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ORIGINAL PAPER

# Invasive plants decrease microbial capacity to nitrify and denitrify compared to native California grassland communities

**Chelsea J. Carey · Joseph C. Blankinship · Valerie T. Eviner · Carolyn M. Malmstrom · Stephen C. Hart**

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**Abstract** Exotic plant invasions are a major driver of global environmental change that can significantly alter the availability of limiting nutrients such as nitrogen (N). Beginning with European colonization of California, native grasslands were replaced almost entirely by annual exotic grasses, many of which are now so ubiquitous that they are considered part of the regional flora ("naturalized"). A new wave of invasive plants, such as *Aegilops triuncialis* (Barb goatgrass) and *Elymus caput-medusae* (Medusahead), continue to spread throughout the state today. To determine whether these new-wave invasive plants alter soil N dynamics, we measured inorganic N pools, nitrification and denitrification potentials, and possible mediating factors such as microbial biomass and soil pH in experimental grasslands comprised of *A. triuncialis* and *E. caput-medusae*. We compared these measurements with those from experimental grasslands containing: (1) native annuals and perennials and (2) naturalized exotic annuals. We found that *A. triuncialis* and *E. caput-medusae* significantly reduced ion-exchange resin estimates of nitrate ($NO_3^-$) availability as well as nitrification and denitrification potentials compared to native communities. Active microbial biomass was also lower in invaded soils. In contrast, potential measurements of nitrification and denitrification were similar between invaded and naturalized

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communities. These results suggest that invasion by *A. triuncialis* and *E. caput-medusae* may significantly alter the capacity for soil microbial communities to nitrify or denitrify, and by extension alter soil N availability and rates of N transformations during invasion of remnant native-dominated sites.

**Keywords** *Aegilops triuncialis* · *Elymus caput-medusae* · Exotic plants · Soil nitrogen availability · Plant invasion · Protozoa

## Introduction

Invasive species occupy many ecosystems worldwide and are considered an important driver of global environmental change (Vitousek et al. 1997; Tylianakis et al. 2008). Understanding the mechanisms by which exotic plants invade, and the ecosystem effects of invasion, are therefore a major priority for invasion ecology and global change research. For decades, the mechanisms (e.g., the diversity-invasibility relationship) and aboveground effects of plant invasion have been extensively studied (reviewed in Mack et al. 2000; Richardson and Pyšek 2008). With a growing appreciation for plant-soil interactions, research has also focused on understanding how key soil processes such as nutrient cycling respond and feed back to influence invasive plant populations (Ehrenfeld 2003; Liao et al. 2008; Gaertner et al. 2014).

Soil nitrogen (N) cycling is a process of particular interest because N is a critical limiting nutrient for plant growth (Vitousek and Howarth 1991) and can have considerable effects on net primary production (NPP), plant community composition (LeBauer and Treseder 2008), water eutrophication, and other ecosystem services. Exotic plant invasion can influence N cycling if traits of the invasive species differ from those of the displaced species. For example, invasive plants can alter N cycling by modifying rooting structure and N uptake patterns, nutrient use efficiency and litter quality, or phenology (Ehrenfeld 2003). Alterations to the soil microclimate (Eviner and Chapin 2003), disturbance regimes (Mack and D'Antonio 1998), bacterial and fungal community composition (Stefanowicz et al. 2016; McLeod et al. 2016), and soil food webs (Clarholm 1985) can also mediate the effects of invasive plants on soil N cycling. Protozoa, for example, are unicellular eukaryotes that can provide top-down control on N cycling by grazing on bacteria. Although not always the case (Meisner et al. 2014), these modifications can lead to plant-soil feedbacks that facilitate the persistence of invasive species (Kulmatiski 2006; Eviner et al. 2010) and potentially inhibit successful re-vegetation of desired species (D'Antonio and Meyerson 2002; Suding et al. 2004a; Corbin and D'Antonio 2011a).

Plant invasion frequently elevates soil N availability by increasing the rates of key N cycling processes, such as N mineralization and nitrification, though the magnitude and direction vary considerably across studies (Liao et al. 2008; Castro-Díez et al. 2014). For instance, McLeod et al. (2016) found that four exotic plant species, *Bromus tectorum*, *Centaurea stoebe*, *Euphorbia esula*, and *Potentilla recta* increased the abundance of ammonia-oxidizing bacteria, and in three of these cases this resulted in rapid increases in soil $NO_3^-$ compared to communities of native plant species. However, the opposite effect is sometimes observed, as in Dassonville et al. (2011), where it was demonstrated that invasion by *Fallopia* spp. lowered potential rates of soil nitrification. Variability among results highlights the need for continued experimental research that unravels the complexity of belowground responses to invasion (Kumschick et al. 2015) by controlling for confounding variables such as pre-invaded site dynamics (Suding et al. 2004b; Kulmatiski et al. 2006) and time since plant establishment (Strayer et al. 2006; Yelenik and D'Antonio 2013). Field-based experiments where plant communities are seeded in a "common garden" provide one promising way to control for these confounding variables and mechanistically capture changes to the soil with exotic plant invasion.

In this study, we compare soil N cycling and microbial biomass across three experimental plant communities that had been established for five years in California. These communities were comprised either of (1) a mix of native species commonly used in California grassland restoration projects, (2) a mix of naturalized exotics that have been dominant in California's grasslands for over 250 years, or (3) two new-wave invasive grasses (*Aegilops triuncialis* and *Elymus caput-medusae*) that are actively invading native and naturalized grasslands in California. Both of these new-wave invasive species, which are winter annuals native to Mediterranean Europe, can significantly

reduce plant diversity and forage production in rangelands (Jacobsen 1929; reviewed in Nafus and Davies 2014). *Aegilops triuncialis* was introduced to the US in the early 1900s (first sighting in 1914; Peters et al. 1996) and is currently spreading throughout California, Oregon, and Nevada (D'Antonio et al. 2007). *Elymus caput-medusae* was introduced in the late 1800s to Oregon (Bossard et al. 2000), infests at least 1 million hectares in the western US, and in 2004 was estimated to increase its areal extent by 12% each year (Duncan et al. 2004).

Previous work has shown that both *A. triuncialis* and *E. caput-medusae* have litter qualities that can reduce decomposition and slow subsequent release of available N into the soil, potentially contributing to a self-sustaining feedback loop (Eviner et al. 2010; Baty 2012). *Aegilops triuncialis* litter, for instance, has high carbon to nitrogen (C:N) and lignin to N mass ratios (Eviner 2004; Drenovsky and Batten 2007). In addition, *E. caput-medusae* litter contains much silica (Bovey et al. 1961; Swenson et al. 1964), which can slow decomposition (Kaneko and Salamanca 1999) by limiting microbial metabolism of plant cell wall constituents (i.e., cellulose, lignin; Street 1974). Although net N mineralization does not scale linearly with litter mass loss (Parton et al. 2007; Manzoni et al. 2008), it is possible that by retarding decomposition, *A. triunicalis* and *E. caput-medusae* slow N release from litter, reduce inorganic N pools, and alter subsequent N transformations in invaded soils. At the same time, the delay in peak spring growth typical of *A. triuncialis* and *E. caput-medusae* (Peters et al. 1996; Drenovsky and Batten 2007; Young and Mangold 2008) leads to a late-spring peak in plant N uptake (compared with earlier peaks for both naturalized and native communities; Baty 2012). This could contribute to prolonged plant uptake of inorganic N in invaded communities, thus removing comparatively more N from the soil over time. Consequently, we hypothesized that *A. triuncialis* and *E. caput-medusae* would reduce N availability, potential rates of nitrification and denitrification, and microbial biomass relative to those in native plant communities (Hypothesis 1), and/or communities of naturalized exotics (Hypothesis 2). To test these hypotheses, we measured soil ammonium ($NH_4^+$) and nitrate ($NO_3^-$), potential rates of nitrification and denitrification, and total active microbial biomass (substrate induced respiration) seasonally in experimental semi-arid grassland communities that had been established for five years. In addition, we measured the abundance of ciliates, flagellates, and amoeba (collectively protozoa) in April to determine whether shifts in N transformations appear to be linked with changes in these bacterial grazers.

