invasive) and soil depth (upper panel 0-15 cm, lower panel 15-30 cm). Values are means ± 1 SE (untransformed; n = 6). Different letters denote significant differences by plant community type at each depth (determined by a Tukey post-hoc test [p < 0.05]). Interactions between plant community type and date were nonsignificant. VWC 15-30 cm values are not presented for October and November 2011 because 0-30 cm TDR probes were not installed at that time.
Figure 2-2 The response of ion-exchange resin (a) NH$_4^+$, (b) NO$_3^-$, and (c) total inorganic N to different plant communities. Bars ± 1 standard error of the mean (untransformed; n = 12). Different letters denote significant differences by plant community type (determined by a Tukey post-hoc test [p < 0.05]). IER-NO$_3^-$ showed a significant treatment by date interaction, with values affected by community type in April.
Figure 2-3 Changes in (a) microbial biomass, (b) nitrification potential and (c) denitrification potential as influenced by plant community type over time. Bars ± 1 standard error of the mean (n = 8). Different letters denote significant differences by plant community type (determined by a Tukey post-hoc test [p < 0.05]). Interactions between plant community type and date were nonsignificant.
Figure 2-4 Plant community effects on the abundance of ciliates, flagellates, amoeba, and total protozoa in April 2011. Values are means ± 1 SE (n = 10). Different letters denote significant differences by plant community type (determined by a Tukey post-hoc test [p < 0.05]).
3 Nitrogen fertilization and defoliation influence soil nitrogen cycling but not the abundance of ammonia oxidizing microorganisms in an invaded grassland community

3.1 Abstract

The ecology of California has seen marked transformations since western colonization. In particular, invasive annual grasses from Mediterranean Europe have replaced many of the native grasslands once dominated by perennial bunchgrasses and annual forbs. Most of these grasslands are grazed extensively by livestock and receive elevated rates of nutrient input through atmospheric deposition. These environmental changes simultaneously affect the landscape; however, little is known about their potential interactive effects on ecosystem structure (e.g., species composition) and function (e.g., nutrient cycling). Our study used experimental grassland plots to measure (1) how soil N cycling responds to changes in plant community composition with invasion by exotic annual grasses and (2) how N additions and defoliation (to simulate one component of cattle grazing: aboveground vegetation removal) affect N cycling individually and in combination. We collected samples seasonally across 2 years and measured instantaneous and accumulated NH$_4^+$ and NO$_3^-$ concentrations (using KCl soil extractions and ion-exchange resin bags, respectively), potential rates of nitrification and denitrification, and total microbial biomass. In order to gain a mechanistic understanding of changes in nitrification—a key process in the N cycle—we also quantified bacterial and archaeal amoA gene abundance, which regulates the first and rate-limiting step of nitrification. We found no evidence that suggests N cycling responds in a non-additive way to the interaction of N fertilization and defoliation. Instead, single factors dominated the response of the system, although some
effects did not persist across years. Invasion, for instance, decreased denitrification potential in the first year and nitrification potential in the second year. Defoliation exacerbated these effects by further reducing nitrification potential and soil NO$_3^-$ availability of invaded grasslands. Fertilization consistently increased nitrification potential and soil NO$_3^-$ concentrations. However, soil NH$_4^+$ concentrations were rarely affected by treatment, including N fertilization, suggesting that NH$_4^+$ is either quickly taken up by plants, immobilized by microorganisms, or nitrified to NO$_3^-$. Surprisingly, changes in nitrification potential were not associated with concurrent shifts in bacterial or archaeal amoA gene abundance.

### 3.2 Introduction

Human-induced global changes, such as climate change (Jones et al., 2009; Bellard et al., 2012), invasion by exotic species (Vitousek et al., 1997; Simberloff, 2011; Stohlgren et al., 2011), increased rates of atmospheric nitrogen (N) deposition (Fenn et al., 2003b; 2010), and land-use change (Asner et al., 2004), are increasingly modifying terrestrial ecosystems worldwide. It is well established that individual global change factors can alter the above- and below-ground structure and functioning of terrestrial ecosystems, such as the composition of plant (Clark & Tilman, 2008; Bobbink et al., 2010), and soil microbial communities (Treseder, 2008; He et al., 2010; Blankinship et al., 2011), and net primary production (NPP; Nemani et al., 2003; Heisler-White et al., 2008). However, much less is known about how interactions among different forms of global change will affect terrestrial ecosystems, despite the fact that individual changes rarely occur in isolation.

In an effort to understand interactive effects, a growing number of studies have manipulated two or more global change factors simultaneously (e.g., Majdi & Öhrvik, 2004; Henry et al., 2005; Niboyet et al., 2011b; Dieleman et al., 2012). Of these, many have focused on the response of soil N cycling (Horz et al., 2004; Barnard et al., 2006; Niboyet et al., 2010; Dijkstra et al., 2010; Niboyet et al., 2011a; Brown et al., 2012; Mueller et al., 2013), given that N limits plant growth in most terrestrial systems (Vitousek & Howarth, 1991; LeBauer & Treseder, 2008). Results of these multifactor global change experiments show wide variation in responses, from non-interactive, to synergistic, to antagonistic. For example, Niboyet et al., (2011c) concluded that global change effects on soil N cycling are primarily additive and as such can be predicted by combining single factor studies. In contrast, Brown et al., (2012) found the presence of a dampening effect with increased multiple global change factors on soil N$_2$O fluxes, suggesting that combining the effects of single factor studies may overestimate the change in N$_2$O production. The variation in observed results displays the complexity of interactive effects, and highlights the need for continued research.

In order to obtain a holistic understanding of ecosystem response to global change, it is critical to discern the underlying mechanisms controlling said responses. For terrestrial N cycling, that requires measuring the abundance, composition, and activity of the soil
microbial community. Microorganisms drive terrestrial nutrient cycling (Conrad, 1996; Hayatsu et al., 2008; Schimel & Scheffler, 2012; Offre et al., 2012) and alterations in ecosystem-level processes are likely linked to microbial abundance and community composition, especially for specialized processes such as nitrification (Schimel, 1995). The accumulating evidence for shifts in microbial community structure with single (Kourtev et al., 2002; Batten et al., 2006; He et al., 2010; Ramirez et al., 2012) and multiple global change factors (Olsrud et al., 2004; Horz et al., 2004; Castro et al., 2010; Gray et al., 2011; Gutknecht et al., 2012; Steinweg et al., 2013) is not surprising, given that microbial communities are sensitive to abiotic (e.g., soil moisture and temperature) and biotic (e.g., plant community composition) variables that are affected by global change. However, there is a need for additional studies that simultaneously measure N cycling processes and microbial community composition within the context of multiple global changes.

Our study addressed two overarching questions: how do invasive plants alter N cycling processes in a California grassland and how do defoliation (‘clipping’, to simulate one component of livestock grazing) and N fertilization (to simulate elevated N deposition) interact to affect N cycling of invaded grasslands? Barb goatgrass (Aegilops triuncialis) and Medusahead (Elymus caput-medusae), the two invasive plants utilized in this study, both have relatively low litter quality (Bovey et al., 1961; Swenson et al., 1964; Eviner, 2004; Drenovsky & Batten, 2007), which may reduce decomposition rates (Bovey et al., 1961; Drenovsky & Batten, 2007) and subsequent N cycling compared to native plant communities (as was demonstrated previously between monocultures at this site). As such, we hypothesized that invaded plant communities (mixtures of natives, naturalized, and invasive species) would exhibit decelerated rates of N cycling compared to native-only communities (hypothesis 1). Defoliation can affect N cycling through a number of mechanisms, including alterations to aboveground litter inputs, root growth, C exudation, and soil microclimate (Bardgett et al., 1998); we hypothesized that clipping would slow N cycling, potentially as a result of reducing aboveground litter inputs into the soil system, or increasing plant uptake and microbial immobilization of N (hypothesis 2). The addition of N fertilizer (as NH₄NO₃) can affect soil N cycling through multiple direct and indirect effects; enhanced N supply can directly affect NH₄⁺ and NO₃⁻ pool sizes, or indirectly affect N availability through changes in plant community composition, plant growth, and decomposition rates (Carreiro et al., 2000; Fenn et al., 2003a; Knorr et al., 2005; Manning et al., 2006). We hypothesized that the direct effects of increasing N availability would predominate at our site, resulting in increased rates of N cycling (hypothesis 3). Finally, we hypothesized that N fertilization and clipping would interact in non-additive antagonistic ways (hypothesis 4).

In conjunction with N cycling measurements, we quantified the abundance of ammonia oxidizing bacteria (AOB) and archaea (AOA) based on ammonia monooxygenase (amoA) genes. Ammonia oxidizing bacteria and AOA perform the first and rate-limiting step of nitrification (i.e., the conversion of ammonium to nitrite), and have shown sensitivity to changes in plant composition (Hawkes et al., 2005; Boyle-Yarwood et al.,
and substrate (NH$_4^+$) availability (Verhamme et al., 2011; Shen et al., 2011; Wessén & Hallin, 2011). In particular, AOB tend to prefer high NH$_4^+$ conditions while AOA tend to do better when NH$_4^+$ availability is low (Verhamme et al., 2011); consequently we expected that soils with elevated N availability would be associated with higher AOB/AOA ratios.

To address our hypotheses, we utilized a well-replicated field experiment comprised of five treatments that had been established three (N fertilization and clipping treatments) to five years (plant communities) in an abandoned agricultural field of California. Samples were collected seasonally over two years, allowing us to determine whether the effects of global change factors are dependent upon time in a Mediterranean climate characterized by large seasonal variations in temperature and precipitation.

3.3 Methods

3.3.1 Site Description & Experimental Design

This study was conducted at an experimental site located in Davis, California (38°32′45.52″N, 121°47′05.37″W). Soils are dominated by the Reiff series (75%, coarse-loamy, mixed, superactive, nonacid, thermic Mollic Xerofluvents) and to a lesser extent the Brentwood soil series (25%, fine, smectitic, thermic Typic Haploxerepts) with a 0–2% slope (USDA Web Soil Survey). The site experiences a Mediterranean climate with a mean annual temperature (MAT) of 15.7°C and mean annual precipitation (MAP) of 485 mm (Figure 3-1).

Treatments are organized in a randomized complete block design and replicated in a factorial manner. Plots are 1.5 m$^2$ and are separated by a 1-m buffer zone. Five treatments were utilized in this study (Native, Invaded, Fertilized, Clipped, Fertilized + Clipped [“Both”]). The native treatment contained plants native to California grasslands (Bromus carinatus, Elymus glaucus, Elymus triticoides, Ascispon americanus, Lupinus bicolor, Stipa pulchra, Poa secunda, and Festuca microstachys). The invaded treatment consisted of native, exotic, and invasive plants (Bromus carinatus, Elymus glaucus, Elymus triticoides, Ascispon americanus, Lupinus bicolor, Stipa pulchra, Poa secunda, and Festuca microstachys, Avena fatua, Bromus hordeaceus, Festuca perennis, and Trifolium subterraneum, Aegilops triuncialis and Elymus caput-medusae). Plant communities were established in 2006 by adding 139 g of seed to each 1.5 m$^2$ plot, with an equal proportion of each species in the native treatment. The invaded treatment was seeded with an equal proportion (33%) of natives, naturalized, and invasive species; within each group there was an equal proportion of the component species. The site was irrigated prior to seeding in order to exhaust the existing seed bank; plants that subsequently germinated were treated with glyphosate. Non-planted species are weeded annually.

