Assessing Cumulative Effects of Climate Change Manipulations on Phosphorus Limitation in a Californian Grassland

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Ⓢ Supporting Information

ABSTRACT: Grasslands throughout the world are responding in diverse ways to changing climate and environmental conditions. In this study we analyze indicators of phosphorus limitation including phosphorus concentrations, phosphorus to nitrogen, and carbon ratios, oxygen isotope ratios of phosphate in vegetation, and phosphatase enzyme activity in soil to shed light on potential effects of climate change on phosphorus availability to grassland vegetation. The study was conducted at the Jasper Ridge Global Change Experiment (JRGCE), California where manipulations mimicking increases in temperature, water, nitrogen and carbon-dioxide have been maintained for over 15 years. We compare our results to an earlier study conducted 3 years after the start of the experiment, in order to assess any change in the response of phosphorus over time. Our results suggest that a decade later the measured indicators show similar or only slightly stronger responses. Specifically, addition of nitrogen, the principle parameter controlling biomass growth, increased phosphorus demand but thresholds that suggest P limitation were not reached. A study documenting changes in net primary productivity (NPP) over time at the JRGCE also could not identify a progressive effect of the manipulations on NPP. Combined these results indicate that the vegetation in these grassland systems is not very sensitive to the range of climate parameters tested.
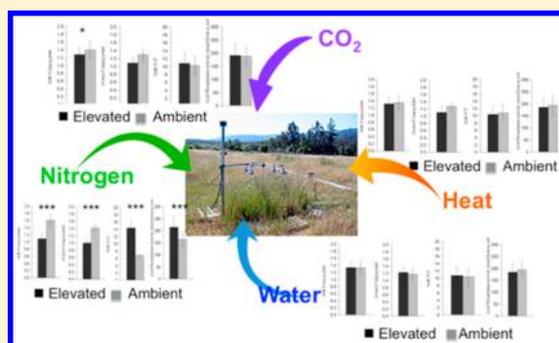
1.0. INTRODUCTION

Phosphorus (P) is an essential macronutrient for life and a major component of plants. It plays a critical role in the structure of DNA, RNA, and cell membranes as well as in transmission of energy in the form of ATP.[1] The abundance and availability of P in soil has the capacity to limit the growth of plants, and thus to affect food supply, plant productivity, and ecosystem structure and function.[2] In many ecosystems, the essential nutrients nitrogen (N) and/or P constrain growth.[3−5] It has been suggested that the influence of anthropogenic global change and specifically the increase in atmospheric N deposition may push more systems toward P limited conditions.[4−9] Recent work has highlighted the role of N and P, and other nutrients in limiting productivity of many grassland systems worldwide.[4,5,9] However, other factors can affect primary productivity, including temperature, water availability, day length, along with other environmental parameters; and actual responses to each environmental variable or combination of variables are difficult to predict.[6] This underscores the importance of experimental investigation in understanding the impacts global change will have on

terrestrial environments, including their response to anthropogenic perturbations and specifically how P dynamics may be impacted by such changes.

The Jasper Ridge Global Change Experiment (JRGCE) is a long-term study on the effects of expected climate and anthropogenic changes on California grasslands. The experiment is designed to test the effects of increased bioavailable N, carbon dioxide (CO_2), temperature, and precipitation and all combinations of these effects on the ecology and biogeochemical cycles in this ecosystem.[10,11] The study site incorporates eight iterations of each of the four amendment combinations resulting in 16 unique treatments and 128 total treatment plots. A study on P biogeochemistry at the site by Menge and Field[7] which was conducted three years after the start of the experiment concluded that in treatments where N was added plants exhibited signs of P limitation. We note that the term P

limitation used in that study as in many other studies including this one does not necessarily imply that NPP is reduced but rather that the biologically available inorganic P is lower than the plants' demand, resulting in physiological changes such as production of enzymes to acquire other P forms or changes in cellular P quotas. Six indices of P limitation were used to evaluate treatment impacts: soil phosphatase activity, P concentration and N:P ratios in green and in senescent leaves of the dominant grass genus, Avena and the total aboveground plant P content. They found that all indices indicated that N deposition, the only factor that stimulated NPP (by a factor of 23% relative to control[12]), increased P demand. The other three factors (CO_2, temperature and water supply), did not stimulate NPP or an increase in P demand by the vegetation. A more recent analysis of changes in NPP response to the climate manipulations over the 15 years of the JRGCE suggested that given the interacting effects of predicted climate change conditions, future conditions will likely result in lower NPP (albeit with large interannual variability).[12] The current study reported here was also conducted 15 years after the start of the experiment and 12 years after the study of P indicators by Menge and Fields,[7] in order to determine if any further progression or decrease in P limitation conditions has occurred and how this may inform other trends observed at the JRGCE.