## Methods

### Site description and experimental design

The experimental site, located in Davis, California (38°32′45.52″N, 121°47′05.37″W), experiences a Mediterranean-type climate, with hot dry summers and cool wet winters. In 2011, summer (June–September) and winter (December–February) mean daily maximum air temperatures were 32.2 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. In 2011 and 2012, annual precipitation was 173 and 216 mm, respectively. Long-term (1981–2010) averages for temperature and precipitation in Davis, California are 16.2 °C and 498 mm, respectively (Arguez et al. 2012). 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 the fall of 2007, experimental grassland communities were established in a fallow field, which prior to 1985 had been cultivated for agriculture. Plots measuring 1.5 × 1.5 m each were arranged in a randomized complete block design (8 blocks total) and seeded as communities of: (1) native species (*Bromus carinatus*, *Elymus glaucus*, *Elymus triticoides*, *Acmispon americanus*, *Lupinus bicolor*, *Stipa pulchra*, *Poa secunda*, and *Festuca microstachys*); (2) naturalized annual species (*Avena fatua*, *Bromus hordeaceus*, *Festuca perennis*, and *Trifolium subterraneum*); or (3) new-wave invasive annual species (*Aegilops triuncialis* and *Elymus caput-medusae*). Experimental communities were seeded to reflect a mix of the most common species in each of these community types. In California grasslands, composition varies greatly from year to year, with dominant species in one year becoming relatively rare in another

(Hobbs and Mooney 1995; Hobbs et al. 2007; Eviner 2016). Our goal in planting the treatments was to introduce key common species of that particular community type, but to allow the relative proportion of these species to vary by treatment and over time.

Prior to seeding, germination of the existing seed bank was stimulated through irrigation, and plants that germinated were treated with the non-selective herbicide glyphosate. In each 1.5 × 1.5 m plot, a total of 139 g of seed was added, with an equal proportion of each species by weight in the mixture. Seed source is provided in Online Resource 1. Plant species were allowed to vary in abundance (Online Resource 1); however, non-planted species were weeded periodically. We assessed species percent cover during the spring of each year, at the time of peak flowering (of the late-season species). Here, vegetation cover in the inner 1 $m^2$ area of each plot was assessed using a modified Daubenmire approach with 10 cover classes (0–5, 5–20, 20–30, 30–40, 40–50, 50–60, 60–70, 70–80, 80–90, 90–100%). Because the same observer assessed all plots across all years, we were confident in the consistency of these smaller cover class bins, which allowed for greater sensitivity to assess changes in cover (Bureau of Land Mangement 1996; Bonham 2013). Native, naturalized, and invaded plant communities were distinct in plant composition from the time of plot establishment through 2012 (Online Resources 2 and 3). In 2012, native, naturalized, and invasive cover of native plots averaged (respectively): 70, 10, and 10% in native plots; 0, 40, and 50% in naturalized plots; 0, 0, 90% in invaded plots. During the year of soil collection, the perennial plant *Elymus glaucus* dominated native plant communities (Online Resource 1), and N-fixing species (*Acmispon americanus*, *Lupinus bicolor*, *Trifolium subterraneum*) were absent or nearly absent from all treatments (minimizing their effect on N dynamics at the time of sampling).

## Sample collection

Semi-arid, Mediterranean-type ecosystems experience strong seasonality in temperature and especially precipitation, thus the relative effects of plant community composition on soil properties may vary considerably throughout the year. Hence, we sampled in April 2011 and four additional times throughout 2011–2012 during periods representative of the major seasons (October—warm and wet; January—cool and wet; April—warm and wet; July—hot and dry). At each sampling date, we took five mineral soil cores (1.9 cm diameter × 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 the <2 mm fraction was retained and stored at 4 °C until analysis. All samples were processed within 3 weeks of collection, with more time-sensitive analyses (e.g., KCl extractions of inorganic N) occurring within 48 h of sampling.

To supplement the collection and analysis of soil cores, we measured a number of in situ soil parameters. Soil temperature was measured every sampling date using a traceable metal thermometer (Control Company, Friendswood, TX, USA) at 7.5 cm depth. We also measured volumetric water content (VWC) 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 per treatment per depth). This design allowed measurement of VWC within 0–15 cm and 15–30 cm soil layers. Finally, we buried ion-exchange resin (IER) bags to estimate N availability over time (see 'Nitrogen Availability' below). Ion-exchange resin bags were prepared by adding 15 mL (approximately 8 g wet weight, 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-mesh stockings that were subsequently tied shut. One bag was buried in each plot at a depth of 7.5 cm in mineral soil (bags were inserted into an undisturbed soil profile face by digging a small access hole) and incubated for an average of 6 weeks (n = 12). After the end of the incubation period, resin bags were removed and exchanged with new resin bags (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 incubation periods; some of the removal and insertion dates corresponded with soil sampling dates.

### Soil properties

Each soil sample was analyzed for gravimetric soil moisture, total carbon (C) and N, and pH. Gravimetric soil moisture was determined by drying soil subsamples at 105 °C until constant mass. Because total carbon (C) and N were not expected to show strong seasonal variation (Binkley and Hart 1989), we ground soil subsamples to a fine powder and combined samples from individual plots over the four sampling dates for these analyses (October 2011–July 2012). These plot composites were then analyzed on an Elemental Combustion System (Costech Analytical Technologies Inc., Valencia, CA, USA). 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 min (the soil slurry was mixed continuously during measurement).

### Nitrogen availability

Soil available N was measured using two indices: instantaneous estimates of KCl-extractable inorganic N pools ($NH_4^+$-N and $NO_3^-$-N) and IER bag time-integrated estimates of in situ inorganic N availability. Measurements of inorganic N pools estimate plant-available N at one point in time, whereas IER bags approximate relative differences in N availability, in situ, over time (Binkley and Matson 1983; Binkley 1984). Prior work has found IER measurements relate well to other soil N transformations such as net N mineralization and nitrification that affect N availability in soil (Binkley and Hart 1989; Lajtha 1988).