Fertilized and clipped treatments were initiated in 2008 on invaded plant communities only, because this mixed community type is a more realistic representation of grassland
composition today. For fertilized treatments, NH$_4$NO$_3$ fertilizer is applied two to three times a year, totaling an annual rate of 4.5 g NH$_4$NO$_3$-N m$^{-2}$ yr$^{-1}$. Clipped treatments are maintained by clipping and removing aboveground vegetation at a height of 5 cm each spring. In 2011-12, the first year of our study, fertilizer was applied on November 29, 2011 and February 16, 2012. Aboveground vegetation was clipped between April 17 and April 27, 2012. In the second year (2012-13), fertilizer was applied on December 4, 2012, February 20, 2013, and March 26, 2013. Plots were clipped between January 11 – 16, 2013 and again on May 2, 2013.

### 3.3.2 Sample Collection

In order to account for the strong seasonal variation at our site that is typical of Mediterranean-type ecosystems, we sampled soil four times per year (eight dates total): once in fall (October/November), winter (January), spring (April), and summer (July) seasons. Within each date, five randomly selected mineral soil cores (2.5 cm diameter x 15 cm deep) were composited per plot (n = 8). Composite field-moist samples were sieved (2 mm) and either immediately frozen at -20 °C for molecular analysis, or stored at 4 °C for all other analyses (maximum 3 weeks).

Ion-exchange resin (IER) bags were used to measure the accumulation of NH$_4^+$ and NO$_3^-$ over time (Binkley & Matson, 1983; Binkley, 1984) and were buried at a depth of 7.5 cm (1 bag/plot) and incubated for 4-7 weeks (n = 12). At the end of each incubation period, resin bags were removed and replaced with new ones using the same hole. The first bags were buried in October 2011 and the last bags were removed in August 2013, totaling 15 consecutive incubations.

Volumetric water content (VWC) was measured at each sampling date using a Time-Domain Reflectometer (TDR; MiniTrase, Soil Moisture Equipment Corp., Santa Barbara, CA, USA). Fifteen-cm long TDR probes were inserted vertically into the soil in October 2011 and 30-cm long probes were installed adjacent to the 15-cm probes in January 2012 (n = 6/depth). This design allowed measurement of VWC within the 0-15 cm and 15-30 cm soil layers.

### 3.3.3 Soil Characteristics

Soil pH was measured on a 1:2 soil to 0.01 M CaCl$_2$ suspension, which was allowed to equilibrate for 30 minutes prior to analysis using a pH electrode (Orion DUAL STAR meter, Thermo Scientific, Waltham, MA, USA). In 2011-12, soil temperature was measured at every sampling date using a traceable metal thermometer (Control Company, Friendswood, TX, USA) inserted 7.5 cm into the soil. In December 2012, we began measuring temperature using HOBO dataloggers (Onset Corporation, Cape Cod, MA, USA) placed at 7.5 cm depth (1 sensor/plot, n = 8) to get a continuous, time-integrated measurement of soil temperature. For the purpose of this dissertation, the temperature data collected from the HOBO sensors was distilled down to three point measurements
that approximated the time of soil sampling (12:00 PM on 1/9/2014, 4/9/2014, and 7/9/2014). Gravimetric water content (GWC) was determined by drying field moist soil at 105 °C to a constant mass. The dried soil was subsequently ground and used to analyze total carbon (C) and N on an Elemental Combustion System (Costech Analytical Technologies Inc., Valencia, CA, USA). Because total C and N were not expected to vary greatly within one year (Binkley & Hart, 1989), samples collected from the same year were composited over time prior to analysis.

### 3.3.4 Nitrogen Availability

Instantaneous measurements of soil NH$_4^+$ and NO$_3^-$ pools were conducted by extracting 15 g field-moist soil with 100 mL 2 M KCl. Samples were shaken for 1 hour on a reciprocating shaker, filtered using pre-leached Whatman No. 1 filter paper, and stored at -20 °C until analysis on a Lachat AE Flow Injection Autoanalyzer (Lachat Instruments, Inc., Milwaukee, WI, USA).

In-situ rates of soil NH$_4^+$ and NO$_3^-$ accumulation over time were approximated using IER bags (Binkley & Matson, 1983; Binkley, 1984). Briefly, 15 mL of cation and anion resin beads (J.T. Baker Mixed Bed Exchange Resin, IONAC NM-60 H$^+$/OH$^-$ Form, Type 1, 16-50 Mesh) were measured into nylon stockings, tied shut, and placed in the field (see Sample Collection for field incubation details). After removal from the field, resin bags were air dried and placed into 125 mL flasks with 50 mL of 2M KCl. Samples were shaken uncovered for 1 hour on an orbital shaker (180 rpm), filtered using pre-leached Whatman No. 1 filter paper, and stored at -20 °C until analysis on a Lachat AE Flow Injection Autoanalyzer.

### 3.3.5 Potential Assays and Microbial Biomass

Potential rates of nitrification were estimated using the shaken soil-slurry method (Hart et al., 1994). Specifically, 15 g of field-moist soil was amended with 100 mL NH$_4^+$ and PO$_4^{3-}$ solution (1.5 mM of NH$_4^+$ and 1 mM of PO$_4^{3-}$, pH = 7.2) in a 250 mL flask and capped with a rubber stopper (containing a hole to allow gas exchange but minimize water loss). Flasks were placed on an orbital shaker (180 rpm) for 24 h. At 2, 4, 22, and 24 h, 10 mL of suspension was removed from each flask and centrifuged at 8,000 x g for 8 minutes. Five mL of supernatant was removed from the centrifuged sample, placed into a disposable polypropylene tube, capped, and stored at -20°C until analysis for NO$_3^-$ on a Lachat AE Flow Injection Autoanalyzer.

Potential rates of denitrification were measured using the protocol described by Smith & Tiedje, (1979). Briefly, 50 g of field-moist soil was combined with NO$_3^-$ and labile C (0.1 mg N-NO$_3^-$ g$^{-1}$ soil, 1 mg C-glucose g$^{-1}$ soil, and 1 mg C-glutamic acid g soil$^{-1}$) in a 250 mL flask, which was subsequently sealed tight with a rubber stopper fitted with a rubber septum. Flasks were alternately evacuated (3 minutes) and flushed with N$_2$ (1 minute) three times to create anaerobic conditions and then injected with 20 mL of acetylene in
order to inhibit the reduction of N\textsubscript{2}O to N\textsubscript{2}. The soils were incubated for 90 minutes on an orbital shaker (180 rpm) and sampled at 30 and 90 minutes by removing 15 mL of the headspace. Gas samples were stored in evacuated Exetainer\textsuperscript{®} until analysis for N\textsubscript{2}O production on a Shimadzu GC-2014 electron capture detector (Shimadzu Corporation, Columbia, MD, USA).

Microbial biomass was determined using substrate-induced respiration (SIR; West & Sparling, 1986), a method that estimates the abundance of active and glucose-responsive microorganisms in the soil (Wardle & Ghani, 1995). Fifteen g of field-moist soil and 30 mL of a glucose solution (30 mg glucose ml\textsuperscript{-1} H\textsubscript{2}O) were added to a 250 mL flask. Flasks were sealed tight with a rubber stopper fitted with a rubber septum and placed on an orbital shaker (180 rpm) for 2.5 h. Fifteen mL of the headspace was sampled at 0.5, 1.5, and 2.5 h; gas samples were stored in evacuated Exetainer\textsuperscript{®} until analysis for CO\textsubscript{2} production on a Shimadzu GC-2014 thermal conductivity detector.

### 3.3.6 Quantification of amoA gene abundance

Between 0.20-0.30 g of soil from each sample was extracted using a MO BIO PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) following the manufacturer’s instructions. Extracted DNA was quantified using a Quant-iT PicoGreen ds DNA Assay Kit (Life Technologies, Carlsbad, CA, USA) and diluted to 1 ng µL\textsuperscript{-1} DNA for subsequent analyses. Quantitative polymerase chain reaction (qPCR) followed Beman et al., (2012). Bacterial and archaeal amoA genes were quantified using the bacterial AmoA1F/amoA2R (Rotthauwe et al., 1997) and archaeal Arch-amoAF/Arch-amoAR (Francis et al., 2005) primer sets; both amplify a broad range amoA sequence types from soils (Hawkes et al., 2005; Di et al., 2009; Stopnisek et al., 2010; Zhou et al., 2014). Twenty-five µL PCR reactions for archaeal amoA contained SYBR Premix F (Epicentre BioTechnologies, Madison, WI, USA), 2 mM MgCl\textsubscript{2}, 1.25 units AmpliTaq polymerase (Life Technologies Corporation, Carlsbad, CA, USA), 40 ng µL\textsuperscript{-1} BSA (Life Technologies Corporation, Carlsbad, CA, USA), 0.4 µM forward and reverse primer, and 1 ng µL\textsuperscript{-1} DNA. Reaction chemistries for bacterial and archaeal amoA were identical, except the reaction for bacteria did not include MgCl\textsubscript{2}.

Serial dilutions (10\textsuperscript{7} to 10\textsuperscript{2} copies) of the archaeal and bacterial amoA standards were used to quantify amoA. Standards were synthesized at Life Technologies (archaeal amoA) or Blue Heron Biotechnologies (bacterial amoA) based on previously cloned and sequenced amoA genes (archaeal amoA clone GOC-G-60-9; GenBank accession no. EU340472; bacterial amoA clone HB_A_0206_GO1; GenBank accession no. EU155190). Cycling conditions for AOB were: 95°C (5 min); 40 × 95°C (45 s), 56°C (30 s), 72°C (60 s); 81°C (7 s); dissociation curve. Cycling conditions for AOA were: 95°C (4 min); 40 × 95°C (30 s), 53°C (45 s), 72°C (60 s); dissociation curve. Efficiencies for AOB and AOA amoA amplification ranged from 0.87 to 0.94 (R\textsuperscript{2} 0.93 – 1.0) and 0.86 to 1.02 (R\textsuperscript{2} 0.91-1.0), respectively.
3.3.7 Statistical Analysis

Two-way repeated measures analysis of variance (ANOVA) was used to analyze all measured variables as a function of treatment and time ($\alpha = 0.05$). In some cases, data was transformed to fit the assumption of normality. To complement the analysis of variance, which tests for treatment interactions that are not additive, we compared observed interactive effects to expected interactive effects. This method provides information on the magnitude and direction of interactions regardless of statistical significance. We used the same method of analysis as Brown et al., (2012), and focused on the additive model. Observed and expected values were calculated using paired samples from within each block, resulting in eight replicates per calculation. The effect size of each individual factor (N fertilization and clipping) was calculated as:

$$X_N = T - C$$

$$X_{CI} = T - C$$

where $T$ is the single treatment value and $C$ is the control value. Observed combined effects of both factors ($N$ fertilization x clipping) were determined as:

$$X_{\text{observed}} = NCI - C$$

where $NCI$ is the observed value for combined treatments and $C$ is the control value. Expected combined effects of both factors based on an additive model were determined as:

$$X_{\text{expected}} = X_N + X_{CI}$$

The interaction term $(I)X$ was calculated by subtracting the expected interactive effect from the observed interactive effect:

$$I(X) = X_{\text{observed}} - X_{\text{expected}}$$

Interaction terms are presented as mean ± 95% confidence intervals (CI); if the CI does not overlap zero, then the observed interaction deviated from an additive model (above zero, the interaction is synergistic; below zero, the interaction is antagonistic).