We use some of the same indices employed by the previous study: total P concentrations and N:P ratios of the bulk aboveground biomass and of green *A. barbata*, and soil alkaline phosphatase activity to evaluate changes in these indices over time. We also use C and N concentrations, C:N and C:P ratios of *A. barbata*, and of the bulk homogenized aboveground biomass, as well as the soil enzyme activities of acid, alkaline, and neutral phosphatases, and β-glucosidase to gain additional information on the nutrient dynamics in this ecosystem. It is expected that as P becomes less available P content the plants will decrease as less luxury uptake and storage will be possible. If N is not depleted then this will also result in higher N:P and C:P ratios in plant tissue. Under such condition plants (and bacteria) will produce phosphatase enzymes that hydrolyze organic P compounds to increase phosphate availability hence an increase in the activity of phosphatase enzymes suggests that there is not enough phosphate to satisfy growth. In addition to these measurements, the oxygen isotope ratio of phosphate ($\delta^{18}O_P$) in *A. barbata* was measured to evaluate if P limitation affects this ratio in living plant leaves. $\delta^{18}O_P$ has not been previously tested as an indicator of P limitation; we hypothesized that when P is scarce cellular P will be extensively cycled resulting in isotopic equilibrium,[13] whereas excess P when available may be stored as polyphosphate which might not be cycled and equilibrated with plant cellular water. This suite of analyses was used to shed light on P cycling and allocation, and changes in the extent of P limitation (e.g., availability vs demand) since the previous study of Menge and Field[7] and how this relates to trends in NPP at JRGCE.

2.0. MATERIALS AND METHODS

2.1. Study Site and Experimental Design. Jasper Ridge Biological Preserve is located in the San Francisco Bay area (37°24′N, 122°14′W). The site experiences a Mediterranean-type climate, with cool, wet winters and hot, dry summers. The soil at the site is a fine grain, mixed Typic Haploxeralf derived from either sandstone or greenstone from the Franciscan complex with a pH ranging from 6.5 and 7.[14] The plant community at the site is dominated by introduced forbs (primarily *Geranium dissectum* and *Erodium botrys*) and grasses (*Avena barbata*, *Avena fatua*, *Bromus hordeaceus*, *Lolium multiflorum*, and *Bromus diadrus*) with few native species.[11]

The four parameters chosen for manipulation in this climate study are atmospheric CO_2 (CO_2), temperature (H), precipitation (W), and nitrate deposition (N). Atmospheric CO_2 is elevated by +275 μmol·mol^{-1}. Temperature is increased by +80 to +250W·m^{-2} (corresponding to 1−1.5 °C above ambient), precipitation is elevated to 150% ambient values, and N deposition is increased by adding 7g NO_3^- m^{-2} yr^{-1} (as a 2 g pulse as liquid $Ca(NO_3)_2$ with the first autumn rains and 5 g as a time-release pellet application (Osmocote) in January of each year) in addition to the background rate of <1g NO_3^- m^{-2} yr^{-1}.[15] The experimental design consists of a four-way split-plot design containing eight iterations of each treatment that form distinct blocks.[16] Each block consists of four plots (3.14 m^2 in area) that are divided into four quadrants, where CO_2 and temperature treatments are administered at the plot level, and precipitation and nitrate deposition are administered at the quadrant level. Further details on the experimental design can be found in Zaveleta et al., 2003 and Gutknecht et al., 2012.[17,18]

2.2. Sample Preparation. Samples for bulk vegetation measurements were taken from the 2012 harvest, whereas *Avena Barbata* measurements were obtained from the 2013 harvest. Using bulk vegetation better represent the ecosystem as a whole; however differences in species composition between plots can affect the measured parameters if the physiological responses are species dependent; using a single species (*A. Barbata*) reduces this potential variability. Whole individuals of annual grasses and forbs in the harvest area (a 141 cm^2 quadrant from each quad and away from infrastructure) were harvested and homogenized to constitute the bulk above ground vegetation. The samples were oven-dried at 60 °C for 1−4 weeks, and stored at ambient temperatures. Bulk above ground vegetation samples were first ground to 1 cm or less than homogenized to a consistent size powder with a ball mill. In cases where there was too little sample, the samples were ground with a mortar and pestle.

The species *Avena Barbata* was selected for analysis due to its high abundance in California grasslands and its presence in most of the treatment plots, ease of identification and direct comparison to published work.[7] It is assumed that changes in *A. barbata* tissue chemistry are representative of other grasses and of the trends in the rest of the vegetation in the respective quadrants. Once separated from the bulk vegetation, samples consisting of all *A. barbata* above ground parts from a sample were dried in a 50 °C oven overnight, and homogenized using a Wiley mill followed by a ball mill grinder to ensure consistent analytical results within each plant sample. In 2014, a smaller harvest of *A. barbata* from three of each of the treatment plots was used to measure oxygen isotopes in phosphate along with N and P ratios.