We estimated instantaneous inorganic N pool sizes by extracting 15 g field-moist soil with 100 mL of 2 M KCl. Samples were shaken on a mechanical shaker for 1 h, filtered using pre-leached (with deionized water) 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). Ion-exchange resin bags, once removed from the field, were immediately air-dried, weighed, and placed into 125 mL flasks, to which 50 mL of 2 M KCl was added. Samples were then shaken uncovered for 1 h on an orbital shaker, filtered, and stored at −20 °C until analysis of filtrate on a Lachat AE Flow Injection Autoanalyzer.

### Potential nitrification and denitrification

Potential rates of nitrification were measured using a shaken soil-slurry method as described by Hart et al. (1994). By creating optimal conditions for (bacterial) nitrification (Ouyang et al. 2016), this 24-h incubation estimates the population size of nitrifying microorganisms in the soil, and therefore approximates in situ nitrification rates likely experienced over longer periods of time. 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 h. Sampling occurred at 2, 4, 22, and 24 h by removing a 10 mL aliquot of the suspension from each flask and centrifuging at $8{,}000 \times g$ for 8 min. This sampling scheme was deemed the most efficient approach for estimating change in $NO_3^-$ over time, given that for a variety of soils the rate of $NO_3^-$ production has been confirmed as linear from 2 h to more than 36 h (Hart et al. 1994). After centrifugation, 5 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 to check that nitrification never became limited by $NH_4^+$.

Similar to nitrification potential, denitrification potential measures the in situ denitrifying enzyme activity of soils. We measured denitrification potential using a protocol developed by Smith and Tiedge (1979). Briefly, non-limiting conditions were created by amending 50 g field-moist soil with $NO_3^-$ and labile C (0.1 g $NO_3^-$N $kg^{-1}$ soil, 1 g glucose-C $kg^{-1}$ soil, and 1 g glutamic acid-C kg $soil^{-1}$) in a 250 mL flask. The flasks were sealed with a rubber stopper and septum and 20 mL of acetylene was added to inhibit the reduction of $N_2O$ to $N_2$. The soils were incubated anaerobically on an orbital shaker (180 rpm) for 90 min; 15 mL of the headspace was sampled at 30 and 90 min. Gas samples were stored in evacuated Exetainer® until analysis for $N_2O$ production on a Shimadzu GC-2014 electron capture detector (Shimadzu Corporation, Columbia, MD, USA). Values of $N_2O$ produced were used to estimate the overall size of the denitrifying microbial community.

### Microbial biomass

Microbial biomass was estimated using two methods that provide complementary information. Substrate induced respiration (SIR) was measured at all four sampling dates from October 2011–July 2012 (n = 8 plots) and microscopic enumeration was measured only in April 2011 (n = 10 plots). By measuring $CO_2$ production, SIR estimates those microorganisms that are active and glucose-responsive (Wardle and Ghani 1995), while direct enumeration allows for the separate quantification of bacteria, fungi, and protozoa (Paul et al. 1999).

Substrate induced respiration was modified from West and Sparling (1986). Fifteen grams of field-moist soil was weighed into 250 mL flasks and 30 mL of a glucose solution (30 g glucose $l^{-1}$ $H_2O$) was added to each. The flasks were sealed with a rubber stopper and septum and placed on a shaker (180 rpm) for 2.5 h. At 0.5, 1.5, and 2.5 h, 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 and Sparling 1986).

In April of 2011, samples (10 g) were shipped overnight to Soil Food Web, Inc. (Corvallis OR, USA) where subgroups of protozoa 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). Soil Food Web Inc. also estimated total bacteria and fungi in the soil samples. Total bacteria was estimated by direct counting using a fluorescein isothiocyanate (FITC) method (Babiuk and Paul 1970) and fungal biomass was calculated by measuring the diameter and length of hyphae (Lodge and Ingham 1991). We converted bacterial and fungal biomass measurements from mg $kg^{-1}$ soil to mg C $kg^{-1}$ soil by dividing by 2, assuming that approximately 50% of microbial biomass is C (Bratbak and Dundas 1984).

### Statistical analysis

A repeated-measures split-plot analysis was used to assess significant effects of treatment ("plant community type") and sampling date on instantaneous and accumulated (IER) inorganic N pools, nitrification and denitrification potential, microbial biomass (SIR), soil water content, and temperature. In this procedure, least square means were calculated, and the model was fit using the Restricted Maximum Likelihood (REML) method. In the model, plant community type was designated as the between-plot effect (fixed), and sampling date as the within-plot effect (fixed). Block was also included as a random effect in each model. A two-way ANOVA was used to assess significant treatment differences in bacterial biomass, fungal biomass, total microbial biomass, and fungi/bacteria mass ratio, because these measurements were sampled only once. When necessary, response variables were transformed for normality and homogeneity of variance. All figures present values as untransformed. A non-parametric Kruskal–Wallis test was used to assess significant treatment differences in protozoan biomass because the assumptions of normality could not be fulfilled through transformations. We used pre-planned contrasts to make comparisons between invaded and native (Hypothesis 1) and invaded and naturalized treatment (Hypothesis 2) means when plant community type in the overall model was significant ($\alpha = 0.05$). We did not compare native to naturalized communities, as this did not address our hypotheses. When date was significant in the overall model, a Tukey HSD post hoc analysis was used to make comparisons among sampling dates. In some cases, a percent difference was calculated between two plant community types to illustrate the magnitude of effect, where the difference (%) = (((Treatment − Control)/Control) × 100).

To further visualize the soil parameters and their relationship to plant community type, we performed a principal components analysis (PCA) using a standardized covariance matrix approach. In addition to the PCA, we performed a multi-response permutation procedure (MRPP) based on Euclidian distance to test for significant differences in soil properties between plant community types (Mielke et al. 1981). The MRPP is a non-parametric procedure that tests the null hypothesis of no difference between groups. We performed MRPP in R Version 3.2.4 (R Core Team 2016) using the Vegan package 2.3-3 (Oksanen et al. 2016) and the 'Strata' function to account for block. All other analyses were performed in JMP Pro 12.0.1 (SAS Institute Inc., Cary, NC, USA).

## Results

### Invaded versus native plant communities

Nitrogen dynamics of invaded and native soils showed several notable differences. During the months of January, February, and April, invaded communities had less plant available (IER) soil $NO_3^-$ than native communities and a lower IER $NO_3^-$ to $NH_4^+$ ratio (Fig. 1a, e, IER-$NO_3^-$ Treatment × Date interaction, $P = 0.012$; IER-$NO_3^-$/$NH_4^+$ Treatment × Date interaction, $P = 0.029$, Online Resource 4). In addition, compared to native plant communities, invaded communities had significantly lower nitrification (percent change: −27.3%) and denitrification (percent change: −40.6%) potentials (nitrification $P < 0.001$, Fig. 2a; denitrification $P = 0.001$, Fig. 2b, Online Resource 4). However, in contrast, pools of extractable $NO_3^-$ did not differ between invaded and native soils (Fig. 1b), nor did pools of extractable $NH_4^+$ or IER-$NH_4^+$ (Fig. 1c, d).