Potential rates of nitrification and denitrification were correlated with bacterial and archaeal amoA gene abundances and soil properties (e.g., $NH_4^+$ and $NO_3^-$ concentrations) using Spearman’s rank correlation coefficients.
3.4 Results

3.4.1 Effects of Invasion

Invaded treatments showed significant differences compared to native treatments in N concentrations, potential rates of nitrification and denitrification, and microbial biomass. Specifically, in 2011-12, invaded treatments had lower IER-NO$_3^-$ values (Table 3-1), reduced rates of denitrification potential in January, and lower soil microbial biomass across time (-10%, $P < 0.001$, Fig. 1a). In addition, invaded treatments had lower VWC from 0-15 cm (marginally significant, $P = 0.06$) and higher VWC from 15-30 cm depth ($P < 0.01$). All other measured variables were statistically similar between these treatments in 2011-12. In 2012-13, IER-NO$_3^-$ was higher in invaded treatments ($P < 0.05$; Table 3-1), which is in contrast to the pattern observed in the previous year. Potential nitrification ($P < 0.001$), microbial biomass (-8%, $P < 0.001$), total C (-3%, $P < 0.001$) and total N (-3%, $P < 0.001$), however, were lower in invaded treatments. Volumetric water content from 15-30 cm depth remained elevated in invaded treatments ($P < 0.01$). No other measured variables were significantly different between these treatments in 2012-13.

3.4.2 Effects of N fertilization, clipping, and N fertilization x clipping

Nitrogen fertilization increased soil NO$_3^-$ pools, as demonstrated by both the instantaneous KCl extractable soil NO$_3^-$ and IER-NO$_3^-$ concentrations. In 2011-12, soil NO$_3^-$ concentrations were unaffected by fertilization except in January ($P < 0.001$; Table 3-2, Figure 3-3); however, IER-NO$_3^-$ showed large responses to fertilization throughout most of the 2011-12 growing season (November – April; N fertilization, +443%, $P < 0.001$; Table 3-2, Figure 3-4). Despite the addition of fertilizer as NH$_4$NO$_3$, neither soil NH$_4^+$ nor IER-NH$_4^+$ concentrations were significantly affected in fertilized plots; however, IER-NH$_4^+$ and soil NO$_3^-$ showed a decrease with clipping. In 2012-13, NO$_3^-$ availability remained elevated in fertilized treatments, with IER-NO$_3^-$ values again showing a more dramatic response (+504%, $P < 0.001$; Table 3-2) than soil NO$_3^-$ concentrations. However, the dramatic effects of fertilization on IER-NO$_3^-$ values were not apparent or statistically significant in October and February. Clipping increased soil NO$_3^-$ concentrations but reduced IER-NO$_3^-$ values. Similar to 2011-12, neither soil NH$_4^+$ nor IER-NH$_4^+$ concentrations were affected by treatment in 2012-13. None of the N availability indices showed a significant interactive response to N fertilization and clipping using repeated measures two-way ANOVA (Table 3-2) and comparison of observed and expected interactive effects (Figure 3-5).

Similar to N availability, fertilization and clipping had large independent effects on N potentials and soil microbial biomass. In 2011-12, N fertilization significantly increased
nitrification potential (Table 3-2) and microbial biomass (Figure 3-6). In contrast, nitrification potential was reduced in clipped treatments, although in April this reduction was only marginally significant. In 2012-13, nitrification potential remained elevated in fertilized plots. Fertilization reduced denitrification potential during January of this year. Similar to 2011-12, clipped treatments showed a significant reduction in nitrification potential in 2012-13. Clipping also increased denitrification potential (Table 3-2) and microbial biomass compared to unclipped treatments (Figure 3-5). Similar to N availability indices, interactions between N fertilization and clipping did not deviate from an additive expectation for either year. This was demonstrated both with the repeated measures two-way ANOVA and by comparing observed to expected interactive effects Figure 3-5. In contrast to effects on potential rates of nitrification, amoA gene copy numbers for AOB and AOA were not significantly affected by N fertilization or clipping in 2012-13 Figure 3-7.

Both fertilization and clipping had moderate effects on other ancillary soil properties (Table 3-3). In 2011-12, fertilization slightly reduced soil pH (-0.6%, P < 0.05) and VWC at 0-15 cm depth (-2%, P = 0.09) and more strongly reduced total N (-6%, P < 0.05) and C (-13%, P = 0.06), although the effects on total C and VWC were only marginally significant. Clipping slightly reduced soil pH (-0.5%, P = 0.05) and induced a stronger, but marginal, reduction on VWC at 15-30 cm depth (-9%, P = 0.06). In contrast, clipping increased VWC at 0-15 cm depth (+9%, P =0.06), but again this effect was marginally significant. Similar to 2011-12, fertilization in 2012-13 slightly reduced soil pH (-0.2%, P = 0.06) and 0-15 cm VWC (-2%, P < 0.05). In July of 2012-13, fertilized treatments had marginally significant higher GWC (+8%, P = 0.08) than plots that were not fertilized. Total C and N showed no significant variation with treatment in the second year of the study. Volumetric (0-15 cm) and GWC in 2012-13 both exhibited a marginally significant interactive response to N fertilization and clipping (P = 0.09 and P = 0.08, respectively). Temperature in 2012-13 showed a significant treatment by date interaction. No significant differences occurred in January; however, in April, clipped treatments were warmer than unclipped and fertilized treatments. In July, the invaded control and fertilized treatment were cooler than plots that were clipped (both the clipped only and fertilized + clipped treatments).

3.4.3 Correlations among response variables

Nitrification potential showed contrasting relationships with AOB and AOA amoA gene abundance, although these correlations were marginal and did not remain consistent across time (Table 3-4). In particular, nitrification potential was positively correlated with AOB abundance in November and July (P < 0.1) and negatively correlated with AOA abundance in July (P < 0.1). Ammonia oxidizing bacterial abundance was negatively correlated with soil temperature in January. Archaeal abundance, however, was negatively correlated with most N availability metrics, but positively correlated with soil temperature, in July. AOA was also positively correlated with soil moisture in November and April.
Potential rates of nitrification and denitrification were correlated with a number of other soil characteristics measured in this study (Table 3-5). In 2011-12 and 2012-13, both potentials tended to show a strong positive correlation with soil microbial biomass. Nitrification potential was also positively correlated with soil NO$_3^-$ concentrations across most dates. IER-NH$_4^+$ and IER-NO$_3^-$ values rarely correlated with potential rates; the only significant relationships occurred in July when nitrification was positively correlated with IER-NH$_4^+$ and denitrification was positively correlated with IER-NO$_3^-$. Soil moisture and pH showed both positive and negative correlations with nitrification and denitrification potentials; however, these relationships were intermittent.

3.5 Discussion

Our study utilized a well-replicated field experiment, and a combination of in situ measurements, potential assays, and molecular tools to address two questions of importance for Mediterranean grasslands in an era of global change: (1) how do invasive plants influence soil N dynamics and (2) how do N dynamics in invaded grasslands respond to the interaction of N fertilization (simulated N deposition) and defoliation (simulated grazing)? We demonstrate that soil N cycling and availability, but not the abundances of ammonia oxidizing microorganisms, are sensitive to each global change factor. Importantly, we also show that N fertilization and defoliation interact in an additive way.

3.5.1 Response of soil N cycling to exotic plant invasion

Consistent with our first hypothesis, invaded plant communities exhibited decelerated rates of potential nitrification and denitrification compared to native communities. These effects, however, did not persist across years and significant changes in denitrification potential (in 2011-12) were decoupled from changes in nitrification potential (in 2012-13). This is likely because these processes are regulated by different factors; for instance, organic matter (C) and NO$_3^-$ are essential for denitrification (Wallenstein et al., 2006), whereas O$_2$ and NH$_4^+$ availability are requirements for nitrification (Chapin et al., 2012). Thus, the transient nature of plant community effects on potential rates may have been a result of changes in relative abundances of plant species within community type (Eviner et al., unpublished), or inter-annual fluctuations in soil moisture availability (Figure 3-2) that may have differentially affected the potential for the soil to nitrify or denitrify.

Reductions in soil total C and N with invasion were not apparent in 2011-12, but became significant in 2012-13, suggesting that *A. triuncialis* and *E. caput-medusae* were beginning to influence total soil C and N pools. There are examples where the presence of invasive plant populations for over 40 years at a site has not resulted in changes to C and N storage capacity of the soil (Svejcar & Sheley, 2001). That *A. triuncialis* and *E. caput-medusae* affected total C and N indicates that the lower litter quality and reduced decomposability (Bovey et al., 1961; Drenovsky & Batten, 2007) of these species has the
potential to transform “slow-changing” soil variables within just a few years of establishment.

Despite the fact that total soil N and nitrification potential were reduced with invasion, ammonia-oxidizing bacteria and AOA amoA gene abundances were unaffected by plant community type. Effects of invasion are thought to be dependent upon the functional distinctiveness of invasive species compared to resident species (Vitousek et al., 1987; Levine et al., 2003; González-Muñoz et al., 2012). As such, it is possible that key traits (e.g., litter quality and N uptake patterns) of *A. triuncialis* and *E. caput-medusae* are not different enough to induce ecologically significant changes in soil variables (e.g., NH$_4^+$ availability, soil moisture) that control the abundance of ammonia oxidizing microorganisms, at least as measured by qPCR of amoA genes.

It is more likely, however, that by comparing native plant communities with mixed communities comprised of native, naturalized, and invasive plants, the effects of *A. triuncialis* and *E. caput-medusae* may have been diluted. Indeed, previous work in an annual grassland showed that AOB gene abundance was significantly increased in soils associated with exotic grasses, and that this increase translated into high gross rates of nitrification (Hawkes et al., 2005). However, these effects were only significant when comparing exotic and native species monocultures; when comparing native communities to mixed communities of native and exotics, changes in AOB abundance and nitrification were no longer significant. Similarly, in a study comparing the effects of exotic annual grass abundance on N cycling in a California grassland, the greatest differences in soil inorganic N, N cycling rates, and microbial biomass occurred between plots dominated by exotic annuals and plots dominated by perennials (Corbin & D'Antonio, 2011). In the same way, it is possible that we would have detected changes in amoA gene copy number in plant communities with varying degrees of invasive species abundance will be important to determine if a there is a threshold after which the growth of ammonia oxidizing microorganisms is altered.

Overall, our results demonstrate that, even when not in monoculture, the presence of *A. triuncialis* and *E. caput-medusae* can reduce soil C and N and the potential for the soil to nitrify and denitrify. Changes in potential rates were associated with lower active (total) microbial biomass, but, surprisingly, nitrification potential was unrelated to changes in AOB or AOA abundance. This suggests that activity or composition rather than abundance of ammonia oxidizing microorganisms may be important in regulating the response of nitrification to exotic plant invasion.

### 3.5.2 Response of soil N cycling to clipping

Results surrounding our second hypothesis that clipping would reduce N availability were dependent upon year. In 2011-12, IER-NH$_4^+$, nitrification potential, and soil NO$_3^-$ were reduced with clipping; in 2012-13, nitrification potential and IER-NO$_3^-$ were reduced, but soil NO$_3^-$, denitrification potential and microbial biomass were increased in
clipped relative to unclipped treatments (soil NO$_3^-$ was increased by 2%, while IER-NO$_3^-$ was reduced by 11% relative to the control). Although potential rates of nitrification responded to clipping, amoA gene abundances remained unaffected.