2.3. Total P in Avena and Above Ground Vegetation. Total P concentrations in dry plant material were derived using a modified method from Aspila 1976.[19] For each sample, 25 mg of dry, homogenized biomass were combusted in an oven at 550 °C for 2 h and extracted with 10 mL of 1 M HCl for 14−16 h. Phosphorus concentrations of the solutions were measured on a PerkinElmer inductively coupled plasma optical emission spectrometer. The detection limit of this procedure is ~10 ppb and the precision is 5% for signals above detection limit. The instrument was calibrated and routinely monitored using 1 and 10 ppm standards. Blanks were processed along
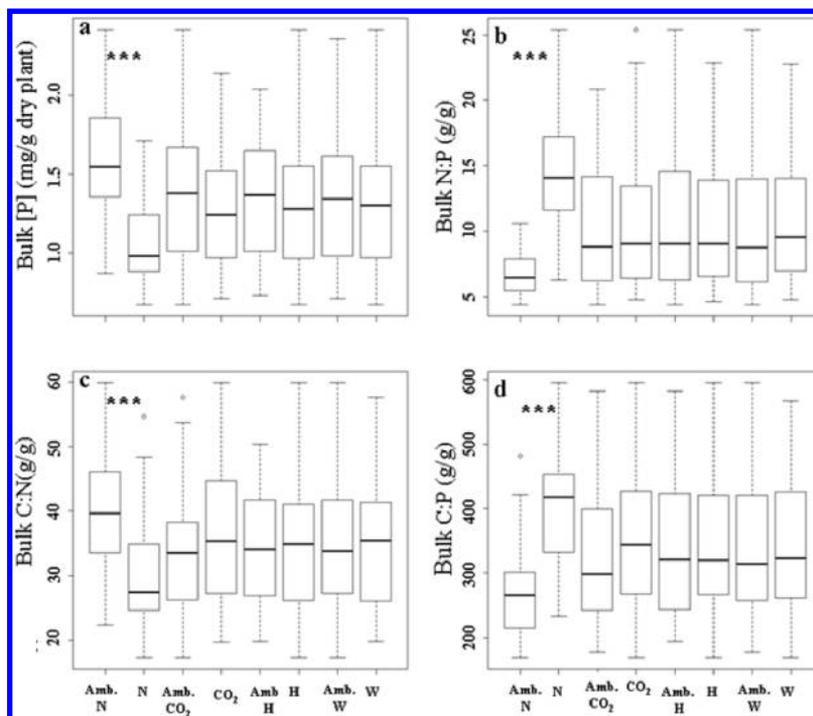
Figure 1. Main effects of N deposition, water, heat, and CO_2 on measurements that reflect changes in phosphorus demand in the JRGCE. Significance of main factors is shown: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. (a) shows P concentration (b) shows N:P ratio (c) shows the C:N and (d) shows C:P of bulk biomass.

with samples and were on average less than 0.2% of sample. P concentrations in the homogenized samples are reported as P per gram of bulk above ground biomass or per gram of Avena. We also calculate the total above ground P at each site by multiplying the P content per gram bulk sample by the above ground biomass measured in each treatment plot.

2.4. Total Nitrogen and Carbon and $\delta^{13}C$ and $\delta^{15}N$. Total N and carbon (C) of plant material (homogenized *A. barbata* or bulk above ground biomass) were analyzed using a CE Instruments NC2500 elemental analyzer coupled to a ThermoFinningan Delta Plus XP isotope ratio mass spectrometer. Dried and ground samples (0.3 mg) were weighed into tin capsules and combusted at a temperature of 1020 °C. The precision of C and N concentrations was better than 1% and for the isotope ratios it was 0.2‰ for $\delta^{13}C$ and $\delta^{15}N$. Standards used were Pugel, Acetanilide, and Oak. Sample isotopic values are corrected for size, drift and source stretching effects.

2.5. $\delta^{18}O$ of Soil and Stem Water. The $\delta^{18}O$ of stem water in Avena was measured using a Picarro L2130-i water isotope analyzer. In brief the method used an induction module periphery, segments of stem material were heated, water completely evaporated, and using dry air the contents introduced to the instrument. One Avena plant sample was chosen from each treatment and assumed to be representative of the $\delta^{18}O$ of stem water for Avena in that treatment. Transects were made along each plant (7 cm increments) in order to account for fractionation within the plant due to evapotranspiration. These values, along with temperature at the site, were used to calculate and expected equilibrium range of $\delta^{18}O$-PO_4 in the *A. barbata* stems.[20] Soil water was extracted from frozen soil plugs sampled during the spring harvest using a modified method from West 2006.[21] Samples were cryogeni-

cally distilled using a vacuum line, then analyzed using a Picarro L230-I water isotope analyzer as discrete samples.

2.6. $\delta^{18}O$ of Plant Phosphate. Phosphate (PO_4) was extracted from samples by shaking 0.3−0.5 g of sample in 100 mL of Milli-Q water for 3 h and the contents filtered using GF/F filters. A magnesium induced co-precipitation of phosphate with brucite was performed by adding 0.2 g of $MgCl_2$ to each sample and raising the pH above 10.5 with NaOH.[22] The precipitate was centrifuged for 5 min at 3500 rpm, the supernatant discarded, and the pellet dissolved with 5 mL of concentrated glacial acetic acid. Once dissolved the solution was 0.2 μm filtered and passed through an Oasis brand filtration column to remove excess organic matter. Phosphate from the solution was purified and precipitated as silver phosphate (Ag_3PO_4) following a modified procedure from McLaughlin 2004.[22] Ag_3PO_4 samples were pyrolyzed and analyzed using Isotope Ratio Mass Spectrometry.[22] Select samples were processed spiked and unspiked with ^{18}O enriched reagents to ensure no artifacts are impacting the data.[22,23] The expected equilibrium values for phosphate in soil and phosphate in the vegetation were calculated using the average high and average low temperatures in soil and in air during the growing season and the range of the soil water and Avena water isotope values, respectively. The equation used for this calculation is T (°C) = 111.4−4.3 $(\delta^{18}O_P - \delta^{18}O_w)$ from Longinelli and Nuti.[24]