Of the microbial measurements, only soil active microbial biomass was significantly affected by plant community type. Specifically, soil active microbial biomass was reduced in invaded compared to native plant communities ($P < 0.001$, Fig. 2c, Online Resource 4). Total fungi and bacteria, measured using direct enumeration, did not significantly differ between native and invaded plant communities (Fig. 3a), nor did the abundances of flagellates, ciliates, or amoeba (Fig. 3b, Online Resource 5). Similarly, other measured physical and chemical soil properties, including gravimetric water content, temperature, pH, and total C and N, remained unaffected by plant community type (Table 1, Online Resource 6). Volumetric water content at 0–15 cm depth tended to be lower in invaded compared to native soils, although this effect was only near significant ($P = 0.06$, Fig. 4a, Online Resource 6). Volumetric water content at 15–30 cm depth was also statistically indistinguishable between the two plant community types (Fig. 4b, Online Resource 6).

Based on the repeated measures split-plot analysis, ion exchange resin-$NO_3^-$ and IER-$NO_3^-$/$NH_4^+$ ratios were the only measurements where plant community type interacted significantly with sampling date (Online Resource 4). Specifically, compared to native communities, invaded soils showed diminished seasonality of IER-$NO_3^-$, such that the IER-$NO_3^-$/$NH_4^+$ ratio in native soils was significantly higher during the winter and spring sampling periods (Fig. 1a, e). However, we found highly significant main effects of season on most measured soil variables (Online Resource 4 and 6). As expected for a Mediterranean-type climate, soils were wettest November through April, driest in August (Fig. 4a, b), and soil temperature increased from January to July (Table 1; Online Resource 7). In addition, soil $NO_3^-$ concentrations were highest in April, whereas $NH_4^+$ concentrations peaked in July (Fig. 1b, d). Potential rates of nitrification and denitrification were highest in January and July (Fig. 2a, b), and July also showed a peak in SIR (Fig. 2c).

For a more holistic and integrated evaluation of soil properties (Sena et al. 2002), we paired a PCA with an MRPP test of significance. The MRPP confirmed that, when all soil parameters were taken into account, invaded and native soils differed more than expected by chance (Fig. 5). This can be observed visually with the PCA, which consistently distinguished invaded soils from native soils (Fig. 5). In October, January, April, and July, the first two axes of the PCA explained 60, 50, 43, and 58% of the variation in the data, respectively. The soil indicators that contributed to separation between native and invaded soils along PC1 in October included nitrification potential, $NH_4^+$ concentration, and VWC at 0–15 cm depth. In January, these indicators included nitrification potential, $NO_3^-$ concentration, and active microbial biomass (SIR). In April quite a few indicators contributed to the separation between native and invaded soils along PC1, including nitrification potential, IER-$NO_3^-$ and $NO_3^-$ concentration, IER-$NH_4^+$, and active microbial biomass. Finally, native and invaded soils separated along the PC2 axis in July, and the soil indicators contributing to this separation included nitrification potential, denitrification potential, $NH_4^+$ concentration, and active microbial biomass.

### Invaded versus naturalized plant communities

During the year we sampled, invasive plants had established in the naturalized plant communities, rendering plant composition between these two community types less distinct than previous years (see "Methods"). Overall, we found few statistically significant differences in N dynamics between invaded and naturalized plots. The IER-$NO_3^-$ and the IER-

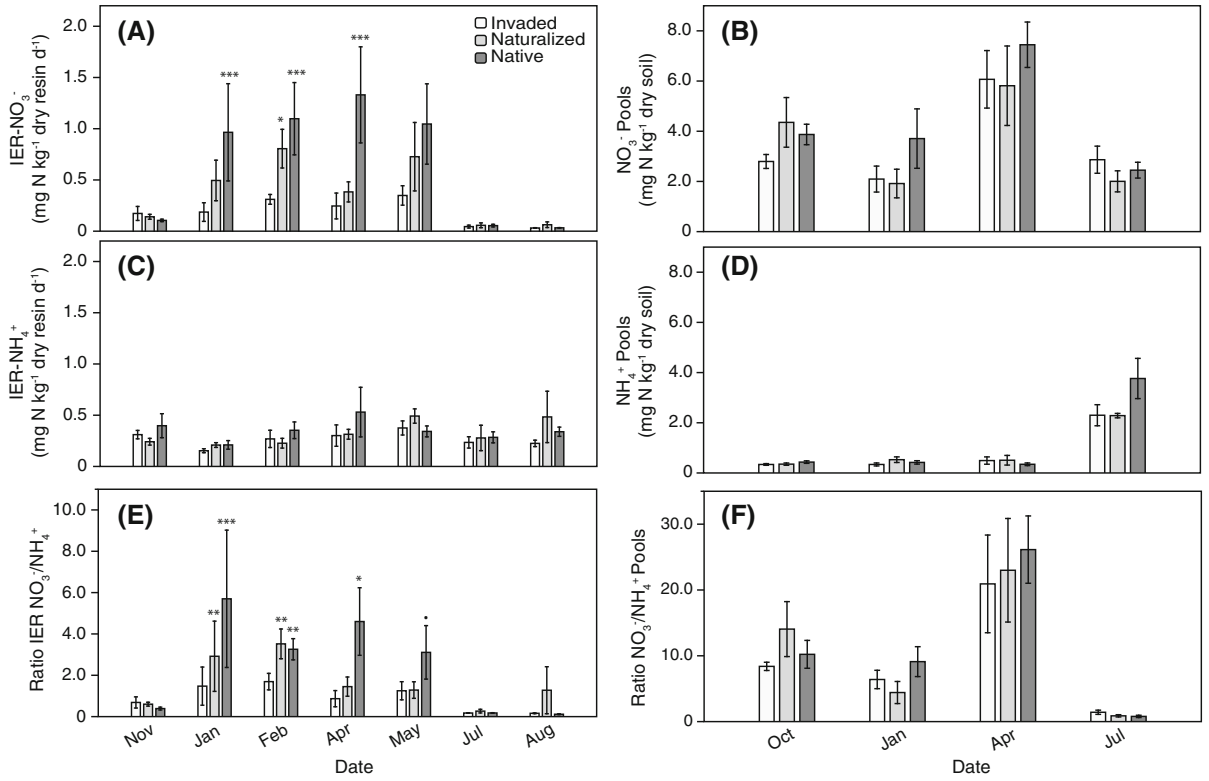


**Fig. 1** Soil inorganic N availability of native, naturalized, and invaded plant communities over time (November 2011–August 2012). **a** Ion-exchange (IER) resin $NO_3^-$-N, **b** extractable $NO_3^-$ N pools, **c** IER resin $NH_4^+$-N, **d** extractable $NH_4^+$-N pools, **e** ratio of IER-$NO_3^-$-N to IER-$NH_4^+$-N, **f** ratio of extractable $NO_3^-$-N to $NH_4^+$-N pools. Vertical bars denote ±1 SE of the mean (IER measurements, n = 12; pools of inorganic N, n = 8). There was a significant plant community type × date interaction for IER-$NO_3^-$ (split plot analysis, $P = 0.012$) and IER-$NO_3^-$/$NH_4^+$ ratio (split plot analysis, $P = 0.029$). *Asterisks above* naturalized or native plant community *bars* indicate significant differences from the invaded community (determined by date-specific contrast analyses for IER-$NO_3^-$ and IER-$NO_3^-$/$NH_4^+$ ratio, $^{\bullet}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Note variations in Y-axis scale

$NO_3^-$/$NH_4^+$ ratio were the only measurements of inorganic N availability that were affected by plant community type. The IER-$NO_3^-$ was lower in invaded compared to naturalized soils only during February (Fig. 1a, IER-$NO_3^-$ Treatment × Date interaction, $P = 0.012$, Online Resource 4). The IER-$NO_3^-$/$NH_4^+$ ratio was significantly lower in invaded compared to naturalized soils only during January and February (IER-$NO_3^-$/$NH_4^+$ Treatment × Date interaction, $P = 0.029$, Online Resource 4). Soil $NO_3^-$ concentrations remained unaffected, as did $NH_4^+$ concentrations and IER-$NH_4^+$ (Fig. 1b–d, Online Resource 4). Moreover, nitrification and denitrification potentials did not differ between soils of invaded and naturalized plant communities across any of the sampling dates (Fig. 2a, b).

