

Intercropping has little effect on root functional traits in a novel perennial grain-legume cropping system

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Abstract

Perennial grain crops are a promising new tool for reducing agricultural impacts on global change because their extensive root systems build soil organic matter and retain nutrients, thereby increasing soil C storage and reducing nutrient export. The potential for advancing sustainability goals is even more pronounced if perennial crops are managed with agroecological principles, such as manipulating species diversity for reduced external inputs. Intercropping with legumes is an ecological nutrient management practice that has the potential to supply organic nitrogen, but this depends on the establishment of the intercrop and associated expression of functional traits. However, the impacts of intercropping on trait expression and ecosystem function in perennial grain systems is poorly understood. To address this gap, we examined variation in root functional traits and soil microbial functions in a field experiment established in 2019 with intermediate wheatgrass (*Thinopyrum intermedium*; IWG) and alfalfa (*Medicago sativa*), a common forage legume. We measured five root functional traits and microbial enzymatic activity in five cropping treatments: (i) an IWG monocrop with fertilizer addition; (ii) an IWG-alfalfa intercrop; (iii) an unfertilized IWG monocrop (iv) an IWG-alfalfa intercrop from which alfalfa shoots were removed throughout the growing season to simulate a dual-use grain and forage system; and (v) an alfalfa monocrop. We found that cropping treatment had little effect on root functional traits and soil microbial enzymatic activity, suggesting that crop life history and genetic controls over belowground form and function were more influential than agricultural management, and that different cropping treatments with IWG and alfalfa provided similar substrates for microbial growth. Root N content and specific root length of IWG in the intercrops were significantly higher than in the unfertilized monocrop, whereas root N was not different between fertilized and intercropped IWG. This suggests intercropping IWG with alfalfa supports a more acquisitive

resource allocation strategy in IWG. Moreover, it suggests that a dual-use intercrop is a viable management strategy for sustaining ecosystem function. This research expands our understanding of root trait expression in novel perennial cropping systems, which will help us refine ecological management strategies that increase agricultural sustainability.

Introduction

Agricultural landscapes, in which annual cropping systems predominate, face persistent challenges to maintaining high levels of ecosystem functioning as a result of low diversity, bare fallows, frequent tillage, and chemical inputs. These practices result in soil erosion, increased water and nutrient exports, loss of soil organic matter, and reduced abundance of associated biodiversity (e.g., pollinators) (Crews et al. 2016, Ryan et al. 2018). This motivates ongoing efforts to breed new perennial grain crops with traits that can mitigate some of the consequences of annual cropping systems (Crews et al. 2016). Crop functional traits, in particular, are the measurable characteristics of organisms that define how they modulate and respond to their environment (Martin and Isaac 2015, Streit and Bellwood 2022, Schipanski et al. 2014). Understanding the range of functional trait expression in perennial cropping systems and its interaction with key ecosystem services is critical to designing multifunctional systems. Yet, to date, the impacts of intercropping on trait expression and ecosystem function in perennial grain systems is poorly understood. The present study addresses this knowledge gap by characterizing variation in root functional traits and microbial community functioning in a perennial grain-legume intercropping experiment.

Through their life-history and functional traits, such as extensive root systems and the translocation of nutrients to roots prior to senescence, perennial crops can enhance ecosystem functions beyond what conservation practices in annual cropping systems can achieve (Crews et al. 2016, Ryan et al. 2018). These effects can be further enhanced by applying principles of ecological nutrient management (ENM), an approach to manage the biogeochemical cycles that underlie agricultural soil fertility (Drinkwater and Snapp, 2022). ENM broadly aims to build soil organic matter, minimize nutrient loss, reduce bare fallows, and diversify cropping systems to

enhance nutrient cycling (Drinkwater and Snapp 2022). For instance, replacing synthetic fertilizer inputs with biological nitrogen (N_2) fixation through intercropping perennial grains with legumes holds promise for further reducing N losses and promoting synchrony between plant N demand and N supply (Crews et al. 2022). Additionally, these intercrops can be managed for both grain and forage production. Known as dual-use systems, this option is of particular interest to farmers as the intercropped legume offers the possibility of reducing fertilizer inputs, as well as an additional source of income.