There are a number of mechanisms by which defoliation can influence soil N cycling, including the reduction of aboveground litter inputs, alterations in root growth and C exudation, and effects on soil microclimate (reviewed by Bardgett et al., 1998). Decomposition of organic matter is a primary pathway by which available N is returned to the soil; as such, annually clipping and removing aboveground vegetation likely had an effect on N availability and microbial-mediated processes. Indeed, the longer-term nature of our study (3 - 4 years) may have increased the relative influence of aboveground litter removal, and could possibly explain why our results contrast with other shorter-term defoliation studies (7 days – 1 year) that reported increases in N availability (Guitian & Bardgett, 2000; Hamilton et al., 2008; Cheng et al., 2011). In addition, accelerated plant uptake (Wallace & Macko, 1993; Bardgett & Wardle, 2003) or microbial immobilization of available N (Magill & Aber, 2000), which can occur in response to defoliation, may partially explain the observed reductions in N availability and potential rates of nitrification in clipped compared to unclipped treatments.

Increases in microbial biomass and denitrification potential during the second year of our study suggest that root exudation of labile C likely influenced the response of soil N cycling to defoliation. Plants can respond to clipping by exuding organic acids, sugars, and amino acids (Bardgett et al., 1998; Hütsch et al., 2002; Hamilton et al., 2008), although this varies by species (Augustine et al., 2011). Thus, root exudation of labile C could explain the observed increases in soil microbial biomass (Holland, 1995; Bardgett et al., 1997) and denitrification potential, given that many microorganisms (including denitrifiers) are heterotrophs. That these increases occurred only during 2012-13, which was a wetter year than 2011-12, intimates that the positive response of microbial processes may be constrained by water availability in this system.

A number of studies have focused on the immediate or short-term responses of soil properties to clipping (Guitian & Bardgett, 2000; Hamilton & Frank, 2001; Hamilton et al., 2008), and others have measured soil N cycling in ungrazed and grazed grasslands (Shariff et al., 1994; Patra et al., 2005; Ingram et al., 2008; Coetsee et al., 2010; He et al., 2011; Hirobe et al., 2013), which cannot necessarily decouple the effects of defoliation, waste excretion, and trampling. Our study subjected experimental grassland communities to annual clipping, and measured soil four times per year (none of which were immediately after clipping). Consequently, we were able to demonstrate that defoliation can have long-term, sustained effects on soil N availability and potential rates of N cycling, which may exacerbate (e.g., N availability and nitrification) or alleviate (e.g., microbial biomass and denitrification) the belowground effects of A. triuncialis and E. caput-medusae invasion.
3.5.3 Response of soil N cycling to N fertilization

Our third hypothesis, which postulated that N fertilization would accelerate the N cycle, was supported by an increase in potential rates of nitrification across both years. Surprisingly, however, no differences in NH$_4^+$ concentrations were observed despite fertilization with NH$_4$NO$_3$. This suggests that nitrifying microorganisms present in the soil were rapidly converting the added NH$_4^+$ to NO$_3^-$, resulting in a substantial increase in NO$_3^-$ availability (443 – 504%). Large accumulations of NO$_3^-$ from deposition of available N could therefore accelerate NO$_3^-$ leaching into groundwater and potentially affect the water quality and ecology of adjacent aquatic ecosystems.

Denitrification potential did not respond to increased NO$_3^-$ availability due to fertilization, suggesting that denitrifying microorganisms may be limited by other factors such as O$_2$ or C. Indeed, denitrification potential was unaffected by fertilization in 2011-12 and was reduced by fertilization in January 2013. This was surprising given that increases in denitrification potential (Niboyet et al., 2010; 2011b), net denitrification rates (Lu et al., 2010) and soil N$_2$O fluxes (Ryden, 1983; Velthof & Oenema, 1995; Aronson & Allison, 2012) have been reported with N additions. While fertilized treatments also exhibited reduced pH and volumetric water content, two soil characteristics known to regulate denitrification (Šimek & Cooper, 2002; Wallenstein et al., 2006), the relatively small magnitude of change (-0.2 to -0.6% pH and -2% VWC) makes it unlikely that these variables influenced potential rates of denitrification. Instead, it is possible that decreases in root growth and biomass (Davidson, 1969; Reynolds & D’Antonio, 1996; George & Seith, 1998) or increases in competition with other heterotrophic microorganisms (Barnard et al., 2005) may have reduced C availability for denitrifiers, thus limiting their ability to respond to increases in NO$_3^-$ with fertilization. An increase in total soil microbial biomass during 2011-12 lends evidence towards the second mechanism. Alternatively, potential rates of denitrification may have been reduced because of shifts in the abundance or composition of denitrifying microorganisms. Accumulating evidence suggests that fungi are predominant denitrifiers in soils (Laughlin & Stevens, 2002; Crenshaw et al., 2008; Hayatsu et al., 2008; Marusenko et al., 2013; Mothapo et al., 2013), and although the effects of elevated N supply on fungal biomass and community composition is equivocal (Bittman et al., 2005; de Vries et al., 2006; Treseder, 2008; Porras-Alfaro et al., 2011; Weber et al., 2013), in some scenarios fungi can be negatively affected. As such, changes in fungal community composition, or suppression of fungal biomass, may be partially responsible for the observed reduction in denitrification potential with fertilization.

Despite marked changes in nitrification potential (which approximates the size of the nitrifying community), the abundances of AOB and AOA were not significantly affected by N additions. The lack of overall response of amoA gene abundance was unanticipated, given that multiple studies have demonstrated increases in AOB abundance to enhanced N supply (Mendum et al., 1999; Hermansson & Lindgren, 2001; Okano et al., 2004; Di et al., 2010; Shen et al., 2011; Wertz et al., 2012). However, we applied N at lower rates
than many of the studies showing a positive response of AOB, which may explain why our results are incongruent with others; for example, Shen et al., (2011) provide evidence that AOB populations only respond to urea fertilization rates above 8 g N m\(^{-2}\) y\(^{-1}\), which is almost double what we applied as NH\(_4\)NO\(_3\) in our study. The observed unresponsiveness of AOA abundance to fertilization in our study corresponds with previous work (Di et al., 2010; Stopnisek et al., 2010; Shen et al., 2011; Wertz et al., 2012) and is consistent with the idea that AOA may prefer to utilize N derived from organic matter mineralization rather than inorganic N fertilization (Levičník-Höfferle et al., 2012). New findings also suggest that AOA growth may be limited by the availability of nutrients such as phosphorus and potassium, thereby restricting their ability to respond to increases in NH\(_4^+\) (Norman & Barrett, 2014).

Nitrification potential and AOB and AOA amoA gene abundances were not strongly or consistently related, suggesting that amoA gene abundance may not be a good indicator of activity in soils, at least as measured by laboratory potential assays. The neutral to negative relationship between AOA and potential rates of nitrification observed in this study have been found elsewhere (Jia & Conrad, 2009; Bernhard et al., 2010; Di et al., 2010; Wertz et al., 2012; de Gannes et al., 2014) and there are a few likely explanations for why this may be. First, AOA may be inhibited by high amounts of substrate added (Martens-Habbena et al., 2009; Bernhard et al., 2010; Verhamme et al., 2011) or physical perturbation (Röling, 2007; Bernhard et al., 2010) typical of a potential assay. In addition, nitrification rates may be influenced more heavily by the composition, and thus the physiology, of AOA rather than the overall abundance of archaeal amoA genes (Röling, 2007; Brankatschk et al., 2010). Lastly, not all AOA may perform ammonia oxidation at a given time (Stahl & la Torre, 2012), which would result in a weak relationship between amoA gene abundance and nitrification potential. Given that a strong positive relationship between AOB abundance and nitrification potential was also lacking suggests that the abundance of amoA transcripts, rather than amoA genes, may be a better indicator of nitrifying activity in these soils.

Overall, our results demonstrate that N cycling of invaded grasslands can be affected by N additions, but that the response of nitrification may be regulated by microbial activity (de Gannes et al., 2014; Graham et al., 2014) that is decoupled from amoA gene abundance. Moreover, similar to findings from agricultural soils, dairy pasture soils, and forest soils (Jia & Conrad, 2009; Di et al., 2009; 2010; Wertz et al., 2012), but in contrast to highly acidic soils (Gubry-Rangin et al., 2010; Zhang et al., 2012), our study suggests that ammonia oxidation in Mediterranean-type grasslands may be largely driven by AOB.
3.5.4 Response of soil N cycling to the interaction of N fertilization and clipping

We found no evidence for the interaction of N fertilization and clipping to affect N cycling in a non-additive way. This means that the impact of clipping on soil N cycling was not dependent upon the nutrient status of the soil (fertilized or unfertilized), and that the impact of fertilization did not differ between clipped and unclipped plots. These findings are consistent with previous studies that suggest interactive responses to global change may be rare (Zavaleta et al., 2003; Zhou et al., 2006; Niboyet et al., 2011c). However, they contrast with many other findings (Shaw, 2002; Henry et al., 2005; Barnard et al., 2006; Castro et al., 2010; Blankinship et al., 2010; Dieleman et al., 2012), including those that have found N fertilization and defoliation to produce significant interactive effects on plant root and shoot biomass and compensation patterns (Li & Redmann, 1992; Hawkes & Sullivan, 2001; Ferraro & Oesterheld, 2002). Whether significant non-additive interactions occur may be dependent upon the system, the response variables measured, and the global change factors that were manipulated. Importantly, our results demonstrate that in Mediterranean-type grasslands, the response of soil N cycling to N deposition is not dependent upon the presence of defoliation, and vice-versa.

3.6 Conclusion

The N cycling variables measured in our study responded to the presence of invasive species, N fertilization, and clipping, but not to the interaction of N fertilization and clipping. Invaded soils had decelerated rates of N cycling compared to native soils, and clipping of aboveground vegetation tended to exacerbate low N conditions. In contrast, N fertilization increased nitrification potential and NO₃⁻ availability, which highlights the possibility for NO₃⁻ leaching to occur in grassland sites that receive elevated rates of N deposition. Importantly, changes in potential rates of nitrification were not consistently associated with shifts in bacterial and archaeal amoA gene abundance, suggesting the likely role of other controlling mechanisms, such as AOB and AOA community composition.