Of the microbial biomass measurements, active microbial biomass was lower in invaded compared to naturalized soils ($P = 0.002$, Fig. 2c, Online Resource 4). Direct count measurements of amoeba ($P = 0.016$, Fig. 3b) and total protozoa (ciliates + flagellates + amoeba; $P = 0.009$, Fig. 4b) were also lower in invaded soils. However, total fungi, bacteria, fungi:bacteria ratio, and other protozoan subgroups were similar between the two plant community types (Fig. 3a, b, Online Resource 5). In addition, soil VWR at 0–15 and 15–30 cm depth did not differ between these two plant community types (Fig. 4a, b, Online Resource 6), nor did any of the measured soil physical and chemical properties listed in Table 1, including soil gravimetric water content, temperature, pH, and total C and N (Table 1, Online

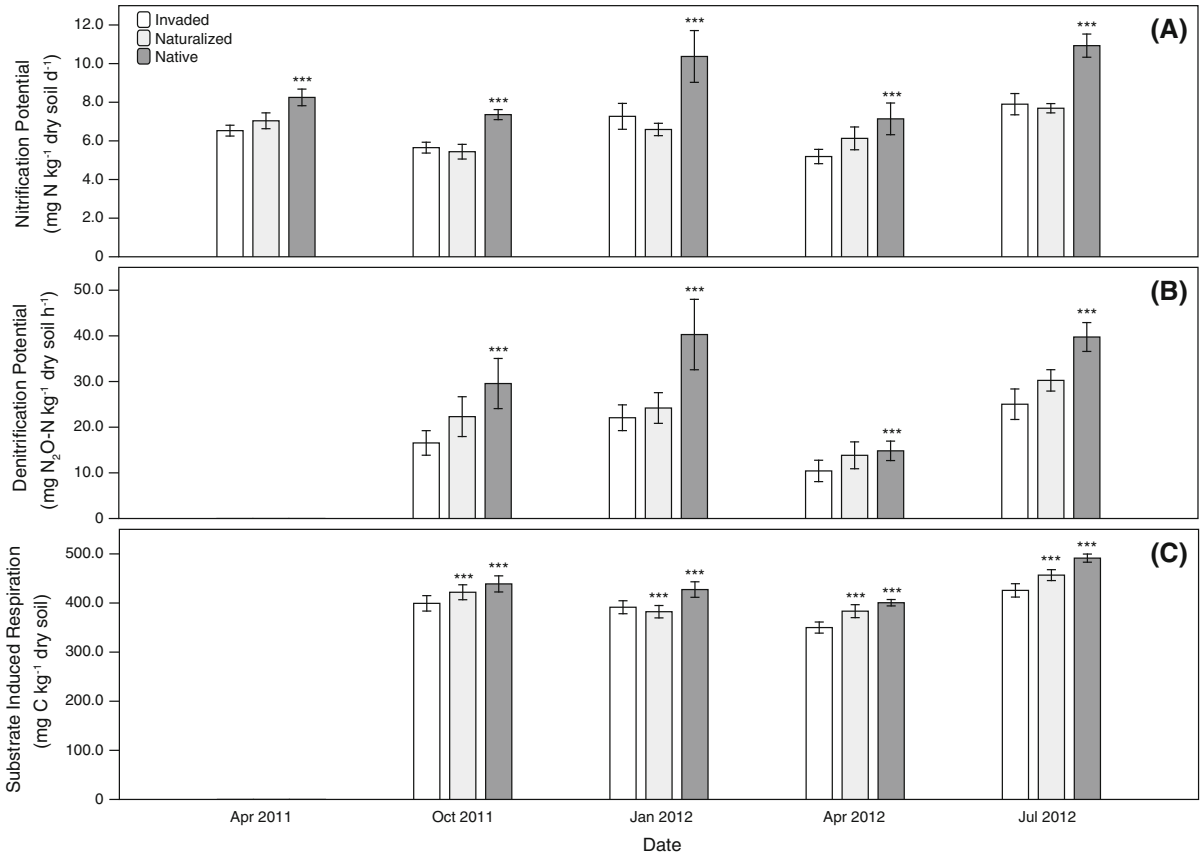


**Fig. 2** Plant community-type effects on **a** Nitrification potential, **b** denitrification potential, and **c** active microbial biomass (SIR) over time. *Vertical bars* ±1 SE of the mean (n = 10 for April 2011; n = 8 for October 2011–August 2012). The effects of plant community type did not depend on time (no significant plant community type × date interactions). *Asterisks above* naturalized or native plant community *bars* indicate significant differences from the invaded community (determined by contrast analyses, $^{\bullet}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Active microbial biomass and denitrification potential were not measured in April 2011. Note variations in Y-axis scale

Resource 6). The PCA and MRPP failed to distinguish between invaded and naturalized soils (Fig. 5), further illustrating the similarity in soil parameters beneath these two plant community types.

## Discussion

### Invaded versus native plant communities

Understanding how invasive plants influence the availability and cycling of N is important given that soil N underlies the provisioning of many ecosystem services (e.g., greenhouse gas production), and that changes in soil N availability can cause invasive species populations to persist at a site (Davis et al. 2000). In this study, we examined how *A. triuncialis* and *E. caput-medusae*, two problematic new-wave invasive species in the western US, influence soil N availability and microbial activity compared to native and naturalized plant communities in Mediterranean-climate California. While the impacts of native versus invasive plants in California grasslands vary by study (reviewed in Eviner and Firestone 2007), we hypothesized that due to greater plant uptake of N (Peters et al. 1996; Drenovsky and Batten 2007) and lower litter quality of *A. triuncialis* and *E. caput-medusae* (Eviner 2004; Drenovsky and Batten 2007), a mixture of these two invasive plants would reduce inorganic N pools, potential rates of nitrification and denitrification, and microbial biomass compared to plant communities that are native to California.