Although much less known than aboveground traits, belowground traits of plants, soil microorganisms, and soil fauna, drive many community- and ecosystem-level processes (Freschet et al. 2021a, Philippot et al. 2013, Bergmann et al. 2020). In agroecosystems, key functions of primary interest to farm-level management—in addition to productivity—are nutrient cycling (Blesh 2018, Emmett et al. 2020), soil moisture regulation, and weed and pest suppression (Schipanski et al. 2014). Root trait expression can influence all these processes; for instance, traits associated with soil space occupancy and root lifespan and turnover are highly important to ecosystem carbon (C) and N cycling as they drive the distribution and frequency of belowground litter inputs to soil (Freschet et al. 2021a). However, basic documentation of root functional traits is generally lacking. Moreover, root traits respond to both abiotic and biotic factors, yet these responses are not well constrained. For instance, specific root length—a trait associated with soil exploration and nutrient acquisition—responds inconsistently to soil fertility levels (Freschet et al. 2021b). For novel perennial grain crops, our knowledge of root functional trait variation is even more limited.

Our current knowledge of root trait variation is to a large degree hampered by the difficulty of sampling specific functional classes of roots—for instance, absorptive, transport, and storage—which is necessary to making comparisons across plant species or environmental conditions

(Freschet and Roumet 2017). Lower root orders (i.e., more distal) are generally thin and N-rich, take up nutrients from soil, and support mycorrhizal colonization. Higher root orders, on the other hand, are generally thicker and longer-lived, performing transport and storage functions (Freschet and Roumet 2017, Valenzuela-Estrada et al. 2008). In woody species, the three most distal orders can serve as a reasonable approximation of the absorptive fine roots, but this has not yet been demonstrated in herbaceous plants (Freschet and Roumet 2017). In herbaceous species, absorptive roots may constitute up to 100% of all roots of diameter equal to or lower than 2 mm (McCormack et al. 2015). This, coupled with their ease of implementation, makes diameter-based cut-offs the most popular way of sampling fine roots (Freschet et al. 2021b). Still, such cut-offs do not adequately capture variation in root functioning and may miss functional differences that arise as the roots of herbaceous perennials age (Zobel 2016).

Plant communities and their functional traits interact with soil microbial communities to drive agronomically-important biogeochemical cycles. Indeed, soil microbial growth relies on the decomposition of plant litter, while the release of bioavailable nutrients in this process feeds plant nutrient uptake (Zak et al. 2011). By altering the biochemical composition and quantity of shoot and root litter that enters the soil food web, agricultural management can change the composition and function of soil microbial communities. For instance, diversified crop rotations increase soil hydrolytic enzyme activity and the rate of organic N cycling (Bowles et al. 2022, Breza et al. 2023), whereas diversification practices like cover cropping and intercropping with legumes increase N mineralization, a microbial function (Stratton et al. 2022). A study with annual wheat and soybean revealed that intercropping is associated with both changes in root traits and biomass allocation, an increase in rhizosphere acid phosphatase activity, and higher root P concentration under P-limited conditions (Bargaz et al. 2017). It is still unknown, however, whether species

interactions mediate similarly coordinated changes in root trait expression, biomass allocation, and microbial metabolic activity in field conditions and in perennial crops.