2.7. Soil Enzyme Activities. Soil samples were collected during the 2013 harvest seasons at the JRGCE and kept frozen until analysis in April 2014. Each soil sample was composed of the homogenized upper 15 cm of two 2.5 cm diameter cores; the average soil moisture content was roughly 10%. We followed a modified method by Sinsabaugh 2003[25] and used pretesting optimization of assay conditions.[26] Although the JRGCE has a neutral pH and enzyme activities have typically been measured at neutral pH, individual phosphatases,

however, are optimized either to alkaline or acidic pH.[27] Because of this, previous studies have offered a generalized analysis of the larger pool of phosphatases, without detailed analysis of different phosphatase classes. Here we chose to go into more detail by separately measuring acid and alkaline phosphatases, and the mixture at neutral pH. A universal buffer was used to maintain the acidic phosphatase (pH 5.2), $\beta-$ Glucosidase (pH 7), and alkaline phosphatase (pH 10) incubation conditions. Phosphatase activity at neutral pH (pH 7) was also performed for comparing results to previous studies.[10,28] Approximately 0.25 g of soil was weighted into bottles and 25 mL of buffer was added. The slurry was shaken for 10 min to homogenize and 200 μL of the soil slurry was added to a 96-well plate in replicates of eight. Following the addition of the soil slurry 50 μL of 200 μM 4-methylumbelliferone linked enzyme substrate was added and allowed to incubate for 1 h, after 20 μL 1 M NaOH was added to each well to improve fluorescence readings. Sample plates were read with a microplate fluorometer with 365 nm excitation and 450 nm emission filters.

2.8. Statistical Analysis. Significant differences between treatment groups for P, C and N concentration, N:P, C:P, and C:N ratios, soil enzyme activities, and the C, N isotopes of bulk organic matter and O isotopes in phosphate were evaluated using mixed effects models using the linear mixed model (lme) package in R.[29] The mixed effects model included all combinations of the manipulation treatments (CO_2, H, W, N) as fixed effects and with block and plot included as random effects. In addition, due to limited number of replicates in some data sets, as well as for comparison to the earlier study, samples were also grouped into ambient or elevated classifications for each parameter and a pair wise analysis was performed to compare the treated relative to the ambient data.

3.0. RESULTS AND DISCUSSION

3.1. P, N, and C Concentrations and Nutrient Ratios in Plant Tissue. P concentrations, and N:P, C:N, and C:P ratios in bulk above ground biomass samples are shown in Figures 1 and 2. In the bulk biomass N addition displays a significant effect across all treatments and data sets while no other treatment is significantly different from the control (Figure 1; Supporting Information (SI) Table S1). Specifically, P concentrations in plant tissue in elevated N plots are lower than the control and all other treatments with ambient N, as is the C:N ratio, whereas N:P and C:P are higher than in plots not receiving N. This is consistent with the observations made over a decade before by Menge and Field.[7]

When comparing results for each of the unique treatment combinations (Figure 2) a trend with N addition is apparent across all data sets. While not statistically significant, the analysis shows that P concentrations are lower in treatments where N was added alone or in combination with other global change treatments. Similarly, the N:P ratios of treatments with N addition are significantly higher (ratios of 10−18) than the control and other treatments to which N was not added (ratios of 5−9). The C:P ratios are also higher for all treatments with N addition (~400 relative to <300 for no N additions) although most results are not statistically significant (SI Table S1). C:N ratios are not significantly different than the control for all treatment groups although the ratios are clearly lower for the treatments with added N (~30 relative to ~40 for no N additions). The consistent results using the two different statistical analyses lend credibility to our interpretation,
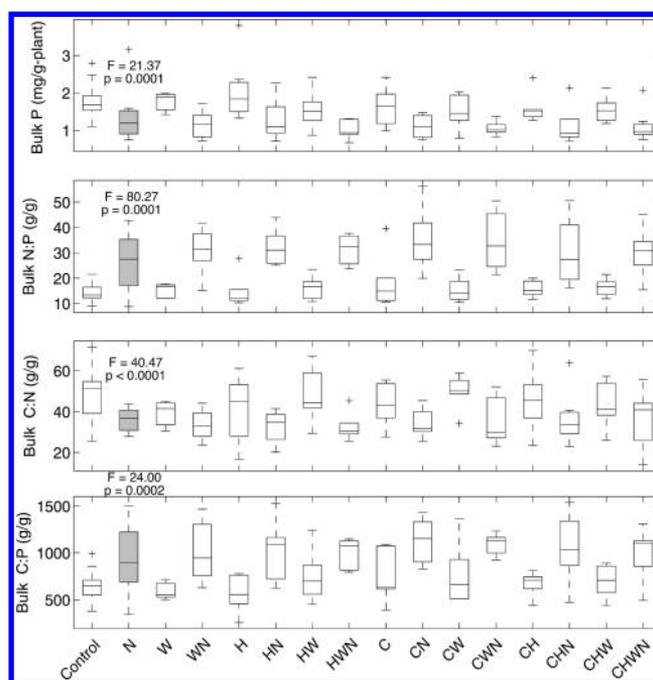

Figure 2. P concentration (mg P/g bulk vegetation), N:P, C:N, and C:P mass ratios (g/g) for bulk above ground vegetation samples.

emphasizing that only N addition consistently resulted in changes in nutrient allocation in the above ground bulk vegetation, similar to previous results from Menge and Field (2007).[7]