3.7 References


### Table 3-1: Effects of invasion on potential rates and N availability indices

<table>
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<tr>
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<th>Nitrification Potential</th>
<th>Denitrification Potential</th>
<th>Soil NH$_4^+$</th>
<th>Soil NO$_3^−$</th>
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<th>IER – NO$_3^−$</th>
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<td><strong>2011-12</strong></td>
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<td>Treatment effects</td>
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<tr>
<td>Invasion</td>
<td>- 6</td>
<td>0.15</td>
<td>- 31</td>
<td>&lt; 0.001</td>
<td>+ 11</td>
<td>0.25</td>
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<tr>
<td>Time</td>
<td>&lt; 0.001</td>
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<td>&lt; 0.001</td>
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<tr>
<td>Invasion x Time</td>
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<td>0.01</td>
<td>0.84</td>
<td>0.06</td>
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<td><strong>2012-13</strong></td>
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<td>Invasion</td>
<td>- 12</td>
<td>&lt; 0.01</td>
<td>+ 4</td>
<td>0.17</td>
<td>+ 44</td>
<td>0.81</td>
</tr>
<tr>
<td>Time</td>
<td>&lt; 0.001</td>
<td>0.08</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
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<tr>
<td>Invasion x Time</td>
<td>0.24</td>
<td>0.84</td>
<td>0.71</td>
<td>0.87</td>
<td>0.48</td>
<td>0.15</td>
</tr>
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</table>

% = percent change, calculated as ((Treatment – Control)/(Control))\*100. Significant responses are highlighted in bold (P < 0.05).
Table 3-2 Effects of nitrogen fertilization and clipping on potential rates and N availability indices

<table>
<thead>
<tr>
<th></th>
<th>Nitrification Potential</th>
<th>Denitrification Potential</th>
<th>Soil NH₄⁺</th>
<th>Soil NO₃⁻</th>
<th>IER – NH₄⁺</th>
<th>IER – NO₃⁻</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>%</td>
<td>P value</td>
<td>%</td>
<td>P value</td>
<td>%</td>
<td>P value</td>
</tr>
<tr>
<td>2011-12</td>
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</tr>
<tr>
<td>Treatment effects</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>N addition</td>
<td>+ 20</td>
<td>&lt; 0.001</td>
<td>- 1</td>
<td>0.72</td>
<td>+ 1</td>
<td>0.98</td>
</tr>
<tr>
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<td>- 15</td>
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<td>- 4</td>
<td>0.99</td>
<td>- 7</td>
<td>0.40</td>
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<tr>
<td>N x Clipping</td>
<td>- 8</td>
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<td>+ 3</td>
<td>0.60</td>
<td>- 3</td>
<td>0.61</td>
</tr>
<tr>
<td>Time effects</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Time</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.001</td>
<td>&lt; 0.001</td>
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<tr>
<td>N x Time</td>
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<td>&lt; 0.001</td>
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<td>0.89</td>
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<td>0.95</td>
<td>0.11</td>
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<tr>
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<td>0.81</td>
<td>0.94</td>
<td>0.55</td>
<td>0.65</td>
</tr>
<tr>
<td>2012-13</td>
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<tr>
<td>Treatment effects</td>
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<td>+ 25</td>
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<td>+ 11</td>
<td>0.57</td>
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<td>+ 20</td>
<td>&lt; 0.05</td>
<td>+ 7</td>
<td>0.95</td>
</tr>
<tr>
<td>N x Clipping</td>
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</tr>
<tr>
<td>Time</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>N x Time</td>
<td>0.62</td>
<td>&lt; 0.05</td>
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<td>0.38</td>
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<td>&lt; 0.001</td>
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<tr>
<td>Clipping x Time</td>
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<td>&lt; 0.05</td>
<td>0.21</td>
<td>0.76</td>
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</tr>
<tr>
<td>N x C x Time</td>
<td>0.42</td>
<td>0.32</td>
<td>0.42</td>
<td>0.80</td>
<td>0.08</td>
<td>0.35</td>
</tr>
</tbody>
</table>

% = percent change, calculated as ((Treatment – Control)/(Control))*100. Significant responses are highlighted in bold (P < 0.05). Abbreviations: N – nitrogen, C – clipping.
Table 3-3 Soil characteristics from 0 – 15 cm depth associated with each treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GWC (%)</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Total C (g kg(^{-1}) soil)</th>
<th>Total N (g kg(^{-1}) soil)</th>
<th>C:N</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011-12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>14.29 (1.05)</td>
<td>6.62 (0.03)</td>
<td>18.53 (1.33)</td>
<td>12.86 (0.28)</td>
<td>1.34 (0.03)</td>
<td>9.63 (0.05)</td>
</tr>
<tr>
<td>Invaded</td>
<td>14.32 (1.00)</td>
<td>6.62 (0.02)</td>
<td>22.21 (4.24)</td>
<td>14.4 (1.41)</td>
<td>1.34 (0.07)</td>
<td>10.62 (0.46)</td>
</tr>
<tr>
<td>N addition</td>
<td>14.64 (0.99)</td>
<td>6.58 (0.02)</td>
<td>17.78 (1.28)</td>
<td>12.54 (0.29)</td>
<td>1.26 (0.02)</td>
<td>9.97 (0.13)</td>
</tr>
<tr>
<td>Clipping</td>
<td>13.90 (1.00)</td>
<td>6.58 (0.02)</td>
<td>18.47 (1.35)</td>
<td>14.06 (1.07)</td>
<td>1.35 (0.05)</td>
<td>10.31 (0.38)</td>
</tr>
<tr>
<td>N x C</td>
<td>13.94 (0.89)</td>
<td>6.57 (0.02)</td>
<td>18.29 (1.30)</td>
<td>12.48 (0.32)</td>
<td>1.22 (0.03)</td>
<td>10.24 (0.26)</td>
</tr>
<tr>
<td>2012-13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>17.07 (1.19)</td>
<td>6.54 (0.02)</td>
<td>16.53 (10.66)</td>
<td>12.20 (0.29)</td>
<td>1.24 (0.03)</td>
<td>9.80 (0.08)</td>
</tr>
<tr>
<td>Invaded</td>
<td>17.85 (1.44)</td>
<td>6.53 (0.02)</td>
<td>18.04 (10.19)</td>
<td>11.83 (0.26)</td>
<td>1.21 (0.02)</td>
<td>9.78 (0.10)</td>
</tr>
<tr>
<td>N addition</td>
<td>18.27 (1.50)</td>
<td>6.52 (0.02)</td>
<td>17.47 (9.81)</td>
<td>12.25 (0.27)</td>
<td>1.24 (0.02)</td>
<td>9.87 (0.13)</td>
</tr>
<tr>
<td>Clipping</td>
<td>18.28 (1.73)</td>
<td>6.53 (0.02)</td>
<td>20.65 (12.29)</td>
<td>12.25 (0.49)</td>
<td>1.23 (0.04)</td>
<td>9.94 (0.10)</td>
</tr>
<tr>
<td>N x C</td>
<td>18.89 (1.42)</td>
<td>6.48 (0.02)</td>
<td>19.97 (12.66)</td>
<td>12.03 (0.38)</td>
<td>1.22 (0.03)</td>
<td>9.83 (0.13)</td>
</tr>
</tbody>
</table>

Values are means ± SE averaged over all four seasons (October/November – July; n = 8). GWC = gravimetric water content; pH measured in 0.01 M CaCl\(_2\) solution; Temperature in 2011-12 was measured at 7.5 cm depth using a traceable metal thermometer, while temperature in 2012-13 was measured using HOBO sensors placed 7.5 cm in the soil (averages from 12:00 PM measurements on soil sampling dates 1/9/2013, 4/9/2013, 7/9/2013); C:N = carbon to nitrogen mass ratio of soil.
Table 3-4 Spearman’s rank coefficients (r) and significance for correlations among ammonia-oxidizing bacteria and archaea and select soil parameters

<table>
<thead>
<tr>
<th>AOB Abundance</th>
<th>November</th>
<th>January</th>
<th>April</th>
<th>July</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>0.31</td>
<td>0.19</td>
<td>-0.03</td>
<td>0.30</td>
<td>0.27**</td>
</tr>
<tr>
<td>Soil NH4+</td>
<td>-0.01</td>
<td>-0.10</td>
<td>-0.07</td>
<td>-0.03</td>
<td>0.18</td>
</tr>
<tr>
<td>Soil NO3-</td>
<td>0.09</td>
<td>0.02</td>
<td>0.10</td>
<td>0.08</td>
<td>-0.13</td>
</tr>
<tr>
<td>IER – NH4+</td>
<td>---</td>
<td>---</td>
<td>0.09</td>
<td>0.05</td>
<td>-0.03</td>
</tr>
<tr>
<td>IER – NO3-</td>
<td>---</td>
<td>---</td>
<td>0.08</td>
<td>-0.10</td>
<td>-0.04</td>
</tr>
<tr>
<td>pH</td>
<td>-0.07</td>
<td>0.02</td>
<td>-0.16</td>
<td>-0.14</td>
<td>-0.04</td>
</tr>
<tr>
<td>Temperature</td>
<td>---</td>
<td>-0.34*</td>
<td>-0.21</td>
<td>-0.18</td>
<td>-0.24**</td>
</tr>
<tr>
<td>Soil Moisture</td>
<td>-0.04</td>
<td>-0.10</td>
<td>-0.12</td>
<td>0.26</td>
<td>-0.09</td>
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</table>

<table>
<thead>
<tr>
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<th>January</th>
<th>April</th>
<th>July</th>
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</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>-0.17</td>
<td>-0.06</td>
<td>-0.18</td>
<td>-0.28</td>
<td>-0.67***</td>
</tr>
<tr>
<td>Soil NH4+</td>
<td>0.13</td>
<td>0.07</td>
<td>-0.24</td>
<td>-0.66***</td>
<td>0.15</td>
</tr>
<tr>
<td>Soil NO3-</td>
<td>-0.16</td>
<td>-0.01</td>
<td>-0.27</td>
<td>-0.28</td>
<td>0.25**</td>
</tr>
<tr>
<td>IER – NH4+</td>
<td>---</td>
<td>---</td>
<td>0.02</td>
<td>-0.32*</td>
<td>-0.34*</td>
</tr>
<tr>
<td>IER – NO3-</td>
<td>---</td>
<td>---</td>
<td>-0.24</td>
<td>-0.25</td>
<td>-0.79***</td>
</tr>
<tr>
<td>pH</td>
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<td>-0.02</td>
<td>0.18</td>
<td>0.32</td>
<td>0.39***</td>
</tr>
<tr>
<td>Temperature</td>
<td>---</td>
<td>-0.03</td>
<td>-0.30</td>
<td>0.43**</td>
<td>0.49***</td>
</tr>
<tr>
<td>Soil Moisture</td>
<td>0.31</td>
<td>-0.04</td>
<td>0.34*</td>
<td>0.26</td>
<td>-0.23*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AOB / AOA</th>
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<th>January</th>
<th>April</th>
<th>July</th>
<th>All</th>
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<tbody>
<tr>
<td>NP</td>
<td>0.25</td>
<td>0.14</td>
<td>0.12</td>
<td>0.37*</td>
<td>0.66***</td>
</tr>
<tr>
<td>Soil NH4+</td>
<td>-0.05</td>
<td>0.06</td>
<td>0.01</td>
<td>0.42**</td>
<td>-0.07</td>
</tr>
<tr>
<td>Soil NO3-</td>
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<td>0.16</td>
<td>0.28</td>
<td>-0.21*</td>
</tr>
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<td>0.06</td>
<td>0.21</td>
<td>0.28*</td>
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<td>-0.06</td>
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<td>-0.31***</td>
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<td>-0.03</td>
<td>-0.46**</td>
<td>-0.65***</td>
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<td>-0.09</td>
<td>-0.26</td>
<td>-0.15</td>
<td>0.31***</td>
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</table>

*** P < 0.001, ** P < 0.01, * P < 0.05, significant responses are highlighted in bold (P < 0.05). N = 40 for each individual sampling date. All = collapsed across sampling dates (N = 160; N = 80 for IER - NH₄⁺ /NO₃⁻). NP = nitrification potential.
Table 3-5 Spearman’s rank coefficients (r) and significance for correlations among nitrification and denitrification potential and select soil parameters