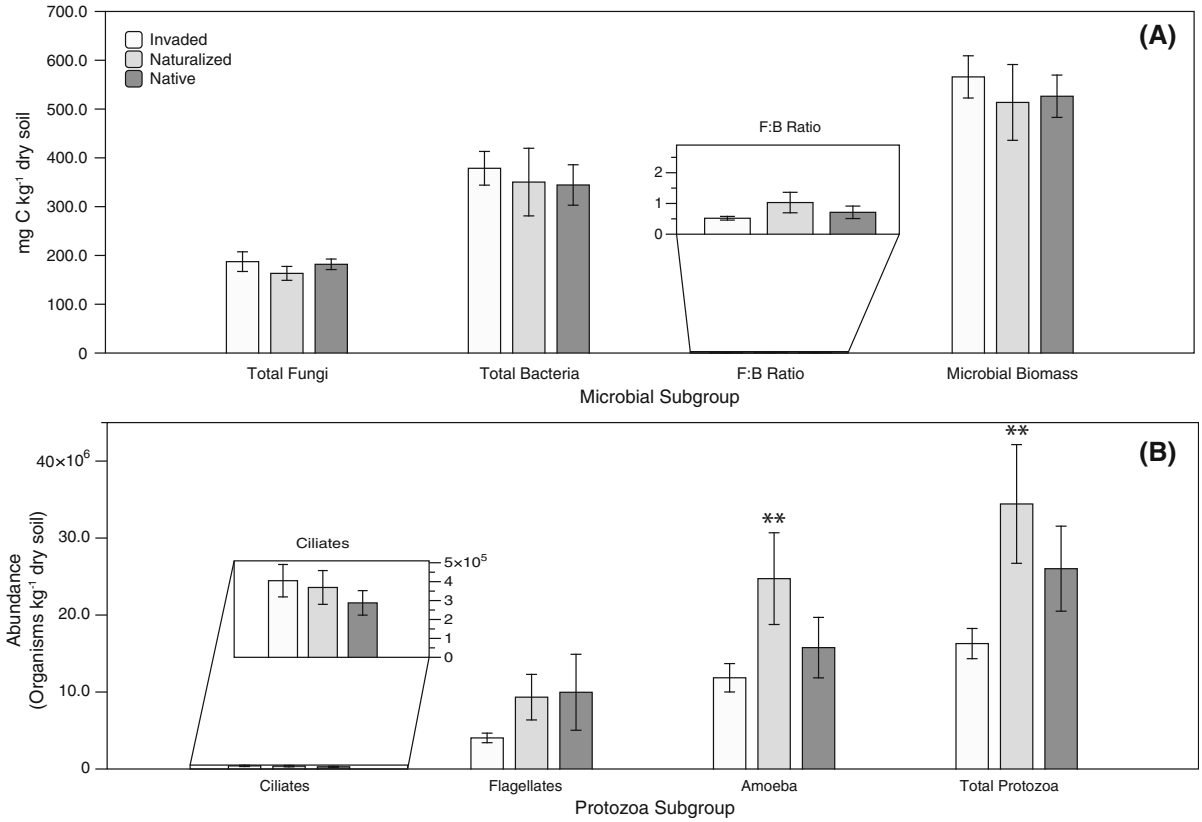


**Fig. 3** Plant community effects on direct count measurements of **a** total fungi, total bacteria, fungi to bacteria ratio (F:B ratio), and microbial biomass (total fungi + total bacteria), and **b** ciliates, flagellates, amoeba, and total protozoa (sum of the three subgroups). Samples were collected in April 2011. Values are means ± 1 SE of the mean (n = 10). *Asterisks above* naturalized or native plant community *bars* indicate significant differences from the invaded community (determined by Kruskal–Wallis post hoc comparison, $^{\bullet}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Note variations in Y-axis scale

In support of this hypothesis, we found that *A. triuncialis* and *E. caput-medusae* decreased IER-$NO_3^-$, potential nitrification rates, and potential denitrification rates compared to native plant communities. However, KCl-extractable measures of $NH_4^+$ and $NO_3^-$ remained constant, indicating that in this ecosystem—like others (Binkley and Hart 1989; Qian and Schoenau 2002)—time-integrated in situ measures such as IER bags can provide greater power to detect relative changes in soil N availability.

Our study was not able to disentangle the relative influences of plant N uptake and litter decomposition on soil inorganic N pools and transformations. However, Baty (2012) measured plant biomass N, decomposition, and litter N loss from the same plots at our site one year prior to our study. Results from that work indicate that plant N uptake may be more important than short-term (<1 year) decomposition in regulating soil inorganic N availability in this system. This is congruent with the idea that N from decomposing litter is often incorporated into soil organic matter rather than released into the soil solution as $NH_4^+$, and that litter quality therefore minimally impacts N availability within a given site (Knops et al. 2002). Regardless of the mechanism(s), our findings agree with prior studies on *A. triuncialis* and *E. caput-medusae* demonstrating reduced decomposition rates, lower soil total N, and a decline in microbial biomass N in invaded soils (Canals et al. 2005; Drenovsky and Batten 2007; Perkins et al. 2011). Our findings further indicate that the effects of invasive plants can ultimately reduce the capacity of the soil microbial community to both nitrify and denitrify.

Although not always the case (Carey et al. 2015; Gornish et al. 2016), invasive plants may indirectly alter N transformations by influencing the size and

**Table 1** Soil characteristics from 0–15 cm depth associated with each plant community type

| Treatment | GWC (%)[a] | | | | pH[b] | | | | Temperature (°C)[c] | | | | Total C (g kg$^{-1}$ soil) | | Total N (g kg$^{-1}$ soil) | | C:N[d] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Oct | | Jan | | Apr | | Jul | | Oct | | Jan | | Apr | | Jul | | | |
| Invaded | 16.8 (0.6) | | 15.5 (0.6) | | 20.0 (0.3) | | 5.8 (0.3) | | 6.6 (0.0) | | 6.6 (0.1) | | 6.7 (0.0) | | 6.6 (0.1) | – | 8.2 (0.3) | 13.0 (0.7) | 29.3 (1.0) | 12.1 (0.1) | 1.3 (0.0) | | 9.5 (0.0) | |
| Naturalized | 15.8 (0.8) | | 14.6 (0.4) | | 21.1 (0.5) | | 5.0 (0.2) | | 6.6 (0.0) | | 6.6 (0.0) | | 6.7 (0.1) | | 6.6 (0.1) | – | 8.0 (0.5) | 13.4 (0.6) | 30.9 (0.7) | 12.3 (0.1) | 1.3 (0.0) | | 9.6 (0.0) | |
| Native | 16.7 (0.8) | | 14.8 (1.0) | | 20.6 (0.4) | | 5.8 (0.4) | | 6.6 (0.0) | | 6.6 (0.0) | | 6.7 (0.0) | | 6.5 (0.1) | – | 8.1 (0.2) | 14.0 (1.0) | 29.7 (1.0) | 12.9 (0.1) | 1.3 (0.0) | | 9.6 (0.0) | |

Values are mean ± SE (n = 8). There were no differences among treatments for any of the soil characteristics ($P < 0.05$)