In this study, we assessed whether variation in root functional traits and soil microbial function, particularly extracellular enzymatic activity, mediate the interaction between agricultural management and ecosystem function in novel perennial grain-legume cropping systems with intermediate wheatgrass (*Thinopyrum intermedium*; IWG) and alfalfa (*Medicago sativa* L.). Specifically, we asked: 1) How does intercropping IWG with alfalfa affect root functional traits and soil microbial functions? And 2) Do these effects vary when alfalfa shoots are removed (i.e., in dual-use intercrops)? We measured a subset of morphological, architectural, and chemical root traits (5 traits total) selected for their relevance to root soil exploration, N acquisition and cycling dynamics, and interactions with soil microbiota (Freschet et al. 2021a). We hypothesized that mean root functional trait values for each species would differ between intercrops and monocrops due to species interactions and varying soil N availability. More specifically, we predicted that root N content of both IWG and alfalfa would be higher in the intercrop than in an unfertilized monocrop, as competition from the grass would induce increased N fixation by the legume, in turn increasing N supply in those plots (Blesh 2019, Schipanski and Drinkwater 2011). We did not predict a direction of change for morphological or architectural traits, because the ecological controls on variation in these traits in herbaceous plants are still largely unknown (Freschet et al. 2021a, Freschet et al. 2021b, Kumordzi et al. 2019). Lastly, we hypothesized that the activity of microbial extracellular enzymes that degrade labile C compounds and acquire organic N would be higher in the IWG rhizosphere in intercrop plots, relative to an unfertilized monocrop, whereas the opposite would be true for the alfalfa rhizosphere. This, we reasoned, would result from higher alfalfa litter N inputs in the intercrops.

Methods

1.1 Experimental design

Samples for this project were collected during the third year of a field experiment planted in September 2019 on a 0.5 ha field at the U-M Campus Farm with a Fox sandy loam soil series. Previously, the plot had been in a 30-year unmanaged fallow with occasional mowing. Prior to establishing the experiment in 2019, a cover crop mixture of sorghum sudangrass and buckwheat was planted to suppress weed growth. Composted poultry manure (Revita Pro 5-4-5; 45 kg N ha⁻¹, 36 kg P ha⁻¹, 45 kg K ha⁻¹) and rock phosphate (22 kg P ha⁻¹) was then applied to all plots ahead of planting, following soil test result recommendations. The experiment was planted in a randomized complete block design with 4 replicate blocks and eight treatments. Of these, five treatments were considered in the present project: i) an intermediate wheatgrass (*Thinopyrum intermedium*; IWG) monoculture, with 57-cm row intervals (IWG-mono); ii) an intercrop of IWG and alfalfa (*Medicago sativa* L.), in which two rows of alfalfa were planted at 19-cm intervals between rows of IWG (IWG+Alf); iii) a monoculture of IWG that was amended with alfalfa shoots 2-3 times in the growing season (IWG+Alf shoots); iv) a dual-use alfalfa-IWG intercrop, where alfalfa shoots were cut and removed (transferred to Treatment 3 plots) 2-3 times in the growing season (IWG+Alf roots); and v) an alfalfa monoculture with 19-cm row intervals (Alf-mono). Table A1 in the Appendix summarizes data collected in the context of the broader experiment.

All treatment plots measured 4 m x 11 m, with a 6 m edge path on all four sides. Treatment 1 (IWG-mono) plots were split pots, where one third of the plot received no further fertilizer inputs following the initial 2019 poultry manure and rock phosphate application. The remaining two-thirds of the plot annually received an additional 80 kg N ha⁻¹ as bloodmeal in early April, which during the year of this project occurred on April 12, 2022. All root and soil samples from Treatment

1 IWG monocultures were taken from the fertilized portion of the split plots, which more closely reflected farmer practices and produced a more agronomically-relevant control. Treatments 3 and 4 allow us to partition the effects of alfalfa above- and belowground biomass inputs on key ecosystem functions, which is important to informing dual-use management systems, where shoot biomass is harvested. As no legume cuttings and transfers had yet taken place at the time of sampling, in this study we consider Treatment 3 an unfertilized IWG monocrop.

1.2 Rhizosphere sampling

Rhizosphere samples were taken on May 23, 2022. Four samples were taken with a 2-cm diameter probe (20 cm depth) immediately adjacent to the stems of representative plants in each plot (in the intercrops, 2 samples were taken adjacent to IWG plants and 2 adjacent to alfalfa plants). Given the high density of roots underneath these perennial crops, we reasoned that soil samples taken immediately adjacent to the plant stem would be, to a great extent, under the influence of roots and substantially different from the soil between rows with lower root density. Push probe samples were homogenized at the species-by-plot level, such that two samples (one for IWG and another for alfalfa) were produced from the intercropped plots. A subsample of unsieved soil was then collected and stored at 4 °C for later analysis of microbial biomass. The remaining soil was sieved in the field with a 2-mm sieve and divided into two subsamples, for DNA extraction (stored at -80°C) and potential extracellular enzyme activity assays (stored at -20°C).