P concentrations, and N:P, C:N, and C:P ratios in Avena samples are shown in Figures 3 and SI Figure S1. Similar trends to concentrations and ratios measured in bulk above ground biomass are seen for Avena but with more scatter, likely due to the smaller sample size for some treatments. When data are grouped by elevated vs ambient status (Figure 3) for each of the four parameters, N addition significantly decreases P concentrations, increases N:P, decreases C:N, and increases C:P. In addition elevated CO_2 has a significant effects on the P concentration (increasing P) and C:P ratio (decreasing the ratio) of the Avena tissue. Heat also has a slight but significant effect, which decreases the C:P ratio of the Avena tissue. When comparing each of the individual treatment responses P concentration for treatments with N additions are all lower, but only N addition alone is significant (S1). For N:P ratios treatment groups with N addition had higher ratios than the control and treatments without N addition (>10 and <10, respectively). C:N ratios in the Avena samples are clearly lower for the treatments with added N (<40 and >40, respectively). For the C:P ratios again all N addition treatments are higher than non- N addition treatments (>400 and <400, respectively). The linear mixed model was inadequate due to small sample size in some treatments, however, despite this limitation the results are consistent with those for the bulk above ground vegetation, suggesting that variability in species abundance between plots does not have a large impact on the nutrient dynamics and that overall all the important species present have responded similarly.

The P concentrations in the bulk above ground biomass and Avena leaves both decreased under N addition by 32% and 30%, respectively, when compared to plots with ambient N (Figures 1 and 3). The N:P ratio increased from 6.8 to 14.4 (an increase of 112%) for the bulk vegetation, and from an average
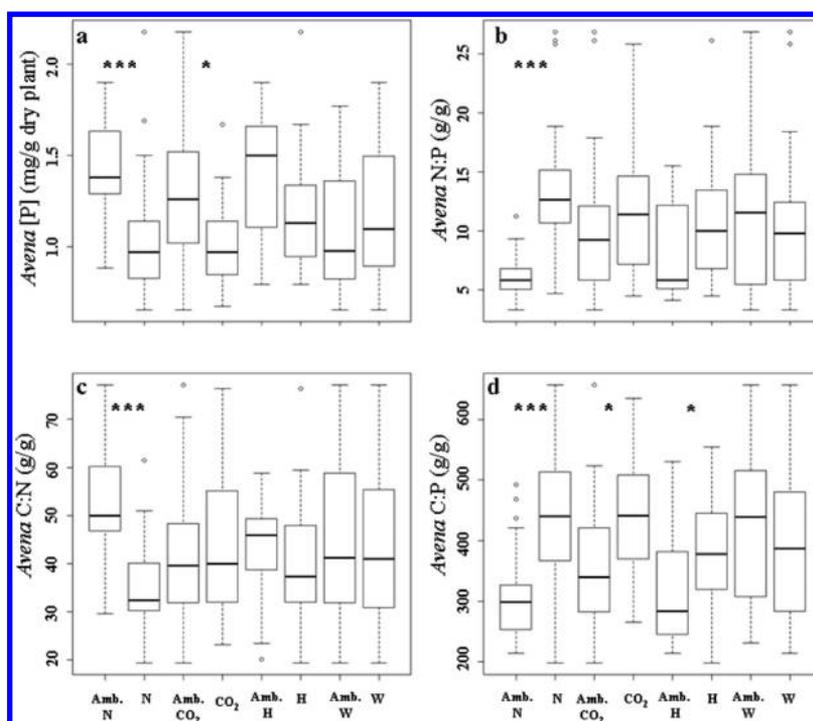
Figure 3. Main effects of N deposition, water, heat, and CO_2 on measurements of indicators of phosphorus demand in the JRGCE. Significance of main factors is shown: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. (a) shows P concentration (b) shows N:P ratio (c) shows the C:N and (d) shows C:P of *Avena* sp. biomass.

of 6.0 to 13.8 (an increase of 129%) for Avena under elevated N conditions when compared to treatments not receiving N (Figures 1 and 3). These shifts suggest a move toward P limitation (lower P availability), as a N:P ratio above 20 for terrestrial foliage or aboveground community biomass[5,30] is generally considered to signify P limitation (although the specific value may vary among species[31]). However, despite the increase in N:P with N addition the ratios are still well below 20 for both measures and hence the system has not reached a P limitation status (consistent with the increase in NPP in the N addition treatments). Our results are similar to those reported in earlier work,[7] conducted 3 years after the start of the JRGCE where treatments with high N deposition significantly decreased P concentrations in Avena tissue across all combinations of treatments that received N (by 40% in green leaves and 44% in senescent leaves). That earlier study also saw a very similar increase in the N:P ratio of Avena green leaves from roughly 5−10 (100%), compared to the current increase of 112% and 129% for bulk and Avena, respectively. The average P concentrations in Avena (~0.7 mg g^{-1} in 2003 and ~1 mg g^{-1} in 2013) and the N:P rations (10 in 2003 and 13.8 in 2013) in plots receiving N additions may suggest a slightly stronger response over time, however when considering all the data of the individual plots the P concentrations and N:P ratios observed in our study are within the range reported previously, suggesting that another decade of continuous N addition did not change the P status of the vegetation any further than what was observed following only 3 years of treatment. This indicates that soon after N addition commenced the vegetation responded and adjusted to the change in available N by lowering P content in the tissues and that this response was rapid and is not a slow cumulative effect. Thus, the degree of plant P limitation (or rather P status, since NPP is not limited but rather physiological changes to increase P uptake and

utilization efficiency) has not increased considerably over the past 10 years. This may suggest that once sufficient N was added some other variable not tested here limits NPP and that the plants likely have optimal growth conditions to which they are adapted.