[a] Gravimetric water content

[b] pH measured in 0.01 M $CaCl_2$ solution

[c] Temperature measured at 7.5 cm depth

[d] Soil total carbon (C) to nitrogen (N) mass ratio. Soil total C and N were determined on samples pooled across all four sampling dates

composition of soil microbial communities (Hawkes et al. 2005; Dassonville et al. 2011; Piper et al. 2015). This includes eukaryotic microorganisms, such as protozoa, which graze on bacteria and archaea (Clarholm 1985; Griffiths 1989). We found that total active microbial biomass was lower in invaded soils (in accordance with our hypothesis); however, the abundances of ciliates, flagellates, and amoeba remained unchanged between invasive and native plant communities even five years after the plant communities were established (Fig. 3). This suggests that *A. triuncialis* and *E. caput-medusae* did not indirectly alter potential microbial activity or available N through their effects on soil protozoa. Only a few other studies have measured how soil protozoans change with plant invasion, and these studies have found results similar to ours (Belnap et al. 2005; Batten et al. 2006). For instance, the abundance of protozoa in a semi-arid desert did not respond to *Bromus tectorum* invasion, even when the site had been invaded for more than 50 years (Belnap et al. 2005). In addition, while *Centaurea solstitialis* increased the PLFA biomarker for protozoa in newly invaded serpentine soils, this biomarker reverted back to uninvaded levels after 2–3 years of *C. solstitialis* establishment (Batten et al. 2006). In the same study, *A. triuncialis* invaded soils showed no significant shift in the PLFA biomarker for protozoa. Although it is possible that other soil fauna (e.g., nematodes) may provide top-down control on soil nutrient cycling (Djigal et al. 2010; Baty 2012), the stability of protozoan biomass between plant communities suggests protozoan grazing on bacteria is not likely responsible for altering nutrient availability or microbial activity of invaded soils.

The disparity in potential nitrification and denitrification of invaded and native soils has implications for restoration because native perennials are often used to re-vegetate sites after the removal of invasive species (Stromberg et al. 2007). If legacies of altered microbial activity persist after the removal of *A. triuncialis* and *E. caput-medusae*, as has been shown with other invasive plants (Corbin and D'antonio 2004; Corbin and D'Antonio 2011a), native species may become less competitive which would inhibit successful reestablishment (Grman and Suding 2010). Recent work suggests that this may indeed be the case. For example, *E. caput-medusae* has been shown to generate soil feedbacks by altering soil nutrient

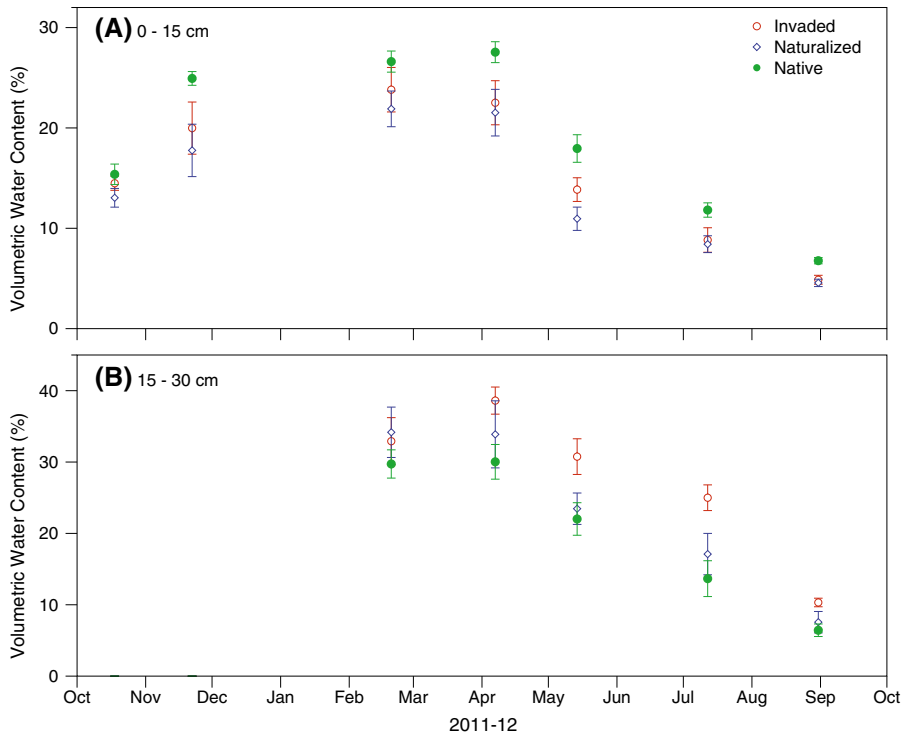


**Fig. 4** Mean soil volumetric water content at **a** 0–15 cm and **b** 15–30 cm soil depth for native, naturalized, and invaded plant communities. Values are means ± 1 SE (n = 6). Volumetric water content 15–30 cm values are not presented for October and November 2011 because 0–30 cm TDR probes were not installed at that time. Note variations in Y-axis scale

availability (in sandy loam soils) and microbial community composition (in clay soils), resulting in their increased performance relative to the native species (Perkins and Nowak 2013; Perkins et al. 2015). In contrast, evidence of feedbacks for *A. triuncialis* have been mixed. Batten et al. (2008) suggested that *A. triuncialis* reduced the success of native plant species by altering the soil microbial community composition (e.g., increasing the biomarker for arbuscular mycorrhizal fungi); however, Perkins and Nowak (2013) found that *A. triuncialis* caused no apparent plant-soil feedbacks affecting con or heterospecific performance. Our work demonstrates that these two invasive plants reduce $NO_3^-$ availability, active microbial biomass, and the potential for soil communities to nitrify and denitrify compared to experimental native plant communities. These differences could possibly help to sustain *A. triuncialis* and *E. caput-medusae* populations to the detriment of restoration efforts. Future work should focus on understanding whether and for how long these changes in soil conditions persist, and the consequences that the observed changes in microbial activity may have for successful establishment of native plant communities.

### Invaded versus naturalized plant communities

We further predicted that the two invasive plants, *A. triuncialis* and *E. caput-medusae*, would reduce inorganic N pools and potential rates of nitrification and denitrification compared to naturalized exotic plant communities. Contradicting this hypothesis, nitrification and denitrification potentials were similar in soils beneath invaded and naturalized plant communities. Most of the other measured soil parameters were also similar, with the exception of IER-$NO_3^-$ (lower in invaded soils during January), and active microbial biomass, amoeba, and total protozoa (lower in invaded soils). Unfortunately, our ability to tease out the effects of these two communities may be limited by crossover of *E. caput-medusae* into the naturalized treatment plots; while the naturalized species had nearly full cover for the first three growing seasons, their cover dropped to 80% in 2011, and 40% in 2012 as *E. caput-medusae* increased in prevalence (Online Resource 1). However, in 2010–2011 when the plant composition of these two communities were still intact (Online Resource 1), Baty (2012) reported findings similar to ours, where invaded and naturalized