<table>
<thead>
<tr>
<th>Nitrification Potential</th>
<th>2011-12</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>2012-13</th>
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<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>October</td>
<td>January</td>
<td>April</td>
<td>July</td>
<td>All</td>
<td>November</td>
<td>January</td>
<td>April</td>
<td>July</td>
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<td>January</td>
<td>April</td>
<td>July</td>
<td>All</td>
<td></td>
</tr>
<tr>
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<td>0.20</td>
<td>0.19</td>
<td>0.18</td>
<td>-0.05</td>
<td><strong>0.49</strong>*</td>
<td>0.25</td>
<td>-0.25</td>
<td>-0.14</td>
<td>0.12</td>
<td>-0.23**</td>
<td>-0.10</td>
<td>-0.25</td>
<td>-0.14</td>
<td>0.12</td>
<td>-0.23**</td>
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</tr>
<tr>
<td>Soil NO$_3^-$</td>
<td>0.01</td>
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<td>0.16</td>
<td>0.03</td>
<td><strong>0.55</strong>*</td>
<td><strong>0.42</strong></td>
<td><strong>0.32</strong></td>
<td>0.00</td>
<td>-0.10</td>
<td>-0.10</td>
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<td>-0.10</td>
<td>-0.10</td>
<td>-0.10</td>
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</tr>
<tr>
<td>IER – NH$_4^+$</td>
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<td>0.13</td>
<td>0.12</td>
<td>0.14</td>
<td>0.14</td>
<td>---</td>
<td>---</td>
<td>0.13</td>
<td><strong>0.40</strong></td>
<td><strong>0.37</strong></td>
<td>-0.01</td>
<td>-0.01</td>
<td>-0.01</td>
<td>-0.01</td>
<td>-0.01</td>
<td></td>
</tr>
<tr>
<td>IER – NO$_3^-$</td>
<td>---</td>
<td>0.15</td>
<td>0.13</td>
<td>0.20</td>
<td>-0.26*</td>
<td>---</td>
<td>---</td>
<td>0.08</td>
<td>0.01</td>
<td><strong>0.65</strong>*</td>
<td>0.01</td>
<td>-0.26</td>
<td>0.06</td>
<td>-0.20</td>
<td>-0.39***</td>
<td></td>
</tr>
<tr>
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<td>0.18</td>
<td>-0.16</td>
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<td>0.25</td>
<td>-0.07</td>
<td>-0.26</td>
<td>-0.25</td>
<td>0.06</td>
<td>-0.20</td>
<td>-0.39***</td>
<td>-0.43**</td>
<td>-0.52**</td>
<td>-0.43**</td>
<td>-0.52**</td>
<td>-0.43**</td>
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</tr>
<tr>
<td>Temperature</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>-0.28</td>
<td>-0.26</td>
<td>-0.01</td>
<td>-0.01</td>
<td>-0.01</td>
<td>-0.01</td>
<td>-0.01</td>
<td>-0.01</td>
<td>-0.01</td>
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</tr>
<tr>
<td>Soil Moisture</td>
<td>0.31</td>
<td>-0.04</td>
<td><strong>0.52</strong></td>
<td>-0.06</td>
<td><strong>0.47</strong></td>
<td>0.02</td>
<td>0.03</td>
<td>-0.30</td>
<td>0.00</td>
<td><strong>0.30</strong></td>
<td>-0.01</td>
<td>-0.01</td>
<td>-0.01</td>
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<td>-0.01</td>
<td></td>
</tr>
<tr>
<td>Microbial Biomass</td>
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<td><strong>0.43</strong></td>
<td><strong>0.52</strong></td>
<td>0.20</td>
<td>0.18</td>
<td>0.11</td>
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</table>

<table>
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<th>Denitrification Potential</th>
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<th>October</th>
<th>January</th>
<th>April</th>
<th>July</th>
<th>All</th>
<th>November</th>
<th>January</th>
<th>April</th>
<th>July</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil NH$_4^+$</td>
<td>0.29</td>
<td>-0.16</td>
<td>0.09</td>
<td><strong>0.44</strong></td>
<td><strong>0.46</strong></td>
<td>0.13</td>
<td>-0.19</td>
<td>0.13</td>
<td>0.29</td>
<td>0.20*</td>
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<td>0.11</td>
<td>0.03</td>
<td>-0.25</td>
<td>-0.20*</td>
<td>-0.13</td>
<td>-0.32*</td>
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<td>0.06</td>
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<td>---</td>
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<td>-0.19*</td>
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<tr>
<td>Microbial Biomass</td>
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<td><strong>0.35</strong></td>
<td>0.28</td>
<td><strong>0.38</strong></td>
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<td>0.20</td>
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<td><strong>0.56</strong></td>
<td><strong>0.62</strong></td>
<td><strong>0.39</strong></td>
<td></td>
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</tbody>
</table>

*** P < 0.001, ** P < 0.01, * P < 0.05, significant responses are highlighted in bold (P < 0.05). N = 40 for each individual sample date. All = collapsed across sampling dates within 2011-12 or 2012-13 (N = 160; N = 120 and 80 for IER - NH$_4^+$/NO$_3^-$ in 2011-12 and 2012-13, respectively.
3.9 Figures

Figure 3-1 (a) Total monthly precipitation and (b) monthly maximum/minimum air temperature (2 m above ground) during the duration of our study (2011-2013). Values are derived from the University of California, Davis Climate Station (http://atm.ucdavis.edu), which is located approximately a mile away from the study site. Vertical dashed line here and in subsequent graphs represents the separation of year 1 (2011-12) and year 2 (2012-13) of sampling.
Figure 3-2 Changes in mean soil volumetric water content over time, as influenced by plant composition, fertilization, clipping, fertilization × clipping and soil depth (upper panel 0-15 cm, lower panel 15-30 cm). Values are means ± 1 SE (n = 6). Volumetric water content at both depths differed by date (P < 0.001), and the relationship between VWC and treatment tended to be only marginally significant (0.05 < P < 0.1). Volumetric water content at 15-30 cm depth is not present for October and November 2011 because 0-30 cm TDR probes were not installed at that time.
Figure 3-3 The response of soil (a) NH$_4^+$ and (b) NO$_3^-$ concentrations to plant composition, fertilization, clipping, and fertilization × clipping over time. Bars ± 1 standard error of the mean (n = 8). Soil NH$_4^+$ differed significantly by sampling date (P < 0.001) but not by treatment. In 2011-12, the effect of N fertilization on soil NO$_3^-$ was dependent upon sampling date. In 2012-13, soil NO$_3^-$ was independently affected by treatment and date (P < 0.01).
Figure 3-4 Ion-exchange resin (a) NH$_4^+$ and (b) NO$_3^-$ values, as affected by plant composition, fertilization, clipping, and fertilization × clipping over time. Bars ± 1 standard error of the mean (n = 12). IER-NH$_4^+$ was significantly affected by treatment and date in 2011-12 (P < 0.01) and by date in 2012-13 (P < 0.001). IER-NO$_3^-$ showed a significant response to treatment and date in both years. The effect of N fertilization on IER-NO$_3^-$ was dependent upon sampling date (P < 0.001).
Figure 3-5 Additive interaction term, calculated as observed – expected, for five key soil measurements (mean ± 95 confidence interval). Solid circles denote measurements made in 2011-12, open circles denote those collected in 2012-13. No values deviate significantly from zero, suggesting that the interactions are additive.
Figure 3-6 Changes in (a) microbial biomass, (b) nitrification potential, and (c) denitrification potential as influenced by plant composition, fertilization, clipping, and fertilization × clipping over time. Bars ± 1 standard error of the mean (n = 8). All three response variables differed significantly by treatment and sampling date. The effect of clipping on nitrification potential was dependent upon sampling date, as was the effect of N fertilization on denitrification potential in 2012-13 (P < 0.05).
Figure 3-7 Changes in (a) AOB amoA gene abundance, (b) AOA amoA gene abundance, and (b) the ratio of AOB to AOA by treatment (native community, invaded community, fertilization, clipping, and fertilization × clipping) and sampling date. Bars ± 1 standard error of the mean (n = 8). Treatment did not affect amoA gene abundance or the ratio of AOB/AOA. All variables were significantly affected by sampling date (P < 0.001).
4 Soil microbial community composition is insensitive to multiple global change factors in experimental grasslands

4.1 Abstract

Soil microorganisms consume and produce trace gases, release and immobilize nutrients, and regulate vegetation dynamics. As such, microbial communities are an essential component of ecosystem response to global change. While a number of studies have sought to understand the effects of single global change factors on microbial community composition and diversity, little is known about how these factors interact. Our study used experimental grassland plots to measure the effect of three primary global changes – invasion by exotic annual grasses, N fertilization, and aboveground vegetation removal (“clipping”, to simulate one component of cattle grazing) – on soil microbial community composition and diversity. We analyzed 16s rRNA using the Illumina MiSeq platform to compare bacterial and archaeal communities across treatments three (N fertilization and clipping) to five years (plant communities) after establishment. Previous work at our site showed that each individual global change factor stimulated a change in the N cycle, although the direction and magnitude across treatments varied. Given that microorganisms are intrinsically linked to N availability and rates of cycling, we expected concurrent shifts in microbial community composition and diversity across treatments. Surprisingly, bacterial and archaeal communities showed very little variation in composition and diversity, suggesting that soil microbial communities are insensitive to exotic plant invasion, N deposition, and defoliation in Mediterranean grasslands, which experience large seasonal changes in temperature, precipitation, and soil N availability. Indeed, these global environmental changes may not produce large enough effects to induce shifts in a microbial community that is already adapted to fluctuating environmental conditions.
4.2 Introduction

Positioned at the interface of the atmosphere-plant-soil system, soil microorganisms drive biogeochemical cycling from the micro- to the global-scale (Wachinger et al., 2000; Falkowski et al., 2008; Madsen, 2011). In their quest to obtain energy and nutrients, microbial communities decompose organic matter (Sinsabaugh et al., 1991), (im)mobilize and transform nitrogen (N; Davidson et al., 1990; Schimel & Bennett, 2004) and phosphorus (P; Frossard et al., 2000), and consume and produce critical trace gases (e.g., CO₂, CH₄, N₂O; Conrad, 1996; Schimel & Guldèged, 1998). Additionally, soil microorganisms influence net primary production (NPP), plant community composition, and the carbon (C) storage capacity of ecosystems (Schimel & Schaeffer, 2012) by regulating the availability of nutrients (van der Heijden et al., 2008) and forming (mutualistic and pathogenic) relationships with plants (Hartnett & Wilson, 1999; Klironomos et al., 2000; Bever, 2003; Reynolds et al., 2003; Raaijmakers et al., 2009).

Because of their ability to affect biogeochemical processes and vegetation dynamics, soil microbial communities likely play an important role in mediating ecosystem response to global change (Swift et al., 1998; Talbot et al., 2008; Zak et al., 2011).