Concentrations of C in the bulk above ground vegetation and in Avena and related C:N and C:P ratios also show significant differences for N addition treatments (lower C:N due to N increase and higher C:P due to P decrease in plant tissue). These ratios, while not utilized regularly as biogeochemical indicators for P stress, can be used to determine if the parameters that were modified in the JRGCE resulted in changes in the nutrient composition of the plant litter, which may affect microbial degradation processes and have long-term implications on the ecosystem.[32]

The $\delta^{13}C$ and $\delta^{15}N$ data of the bulk vegetation and of Avena reflect the background values and the source values of the treatment additions. Ambient values of $\delta^{13}C$ and $\delta^{15}N$ ranged between −27‰ to −31‰, and 20‰ to 30‰, respectively, while the plots receiving CO_2 had $\delta^{13}C$ of −35‰ to −40‰, and the plots receiving N had $\delta^{15}N$ values in the range of 11−15‰ (SI Figure S2).

The previous study[7] reported that some of the treatments with elevated precipitation showed slight but significant responses toward a decrease in P demand (in both a decrease in enzyme activity and an increase in aboveground P). We did not observe a response of P limitation indicators to precipitation, possibly due to the extended drought conditions during the period of our study when even the increased precipitation treatment (150% over ambient) was relatively low and did not increase NPP. In our study we observed a small response to CO_2 addition; comparing ambient and elevated CO_2 treatments for *Avena* sp showed a small increase in tissue C:N and C:P ratios (more C and less P incorporation into the
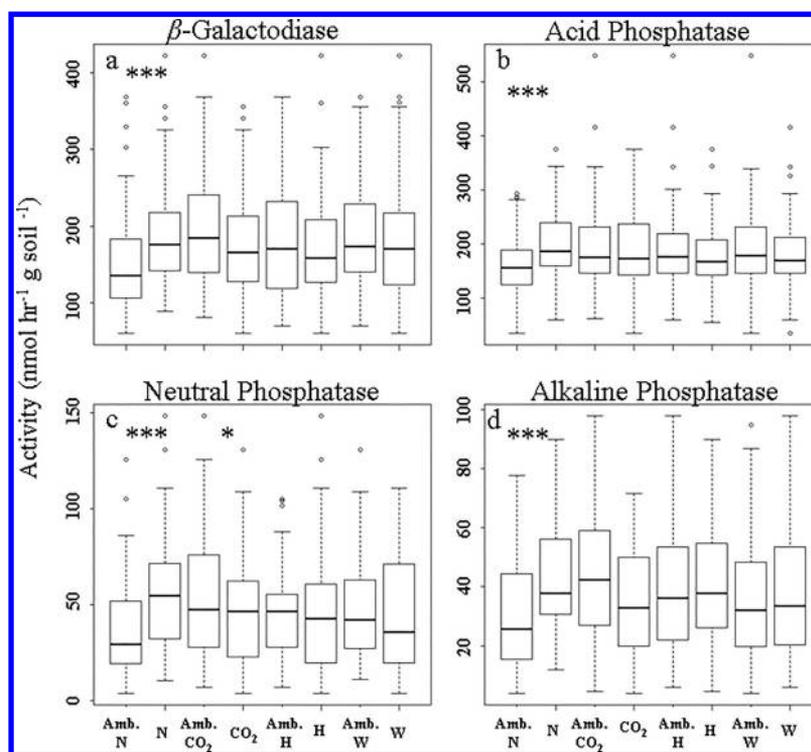
Figure 4. Soil enzyme activities at the JRGCE. Significance of main factors is shown: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. (a) shows β-Glucosidase activity, (b) acidic phosphatase activity, (c) neutral phosphatase activity, (d) alkaline phosphatase activity.

cell biomass in high CO_2), indicating that C fixation was not limited by P availability and pointing toward some plasticity in nutrient requirements. However, the response of nutrient concentrations and ratios to CO_2 is small relative to the larger and clearer response observed with the addition of N. These results indicate that N availability is a larger driver of C:nutrient ratios of both bulk vegetation and *Avena* sp. Other work has documented reduced plant P concentrations in response to elevated CO_2 concentrations.[33] However, in our study this response was limited to Avena and the bulk aboveground biomass showed no significant response to anything other than N addition across all indices. These data indicate that when CO_2 is added in addition to N, the move toward P limitation caused by N deposition may be enhanced in *Avena* sp., but not in the community as a whole. Thayer (2008)[34] investigating P limitation in *Geranium dissectum*, the dominant dicot at the JRGCE, also noticed that foliar P levels were suboptimal for growth under combined elevated CO_2 and elevated N treatments. As a whole, the role of CO_2 on P limitation appears to be small compared to that of N deposition, restricted to select species, and may be responsive to interactions with other factors.