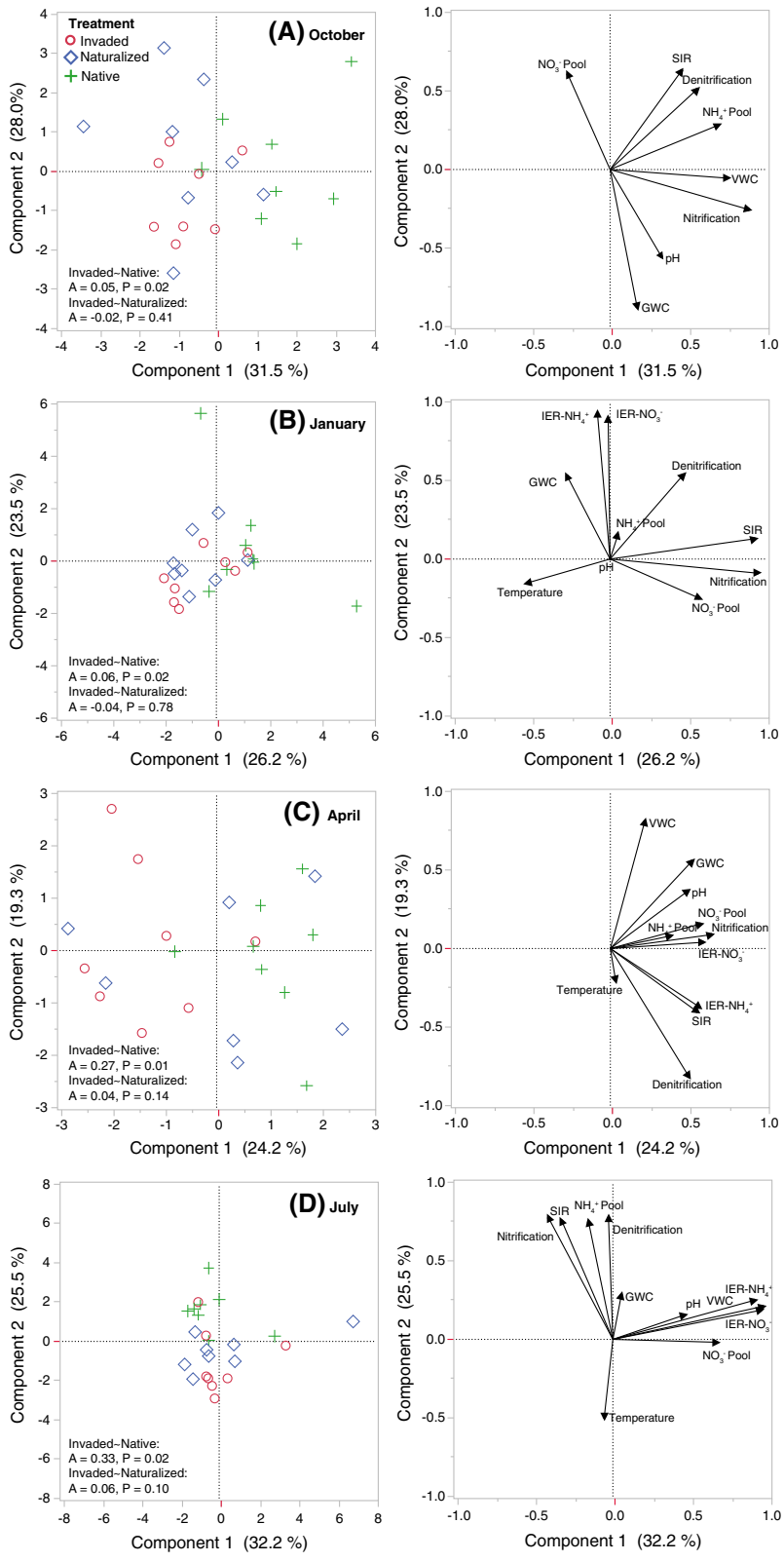


**Fig. 5** Principal components analysis (PCA) of plant community type (*symbols*; *left panel*) and soil parameters (*vectors*; *right panel*) separated by sample period (**a**–**d**). *Red circle* invaded plant community; *green cross* native plant community; *blue diamond* naturalized plant community. *SIR* substrate induced respiration, *VWC* volumetric water content; *GWC* gravimetric water content; *IERNH*$_4$ *and IERNO*$_3$ ion-exchange resin $NH_4^+$ and $NO_3^-$ availability, *NH*$_4$*Pool and NO*$_3$*Pool* KCl extractable concentrations of soil $NH_4^+$ and $NO_3^-$. The soil indicators that contributed to separation between native and invaded soils along PC1 in October included nitrification potential, $NH_4^+$ concentration, and VWC at 0–15 cm depth. In January, these indicators included nitrification potential, $NO_3^-$ concentration, and active microbial biomass (SIR). In April, PC1 indicators included nitrification potential, IER-$NO_3^-$ and $NO_3^-$ concentration, IER-$NH_4^+$, and active microbial biomass. Finally, native and invaded soils separated along the PC2 axis in July, and the soil indicators contributing to this separation included nitrification potential, denitrification potential, $NH_4^+$ concentration, and active microbial biomass. Results of MRPP are also presented, where A = percent change and *P* = *P* value. Note variations in X and Y-axis scales

treatments had similar rates of net N mineralization and net nitrification. This suggests that our findings are robust, and that other explanations for these similarities may exist beyond crossover between treatment plots.

One possible explanation is that differences in important morphological or physiological characteristics between these two particular plant community assemblages were not large enough to induce changes in N pools and transformations. Indeed, the effects of an invasive plant can depend on how functionally distinct it is from the resident species (Castro-Díez et al. 2014), and in this case *A. triuncialis* and *E. caput-medusae* shared some ecologically important traits with species comprising the naturalized plots. For example, both *A. triuncialis* and *E. caput-medusae* have similar litter C:N ratios as *Bromus hordeaceus*, the dominant plant in naturalized plots at the time of sampling (Eviner 2004). Furthermore, because both the invasive and naturalized plants are annuals, they likely share similar rooting depths and may consequently use inorganic N and water from the same spatial niche (Holmes and Rice 1996), illustrated here by similarities in soil moisture (Fig. 4). In addition, because *A. triuncialis* has been found to differ more significantly from naturalized grasses than *E. caput-medusae* (in terms of field rates of net nitrification; Eviner et al. 2006), it may be that invaded soils would have differed from those of naturalized soils if *A. triuncialis* dominated the invaded plots instead of *E. caput-medusae* (Online Resource 1).

It is conceivable that five years was not enough time for detectable differences to emerge between the invaded and naturalized plant communities, especially because our site was previously cultivated—a practice which may produce significant legacies in the soil (Buckley and Schmidt 2001). However, we measured differences between invaded and native plant communities within the same time frame, and other comparable studies have demonstrated significant shifts in belowground parameters (e.g., N mineralization, nitrification) within five years of plant establishment (Hawkes et al. 2005; Corbin and D'Antonio 2011b). While this suggests that sufficient time had accrued, the degree to which species relative abundance, time since establishment, and site history interact to influence soil N pools and transformations in invaded areas is an important area of future research.

## Conclusion

We have demonstrated that—compared to native plant communities—the invasive species *A. triuncialis* and *E. caput-medusae* reduce soil inorganic $NO_3^-$ availability and the abundance of microbes involved in nitrification and denitrification. It follows that gaseous ($N_2O$, $N_2$) or aqueous ($NO_3^-$) loss of N may be lower in invaded soils, and that changes to N availability by these invasive species may inhibit native plant restoration. In contrast, very few differences were detected between invaded and naturalized soils, suggesting the belowground effects of *A. triuncialis* and *E. caput-medusae* during invasion of naturalized grasslands may be less pronounced than previously expected.

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