Most primary global change factors, such as N deposition and exotic plant invasion, involve changes to abiotic (e.g., soil moisture, inorganic N availability) and biotic (e.g., plant composition) variables that can directly and indirectly affect soil microbial community composition and diversity. Microorganisms vary in their functional traits (Schimel & Schaeffer, 2012; Martiny et al., 2013), metabolic requirements (Falkowski et al., 2008) and response to stress (Schimel et al., 2007); as a result, environmental change can favor certain groups of microorganisms over others, with possible implications for ecosystem function. For example, changes in C and N availability can affect the relative abundances of copiotrophic (r-selected; e.g., Actinobacteria, Bacteriodetes and Proteobacteria) and oligotrophic taxa (K-selected; e.g., Acidobacteria and Verrucomicrobia; Fierer et al., 2007; Ramirez et al., 2012; Fierer et al., 2012). Additionally, ammonia-oxidizing bacteria (AOB) generally respond more significantly to increases in NH₄⁺ availability than ammonia-oxidizing archaea (AOA; Di et al., 2010), resulting in higher AOB/AOA ratios with elevated N supply (Shen et al., 2011). While variations in microbial physiology at the population level provides the opportunity for differential responses to occur, it is still unclear whether this necessarily translates into broad-scale alterations of microbial community composition under all global change scenarios.

To better understand microbial responses to global change, a growing number of experiments have monitored community composition under single (Frey et al., 2004; Zhang et al., 2005; Batten et al., 2008; Ingram et al., 2008; Lesaulnier et al., 2008; Campbell et al., 2010; Ramirez et al., 2012; Coolen et al., 2013) and combined (Horz et al., 2004; Chung et al., 2007; Castro et al., 2010; Gray et al., 2011) treatments. Of these studies, many have reported significant responses in soil microbial community composition (e.g., Shade et al., 2012); however, others have shown resistance to change.
(Waldrop & Firestone, 2006; Cruz-Martínez et al., 2009; Allison et al., 2010; Marshall et al., 2011; Shade et al., 2012). For example, bacterial and fungal composition measured using phospholipid fatty acid analysis (PLFA) was insensitive to five years of altered plant functional identity in a grassland ecosystem (Marshall et al., 2011). Furthermore, the composition of fungi subjected to warming in a boreal forest also showed no significant differences compared to the unwarmed control (Allison et al., 2010). Variations in observed sensitivity may be dependent upon the global change factor, ecosystem type (Waldrop & Firestone, 2006; Shade et al., 2012; Griffiths & Philippot, 2013), intensity and duration of the treatment, and physiological plasticity of the resident microorganisms (Shade et al., 2012). Continued research that focuses on the response of soil microbial communities to global change factors and their interaction will help build a more comprehensive understanding of what governs microbial community sensitivity to change.

We examined soil bacterial community composition in experimental Mediterranean-type grasslands subjected to multiple global change manipulations. Treatments consisted of altered plant community composition (invaded and uninvaded grasslands), N fertilization, and defoliation, which represent three major global environmental pressures affecting grasslands worldwide (Sala et al., 2000). Nitrogen fertilization and defoliation were manipulated in a factorial manner to determine whether their interaction affected soil microbial communities in an unexpected way. Given that changes in N cycling and availability had previously been demonstrated to occur in response to treatments at our site (Chapter 2), we sought to understand whether there were any accompanying changes in soil microbial community composition.

4.3 Methods

This study was conducted at an experimental site located in Davis, CA. Climatic conditions and soil classification at this site, as well as the experimental design, are described in chapter two of this dissertation.

4.3.1 Sample Collection, DNA Extraction, and Quantification

Soil samples were collected from five treatments (Native, Invaded, Fertilized, Clipped, Fertilized + Clipped [‘Both’]) in April 2013. Each treatment had eight replicates, where one replicate was a composite of five soil cores (2.4 cm diameter x 15 cm deep), each taken at random locations from within the plot. Composite field-moist samples were sieved (2 mm) and stored at -20 °C prior to analysis. Microbial DNA was extracted from 0.20 – 0.30 g of soil using a MO BIO PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) following the manufacturer’s instructions. Extracted DNA was quantified using a Quant-iT PicoGreen ds DNA Assay Kit (Life Technologies, Carlsbad, CA, USA) and diluted to 1 ng uL⁻¹ DNA.
4.3.2 Barcoded Amplicon Sequencing on the Illumina MiSeq Platform

DNA extracts (10 µL per sample) were shipped overnight on dry ice to Argonne National Laboratory (Lemont, Illinois, USA) for PCR amplification and sequencing using the Illumina MiSeq2000 platform. The PCR amplification of 16S rRNA was conducted on triplicate samples using primers (F515/R806 barcoded) targeting the V4 region (Caporaso et al., 2012). Cycle conditions followed the Earth Microbiome Project protocol (Caporaso et al., 2011): 3 min @ 94 °C (denaturation); 35 cycles of 45 s @ 94 °C, 60 s @ 50 °C, 90 s @ 72 °C (amplification); and 10 min @ 72 °C (extension). Each 25 µL reaction mixture included 10 µL 5 PRIME Hot Master Mix (5 PRIME, Inc., Gaithersburg, MD, USA), 0.2 µM forward and reverse primers, 1 µL template DNA, and 13 µL PCR grade water (MoBio Laboratories, Inc., Carlsbad, CA, USA). After amplification, the triplicate PCR products were combined for each sample into a single volume, which was subsequently run on an agarose gel and quantified using a Quant-iT PicoGreen ds DNA Assay Kit. Equal amounts of amplicon from each sample were pooled, purified using a MoBio UltraClean PCR Clean-Up Kit, and sequenced using the Illumina MiSeq2000 platform.

4.3.3 Sequence Analysis

We used default parameters in Quantitative Insights into Microbial Ecology (QIIME; Caporaso et al., 2010) for quality control. Specifically, reads were excluded if there were more than three consecutive low quality base calls, if less than 75% of the read length was consecutive high quality base calls, if a Phred score was below three, if one or more ambiguous calls were present, or if the length was less than 75 bases (Bokulich et al., 2013). After forward and reverse reads were joined and demultiplexed, we picked operational taxonomic units (OTU) at 97% similarity using open reference UCLUST against the 13 8 release of the Greengenes database. Reads that did not match any sequences in the database were clustered de novo.

Prior to conducting diversity analyses, OTUs were rarefied to 12,000 reads per sample. Alpha diversity was measured using a variety of approaches: Shannon diversity index, Chao1 richness, Simpson’s D, and Faith’s phylogenetic diversity. Diversity indices were correlated with select soil characteristics (NH\textsubscript{4}\textsuperscript{+}, NO\textsubscript{3}\textsuperscript{-}, microbial biomass [SIR], pH, and soil moisture) using Spearman’s rank correlation. Beta diversity was visualized using principle coordinate analysis (PCoA) from unweighted and weighted Unifrac distances; analysis of similarities (ANOSIM) and perMANOVA were used to determine if beta diversity differed significantly among treatments. Unifrac uses the overlap in branch lengths to estimate phylogenetic distance, or relatedness, between pairs of bacterial communities (Lozupone & Knight, 2005). Treatment effects on the relative abundances of select taxa were examined using analysis of variance (ANOVA, \(\alpha = 0.05\)). Interactions between N fertilization and clipping were determined by comparing expected additive effects to observed effects (same methods as Chapter 2). Models were checked for normality using a Shapiro-Wilk test and were log transformed when necessary. In addition, we utilized a distance-based redundancy analysis (db-RDA; Legendre &
Anderson, 1999) to determine which soil characteristics best explained the variation in microbial community composition across treatments.

4.4 Results

Sequencing of 40 samples (5 treatments with 8 replicates each) resulted in approximately 2.1 million reads. After quality control, 1.1 million reads remained, averaging 29,441 reads per sample (average sequence length = 253 bp). All samples were subsampled to 12,000 reads prior to running diversity analyses (Figure 4-1).

The dominant phyla were similar across all treatments and replicates (Figure 4-2), with Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, and Gemmatimonadetes accounting for approximately 80% of sequences from each sample (range 80 – 82%). There were 26 additional phyla that were present in all soils but at lower abundances, and 10 other low-abundance phyla that were not consistently present across soils (Figure 4-3). The interaction of N fertilization and clipping affected the relative abundances of individual bacterial and archaeal taxa more often than main treatment effects (Table 4-1; Figure 4-4). Of the top 10 most abundant phyla (Fig. 2), two showed marginally significant responses to main treatment effects: Proteobacteria (P = 0.07) were reduced and Verrucomicrobia (P = 0.09) were increased in clipped relative to unclipped treatments. In contrast, neither N fertilization nor clipping individually affected Actinobacteria, Planctomycetes, Chloroflexi, and Crenarchaeota; however, these phyla responded antagonistically to the interactions of N fertilization and clipping (N fertilization x Clipping, P < 0.05).

The relative abundances of nitrogen-cycling microorganisms were also compared. Nitrosomonadaceae, a family containing ammonia-oxidizing bacteria, was significantly reduced in invaded relative to native treatments (P < 0.05, Figure 4-5) and demonstrated a marginally significant reduction with clipping (P = 0.09). In addition, Nitrososphaeraceae, a family of ammonia-oxidizing archaea, was affected by the interaction of N fertilization and clipping (Table 4-1), while Nitrospirae, a phylum of nitrite oxidizing bacteria, was also detected but showed no significant response to treatment.

Bacterial and archaeal community diversity and overall composition were unaffected by the global change factors manipulated in our study. Specifically, community richness, evenness, and phylogenetic diversity were statistically indistinguishable among treatments, and did not correlate with any of the soil characteristics listed in Table 4-2. Principle Coordinates Analysis of the UniFrac distance illustrated a lack of distinct clustering by treatment (Figure 4-6), which was confirmed using ANOSIM (R = 0.04, P = 0.09, permutations = 999), perMANOVA (Pseudo-F statistic = 1.01, P = 0.23, permutations = 999), and ADONIS (R2 = 0.11, P = 0.23, permutations = 999). Additionally, select soil characteristics (available N, soil pH, and soil moisture) failed to explain any variation in microbial community composition (P = 0.46, permutations = 99).
4.5 Discussion

Using a well-replicated field experiment, we asked whether microbial community composition was affected by changes in plant community composition, aboveground vegetation removal (‘clipping’), and N fertilization in a Mediterranean-type grassland. The vegetation treatments in our study represented grassland communities native to California, and grassland communities (native and naturalized species) invaded by two exotic species, Aegilops triuncialis and Elymus caput-medusae. Invaded communities were subjected to clipping and N fertilization in order to simulate aboveground vegetation removal via livestock grazing and N deposition, which are two global change factors that affect California grasslands simultaneously. Our results demonstrate that microbial composition was largely insensitive to each treatment, suggesting a great deal of resistance or resilience in these soil microbial communities.

We found no differences in broad-scale microbial community composition or diversity in invaded compared to native plant communities. This was unexpected, given that changes in some abiotic soil characteristics (e.g., inorganic N, water availability, temperature) were observed, which could affect soil microbial community composition (Wallenstein et al., 2006; Ramirez et al., 2012). We did find that the relative abundance of Nitrosomondaceae, a family of ammonia-oxidizing bacteria, was reduced with invasion, which is consistent with reductions in nitrification potential in these treatments. However, the relative abundance of Nitrosomonadaceae failed to significantly explain variation in potential rates of nitrification (Linear regression; $R^2 = 0.01$, $P = 0.28$; $N = 8$); this could be due to differences in per cell nitrification rates and varying contributions of other ammonia oxidizing microorganisms, such as ammonia oxidizing archaea.

There are at least three reasons that could explain the lack of response in microbial community composition to invasion. First, it is possible that the effects of invasion were diluted, given that we compared native communities to mixed communities comprised of native, naturalized, and invasive plants. Second, the treatments may not have been established long enough to induce changes in microbial community composition. Potthoff et al., (2006) found that soil microbial communities were similar between exotic annual and restored native perennial grasslands, and suggested this had to do with the relatively recent establishment (four years) of the perennial bunchgrasses. Finally, microbial communities at our site may be resistant or resilient to global change.