1.3 Root sampling

Fine root samples were collected on May 31, 2022, when IWG was flowering and its demand for nutrients was expected to be highest. Samples were collected one week prior to the first scheduled mowing of alfalfa shoots. Legume mowing likely affects patterns of belowground

allocation and, while it may affect species interactions in the intercrops following mowing, testing this effect was beyond the scope of the present study.

Roots were collected by taking 8-cm diameter cores directly over target plants to 20 cm depth (Freschet et al. 2021b). Three plants of each species present were sampled in all plots (“root trait samples”). Three additional cores were taken between rows in the IWG and alfalfa monocrops; roots collected from these cores were used to estimate plot-level root biomass only (“biomass samples”). Even though alfalfa and IWG roots extend from 2 to close to 4 m depth (Clément et al. 2022, Rasmussen et al. 2021), we sampled to 20 cm as previous research suggests that the majority of rhizosphere impacts on soil C and N cycling occur in the topsoil (Finzi et al. 2015). Roots were washed with a wet sieving method and separated into two size classes, captured on 2-mm and 0.5-mm sieves. Coarse roots, including alfalfa tap roots and lignified IWG roots greater than approximately 5 mm in diameter, were removed from the root trait samples and dried and weighed separately for plot-level root biomass. Then, 10 and 3 g subsamples of root material were taken from the 2-mm sieve and 0.5-mm sieve and frozen at -20°C for subsequent imaging and analysis of root traits. The remaining roots were dried at 60°C, weighed, and analyzed for C and N concentration in a LECO TruMac CN Autoanalyzer.

1.4 Root imaging

Frozen samples were thawed and one random subsample of each was taken for analysis on a WinRhizo scanner. Samples captured on the 2-mm sieve were suspended in water in a glass case and cleaned of debris. All samples (from 2-mm and 0.5-mm sieves) were then suspended in water in an acrylic case and arranged to minimize overlap. Scanned images were analyzed with WinRhizo software to determine root diameter (RD, mm) and specific root length (SRL, cm g⁻¹). SRL was calculated as the average root length in a sample (from WinRhizo output) divided by the

dry mass of roots in the sample (Freschet et al. 2021b). Root branching density (RBD, cm^{-1}) was measured on root segments of 0.5 to 0.6 mm in diameter (as determined by WinRhizo) in the 2-mm sieve samples. Briefly, we used ImageJ software to measure the length of a root segment from the point where the first lateral intersects it, through to the end of the segment. For consistency, the shortest “end” (portion of parent root segment past the last lateral intersection) was the one included in the length measurement. RBD was then calculated as the number of laterals per length of parent root. RBD was measured on 5 root segments per sample. Following scanning, both the scanned and unscanned portions of the subsamples were separately dried at 60 °C and weighed.

1.5 Rhizosphere microbial functions

To assess the effect of cropping treatment and rhizosphere microbial functions we measured potential extracellular enzyme activity (EEA) of three hydrolytic enzymes produced to degrade cellulose C (β -1,4,-glucosidase, BG) or access organic N (leucine aminopeptidase, LAP, and β -N-acetyl-glucosaminidase, NAG) (Emmett et al. 2020, Blesh and Ying 2020). Our aim in performing this assay was to determine whether the decomposition of organic N by soil microbial communities is associated with N cycling dynamics at the plot level. Extracellular enzyme activity was measured by making soil slurries with 1 g soil and 40 mL 50 mM sodium acetate buffer, which was previously adjusted to match the average plot-level soil pH (\pm 0.5). Slurries were homogenized with a blender, then pipetted into 96-well black plates, along with the respective enzyme substrates and fluorescing agents. Plates were incubated in a dark space for 4 hours (for BG and NAG) and 20 hours (for LAP). We calculated enzyme concentrations based on fluorescence, which was measured using a Synergy HT microplate reader (Gen5, version 2.00.18, BioTek, Winooski, Vermont, USA) at 365 nm. Finally, we calculated the ratio of BG to the sum

of NAG and LAP concentrations, to better understand the microbial communities' relative energy and nutrient demands.