Calculated above ground total P (e.g., P concentrations we measured multiplied by the total above ground biomass in each plot) ranged from 0.43 to 0.89 g m^{-2} when using the Avena P concentration data for the calculation (for comparison to earlier study). The average ambient value was 0.72 g m^{-2}, while treatments including N addition averaged 0.76 g m^{-2}. These values are twice as high as the values reported by Menge and Field (0.35 g m^{-2} for no N addition and 0.30 g m^{-2} for N addition treatments). The difference in the total above ground P between these two studies may indicate a progressive transfer of P from the soil to the biomass, however this cannot be determined based on only two time points and may instead

result from considerable year to year variability in both P concentrations in Avena (average ~0.7 mg g^{-1} in 2003 and ~1 mg g^{-1} in 2013) and in total above ground biomass (average ~0.42 g m^{-2} in 2003 and ~0.76 g m^{-2} in 2013); more work should be done to investigate this. When calculating the aboveground total P using the bulk above ground biomass P content to determine total biomass P, the average for plots without N addition P content is 0.86 g m^{-2}, and the N addition treatments were almost identical averaging 0.84 g m^{-2}. The similarity in total biomass P for each plot for treatment with or without N addition, using either the average Avena concentrations or bulk above ground vegetation concentrations (e.g., g P in all biomass in a plot normalized to m^{-2}) suggest that the plots with vegetation that had lower P per gram plant material (e.g., receiving N addition) had higher biomass per plot, likely due to the N fertilization effect which increased the biomass per plot (a trend observed throughout the study).[12] It is possible that the fertilization effect on biomass production reflects changes in community structure and functioning or soil processes, rather than the direct growth response of plants to nutrient availability and nutrient ratios. This suggests that P overall had minimal effect on the biomass yield, consistent with our conclusion that the system has not reached P limitation status and that the plants in this ecosystem have considerable plasticity in modulating cellular nutrient ratios to match availability and maximize NPP and growth. This is also consistent with our observation that there was no evidence of progressive P limitation, likely because the increase in NPP did not require more P. P concentrations indicative of P limitation (<0.7 mg g^{-1}[35]) and other signs of P limitation such as the accumulation of carbohydrates, the inhibition of photosynthesis, a reduced N content, and accelerating leaf senescence[30] are also not observed at the JRGCE.

3.2. δ^{18}O of water and *A. barbata* phosphate. δ^{18}O of soil water extracts range in value between −4.29 to −7.24 (average −5.58‰) and did not differ between the treatments. Values of δ^{18}O of phosphate ($\delta^{18}O_P$) in *A. barbata* ranged from ∼25−31‰ (SI Figure S3) and there was no significant differences among treatments mostly due to the large variability among replicates within each treatment.

Investigation of the $\delta^{18}O_P$ of *Avena* tissue was performed to see if differences between treatments and specifically P availability could be observed in this isotope ratio. Although intracellular dissolved phosphate undergoes fast and reversible hydrolysis catalyzed by the enzyme pyrophosphatase (PPase) resetting the $\delta^{18}O_P$ value of phosphate taken up from the environment to a new value in equilibrium with ambient temperature and the cellular water isotopic composition[13] it may be possible that when P is available in great excess to demand (e.g., replete conditions) the intracellular PPase mediated reaction would be slower than P uptake rates from the environment resulting in offsets from equilibrium toward environmental values (e.g., values closer to equilibrium at P limited conditions). All $\delta^{18}O_P$ of *Avena* tissue fall well above the equilibrium range calculated for soil water and soil temperatures during the growing season and within equilibrium with leaf water and the growing season temperatures. This is consistent with other studies[36] and suggests that P cycling within the living cells is fast and not affected by the degree of P availability in the soil relative to P demand by the plants and that the P content of the plant tissue does not have a measurable effect on the isotope ration of P in these plants (at least within the values measured at our study site). We did not see a clear systematic impact of the increased temperature on the biomass $\delta^{18}O_P$, but the expected effect of 1−1.5 °C temperature increase on $\delta^{18}O_P$ is relatively small and within the analytical and calibration errors and could be masked by other effects like the range in leaf water isotopes, plant composition in the plots and other variables.[37] Hence at least for the JRGCE the plant phosphate oxygen isotope ratios are not useful indicators of P status.

3.3. Soil Enzyme Activity. When considering all ambient versus elevated interactions together, N addition caused a significant increase in activity across all enzymes measured (Figure 4). Additionally elevated CO_2 shows a slightly significant increase in the activity of neutral phosphatase. This present study measured three phosphate utilizing enzymes and one carbon utilizing enzyme, and found activity by all four enzymes increased by 22−48% in treatments with elevated N. The majority of the observed activity was attributable to acidic phosphatase and β-Glucosidase, which had increased activities of 22% and 24%, respectively. Menge and Field[7] also measured phosphatase enzyme activity, and observed a 14% increase in the treatments containing elevated N. Despite differences in methodology, the large increase in activity (from 14% to >20%) indicates a real and consistent increase in activity since the initial study. Enzyme activities are an indicator for microbial nutrient demand, and are also sensitive to environmental conditions.[38,39] This increase in phosphatase enzymes might suggest that to sustain cellular P demand as N increased over the past decade, more intensive hydrolysis and use of organic P compounds in the soil is needed by plants, microbes, or both. For example, it is possible that the increased P uptake by plants could indirectly be driving microbial P demand, even if N addition does not directly alter microbial P demand. Having said that, there has been consistent evidence at the JRGCE that

the microbial community is very responsive to N demand.[17,28,40−42] The consequence then of an N deposition driven chronic increase in microbial P demand could be an increasing shift where more of the soil available phosphorus is bound in unavailable microbial biomass. Depending on rates of microbial turnover, this could further lead to plant phosphorus limitation.