Our results agree with a number of studies that have found small or insignificant effects of plant identity on microbial community composition compared to other variables such as soil type (Buyer & Roberts, 2002; Singh et al., 2007) or plant productivity (Marshall et al., 2011; De Deyn et al., 2011), but contrast with studies that have demonstrated significant responses in microbial composition to exotic plant invasion (Belnap & Phillips, 2001; Hawkes et al., 2005; Batten et al., 2006; 2008; Zhang et al., 2013). Together, the inconsistency in effects between our study and others suggest that the impact of plant invasions on soil microbial communities may be dependent upon site,
functional differences between the native and invasive species (Vilà et al., 2011), and duration of the invasion process (Kulmatiski & Beard, 2011).

In contrast to other studies that tend to focus on the immediate or short-term response of microbial communities to defoliation (Mawdsley & Bardgett, 1997; Bardgett et al., 1998), we observed minor effects of three years of clipping on microbial community composition. Indeed, microbial diversity remained unaffected, although clipping reduced the abundance of Proteobacteria and increased Verrucomicrobia, which are copiotrophic and oligotrophic taxa, respectively (Fierer et al., 2007; Nemergut et al., 2008). There are a number of mechanisms by which defoliation can influence soil microbial communities, including changes in soil temperature, moisture, and nutrient and C availability (Bardgett et al., 1998; De Graaff et al., 2010). Previous work at our site demonstrated reduced N availability in clipped treatments (Chapter 2), which likely contributed to the relative increase in Proteobacteria and decrease in Verrucomicrobia. Despite these rather minor changes, our results suggest that microbial community composition of invaded grasslands is largely insensitive to aboveground vegetation removal.

Our results that N fertilization did not significantly change in microbial community composition or diversity were surprising. Elevated N supply is a particularly well-studied global change factor, and has been shown to reduce microbial diversity and alter composition by favoring copiotrophic phyla such as Actinobacteria and Firmicutes (Nemergut et al., 2008; Campbell et al., 2010; Ramirez et al., 2012; Fierer et al., 2012; Coolon et al., 2013). We had previously shown drastic changes in N availability (up to 504% increase) and potential rates of cycling with fertilization (Chapter 2); as such, we expected to observe increases in the relative abundances of copiotrophic taxa, leading to overall shifts in community composition and reduced diversity. Instead, our results indicate that changes in N availability and rates of cycling occur independently of changes in broad-scale community composition, and so likely represent microbial physiological response to change.

The observed insensitivity of microbial communities to each treatment may be due to the large seasonal variability at our site. California grasslands experience a Mediterranean climate that is marked by strong seasonal changes in soil N availability, moisture, and temperature – changes that are large compared to those observed between treatments (Figure 4-7). Microorganisms within this environment should be adapted to cope with significant seasonal changes in edaphic variables – for example, through individual metabolic capabilities, gene expression, and mixotrophy (Shade et al. 2012). As a result, the community is able to withstand comparatively minor effects of treatment without changing composition. Indeed, Waldrop & Firestone, (2006) found that microbial communities in grassland soils were insensitive to change when transplanted for two years into an oak-dominated environment, and concluded that this was likely because the new environmental conditions were not outside the “life history” of the microbial community. Cruz-Martinez et al., (2009) also found that seasonal changes in microbial composition trumped the effects of altered precipitation at any single sampling date in a
coastal grassland, lending further evidence to the idea that microorganisms in Mediterranean soils have the physiological capability to withstand compositional changes, at least in response to experimental global change treatments. Differences in background variation experienced by resident microorganisms may explain the discrepancy between our results and other studies that reported significant changes in response to global change factors.

Experimental limitations may have also contributed to the lack of response observed in microbial communities. It is possible that the effects of fertilization and clipping (which can be considered as “pulse” events) were transient and disappeared before our sampling effort. If this were the case, the microbial community would actually be resilient, not resistant to change. In addition, the effect of treatment may be dependent upon time, so sampling at more than one time point would increase the likelihood of picking up compositional differences within microbial communities. However, the influence of treatment on microbial activity (potential nitrification and denitrification) and edaphic variables was rarely dependent upon time, so this possibility seems unlikely (Chapter 2). Finally, it is possible that we would have captured ecologically relevant changes in microbial composition by sequencing key functional genes, such as amoA (ammonia oxidation) or nirS and nirK (denitrification), instead of using a universal gene such as 16 rRNA.

Overall, microbial communities in Mediterranean grassland soils appear to be insensitive to multiple global change factors. While individual taxa showed some variation by treatment, overall community composition and diversity did not change. The lack of sensitivity observed in our study may be a result of the historical exposure of microorganisms in these soils to large seasonal variations in key edaphic factors. These results, coupled with prior work at this site demonstrating large changes in N cycling by treatment (Chapter 2), suggest that microbial functioning may be decoupled from composition in these soils.

4.6 References


Batten KM, Scow KM, Davies KF, Harrison SP (2006) Two Invasive Plants Alter Soil


Mawdsley JL, Bardgett RD (1997) Continuous defoliation of perennial ryegrass (Lolium perenne) and white clover (Trifolium repens) and associated changes in the composition and activity of the microbial population of an upland grassland soil. *Biology and Fertility of Soils, 24*, 52–58.


### 4.7 Tables

Table 4-1 Main and interactive effects of N fertilization and clipping on the top 10 dominant phyla across samples and nitrifying taxa. Significant (P < 0.05) and marginally significant (P < 0.1) values are highlighted in bold.

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Table 4-2 Spearman’s rank correlations ($r$) between diversity indices and soil characteristics ($n = 39$). No significant correlations were observed. SIR = substrate induced respiration (measure of microbial biomass); SM = soil moisture.

<table>
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4.8 Figures

Figure 4-1 Rarefaction curve of OTU richness by treatment (purple – native community; green – invaded community; orange – fertilized; blue – clipped; red – fertilized x clipped). Samples were rarefied to the lowest common denominator: 12,000 reads.
Figure 4-2 Relative abundances (frequency) of the dominant bacterial phyla across treatments. “Other” indicates the combined relative sequence abundance of the additional, rare phyla (28 phyla).
Figure 4-3 Rank abundance curve. Mean proportional abundance of each phylum by treatment.

Figure 4-4 Additive interaction term, calculated as observed – expected (mean ± 95 confidence interval), for select taxa that showed significant interaction terms based on a two-way ANOVA. All values fall below zero, suggesting that the interactions between N fertilization and clipping are antagonistic.
Figure 4-5 Relative abundances (percent) of key taxa involved in nitrification. *Nitrosomondaceae* is a family containing ammonia-oxidizing bacteria, *Nitrososphaeraceae* is a family containing ammonia-oxidizing archaea, and *Nitrospirae* is a phylum containing nitrite-oxidizing bacteria.
Figure 4-6 Principal coordinate analysis of 16S community composition based on weighted UniFrac distance matrices. Colors are coded by treatment (purple – native community; green – invaded community; orange – fertilized; blue – clipped; red – fertilized x clipped). Together, the first two principle coordinates explain 42.55% of the variation in microbial community composition. Unweighted UniFrac (not shown) demonstrated qualitatively similar patterns.
Figure 4-7 Seasonal (black bars) and treatment (gray bars) variation in select soil characteristics. VWC = volumetric water content, GWC = gravimetric water content. Seasonal variation is larger than treatment variation for all displayed characteristics.
5 Conclusion

The overall aim of my dissertation was to quantify the response of soil N cycling to single and combined effects of three principle global change factors affecting Mediterranean grasslands today: exotic plant invasion, aboveground vegetation removal (one component of cattle grazing), and N deposition. Because soil microorganisms largely regulate N cycling, my dissertation also measured the influence of each global change factor on soil microbial community composition, diversity, and the relative abundance of a functional gene (amoA) responsible for the first and rate-limiting step of nitrification.

In the first chapter, I found that soils invaded by A. triuncialis and E. caput-medusae had significantly lower inorganic N availability and potential rates of nitrification and denitrification than native communities, and that reductions were often associated with lower microbial biomass and volumetric soil moisture in surface soil. This observed disparity in N cycling between native and invaded soils could have implications for restoration, given that native perennials are often used to re-vegetate sites after the removal of invasive species; if legacies of altered N cycling persist, the competitive edge may be shifted away from natives and successful re-vegetation may be hindered. In contrast, soil N cycling did not differ between invaded and naturalized plant communities, suggesting that invasion of naturalized grasslands by A. triuncialis and E. caput-medusae may not induce changes in soil N cycling, and that alterations in N availability are not a mechanism by which these particular exotic species invade.

In the second chapter, invaded grasslands were subjected to factorial replication of aboveground vegetation removal and N fertilization. Cattle grazing and mowing are often used to control invasive plant populations and given that changes in N availability may affect re-vegetation of native plants (as has been observed in other species), it is important to understand how these management tools influence N dynamics of invaded sites. In this case, removal both exacerbated (e.g., N availability and nitrification) and alleviated (e.g., microbial biomass and denitrification) the belowground impacts of A. triuncialis and E. caput-medusae invasion. Importantly, the response of denitrification potential and active microbial biomass seemed to be dependent upon soil water availability, with wetter years allowing for a larger increase in denitrification potential and microbial biomass.
Not surprisingly, fertilization with NH$_4$NO$_3$ consistently increased nitrification potential and soil NO$_3^{-}$ concentrations of invaded grasslands. However, NH$_4^{+}$ concentrations remained unaffected, demonstrating that NH$_4^{+}$ is quickly taken up by plants, immobilized by microorganisms, nitrified to NO$_3^{-}$, or some combination of these processes. Dramatic increases (443 – 504%) in NO$_3^{-}$ were observed, suggesting atmospheric deposition of inorganic N could accelerate NO$_3^{-}$ leaching into groundwater and potentially affect the water quality and ecology of adjacent aquatic ecosystems. This is even more likely given that denitrification potential was concurrently reduced, possibly because of C limitation or reductions in the abundance of fungi that denitrify.

It is important to go beyond measuring ecosystem response to single global change factors and consider whether vegetation removal and N fertilization interact to produce unanticipated effects when co-occurring on the landscape. I hypothesized that vegetation removal and fertilization would interact antagonistically; however, results from this experiment show that they always interact in an additive way and thus their combined effects can be predicted based on single factor studies.

Finally, my second and third chapters show that soil microbial community composition and diversity were unaffected by each global change factor. This observed insensitivity may be due to the large seasonal variability at our site, which is typical of a Mediterranean-type climate. Indeed, seasonal changes in key soil variables such as inorganic N, moisture, and temperature, are much larger than those observed among treatments. Consequently, the resident microbial community may be able to withstand the comparatively minor effects of treatment through malleable metabolic capabilities, gene expression, or mixotrophy. These results have important implications for our understanding of microbial controls on N cycling in Mediterranean grasslands.

Overall, my dissertation demonstrated that microbial community composition was insensitive to exotic plant invasion, aboveground vegetation removal, and N fertilization. However, shifts in total active microbial biomass, N availability, and potential rates of N cycling were observed in response to each treatment. These results challenge the idea that microbial composition, at least as measured by qPCR of the amoA gene and next-generation sequencing of 16s rRNA, determines N cycling dynamics; instead they point towards a more nuanced relationship where microbial activity is decoupled from community composition.