Soil enzyme activity by treatment for acidic phosphatase, alkaline phosphatase, a comparative assay of phosphatase activity at neutral conditions, and β-Glucosidase are shown in SI Figure S4. All enzyme activities showed a significant response to N addition.

The response of phosphatase tested at neutral pH (a mix of acid and alkaline phosphatase activities) to CO_2 is interesting and along with the response of a decrease for some plant species in foliar P concentrations indicates that in some circumstances, the elevated CO_2 can also play a role in impacting P demand and utilization by some of the plants. This is not surprising as multiple factors influence enzyme activity, such as soil moisture and temperature.[28,43,44] In regards to soil moisture, the year Menge and Field sampled the Avena and aboveground biomass (2001) was an average rain year, whereas the years monitored in this study were during a drought period with lower rainfalls (roughly 50% of average in 2011−2012 rain year and 60% of average in 2012−2013) which would if anything result in lower enzyme activities (enzyme activity has been shown to be very responsive to precipitation both in the JRGCE[28] and elsewhere[43,44]). We note that enzyme activities have been measured multiple times over several years at the JRGCE, in 2002,[7] 2004−2006,[28] and here in 2013. Based on this cumulative evidence and persistent trends we conclude that an elevated production of phosphatase enzymes is not due to precipitation effects and appears to be persistent and increasing over time in treatments receiving elevated N suggesting that elevated N induced physiological responses to increase P utilization to maintain NPP levels.

3.4. Implications. Our analyses of indicators for P status suggest that N addition is the only consistent factor that affects P dynamics in this grassland system and that there is only a very small cumulative effect of N deposition on P status, as the indicators measured have changed little after more than a decade of continuous N addition since the last study. Indeed, Zhu et al. (2016)[12] observed no cumulative effects of the manipulations on NPP in the JRGCE over the 16 years of treatment, and concluded that, unlike observations at other sites that indicated cumulative effects, factors unique to this location may be in play at Jasper Ridge, such as the large interannual variability in weather which gives advantage to species that have a wide tolerance range to environmental conditions.[12] An additional factor that may limit the response to cumulative N deposition at Jasper Ridge is colimitation by multiple nutrients, (e.g., the need for another micronutrient to enhance growth further), such that N additions alone only have a limited and finite effect. This work further demonstrates the complexity of predicting how ecosystems will respond to changing environmental conditions as interactions between different parameters (manipulated or natural) make it difficult to predict the response of any one species or the community as a whole to any one or multiple variables.

The measurements made in this study are consistent with previous work suggesting that N deposition increases P demand and changed plant nutrient quotas but we also show that indicators of P limitation of the system have increased only

slightly since the work done by Menge and Field.[7] These results suggest that the system responded quickly to N additions after the start of the experiment by increasing enzyme activity and lowering the P content of the biomass, which is reasonable given that many N and P addition experiments in grasslands observe rapid responses to increased nutrients.[5,45] Moreover we see that the changes in P dynamics after this initial response are slow. It is interesting and worthwhile to note that, despite the duration of the experiment, the relative abundance of the main plant functional groups seems minimally affected by the altered conditions.[12] This may be due to the weedy and/or annual nature of many of the dominant species at this site. Treatment effects on $\delta^{18}O_P$ of *A. barbata* were not observed, indicating that $\delta^{18}O_P$ in the water leachable pool of *A. barbata* quickly reach equilibrium with leaf water and is not sensitive to plant P availability, hence limiting the applicability of $\delta^{18}O_P$ as an indicator of P limitation.

The changes we record following N fertilization (high N:P and C:P, and low P and C:N), while not indicative of severe P limitation, are likely to affect the functioning of terrestrial vegetation both directly (through the physiological responses of plants) and indirectly, through responses of herbivores and decomposers, which can feed back on nutrient availability to plants, and through changes in plant−microbe interactions. For example plants with a high N:P ratio generally allocate less biomass to roots than plants with similar growth rate but a low N:P ratio[30,46] impacting soil microbial processes. Moreover, in the fertilized plots the species composition may change[47] affecting the total biomass as well as the herbivores community with potential effects on food-web dynamics. It has been shown that over longer time scales if P becomes limiting species with lower P demand or those with inherently high N:P ratios will become more dominant.[48] These changes may correspond to a reduction in species richness since a negative correlation between species richness and grasses biomass has been reported.[49] While these effects have not been observed at this location, they must be taken into consideration in other systems. These effects are expected to interact with other effects of global climate and environmental change and with effects of ecosystem management and should be considered in future research.

ASSOCIATED CONTENT

⑤ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b04362.

Boxplots, correlation tables and the ANOVA analysis of the four individual enzyme data (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Overall project initiation, development and coordination were headed by A.P. Sample collection was done by T.M., C.S., Nona Chiariello, and JRGCE staff. Sample processing was performed by T.M., C.S., K.D., and J.G. Data analysis was performed by T.M., A.L., D.D., K.R., J.G., A.P. Study site, design, maintenance, and auxiliary information provided by C.F.

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