To enable comparisons of microbial activity across treatments, we measured microbial biomass through direct chloroform extraction using the same rhizosphere soil samples (Gregorich et al. 1990). Briefly, two replicate soil slurries per sample were prepared with 10 g of soil and 40 ml 0.5 K₂SO₄. 0.5 ml chloroform were then added to one of these slurries and all jars (including chloroformed and non-chloroformed replicates) were shaken at 150 revolutions for 4 hours, then centrifuged at 453 g for 10 minutes. Samples were then filtered through Fisher brand Q2 filters, bubbled for 20 minutes, then stored at -20°C until further processing. Once thawed, all samples were filtered with a Whatman puradisc 0.45µm syringe filter and analyzed on a Shimadzu TOC-TN (Shimadzu Scientific Instruments, Columbia, MD).

1.6 Soil properties

Because root traits are influenced by both environmental and management factors, in our analyses, we used data on soil chemical and biological properties collected in 2021 and 2022 for the broader experiment. These included potentially mineralizable carbon (PMC; i.e., an indicator of microbial activity), measured in samples taken in August 2021, and cumulative N mineralization rates over the three weeks during which root and rhizosphere sampling took place for this experiment.

PMC was measured as the flush of CO₂ during a 1-day incubation of rewetted soil, adapting the method in Franzluebbbers et al. (2000). Ten g samples of air-dried soil were weighed into 50 mL centrifuge tubes with airtight lids and brought to approximately 50% water-filled pore space (WFPS) with deionized water. The CO₂ concentration was measured by sealing the tubes and immediately taking a 0.5-mL sample of headspace gas, which was analyzed in a Li-Cor LI-820

infrared gas analyzer (Li-Cor Biosciences, Lincoln, NE). The sealed tubes were then incubated for 24 hours in the dark at 25 °C. The CO₂ concentration was measured again at the end of the incubation and one-day CO₂-C was determined as the difference between the time zero and day one concentrations.

Nitrogen mineralization was measured through an *in-situ* incubation. Briefly, six 2-cm push probe soil samples were randomly collected from each plot to 20-cm depth, then homogenized and sieved to 2 mm in the field. Triplicate 10-g subsamples were immediately extracted with 2 M KCl and frozen at -20 °C for later analysis of extractable inorganic N (nitrate and ammonium) in a discrete analyzer (AQ2, Seal Analytical, Mequon, Wisconsin, USA). Then, an additional six cores were collected from each plot, placed immediately in polyethylene bags (one sample in one bag), and buried near the sites where they were taken at a depth of approximately 5-cm for a three-week incubation. At the end of the incubation period, bags were recovered and any bags that were seriously damaged discarded. Soil samples were homogenized at the plot level, sieved to 2 mm and extracted with 2 M KCl for extractable inorganic N analysis, as was done with the baseline samples. Cumulative N mineralization over the three-week period was calculated by subtracting the initial amount of inorganic N from the amount measured at the end of the incubation.

1.7 Data analyses

Treatment effects on root traits, microbial biomass C and N, and extracellular enzymatic activity (EEA) were analyzed with mixed effects ANOVA models in R using the *nlme* package. EEA measurements were transformed with the natural logarithm prior to analysis due to non-normality. As we were primarily interested in comparing root traits and rhizosphere functions within a species (e.g., comparing monocropped IWG to intercropped IWG), we ran separate

ANOVA models for IWG and alfalfa samples. All models were run with treatment as a fixed effect and block as a random effect. Post hoc tests were run with the *emmeans* package (“emmeans” function) in R.

To probe patterns in root functional trait variation, we conducted principal components analyses (PCA) to separately describe patterns of covariation among traits in the 2-mm and 0.5-mm sieve classes. We repeated this analysis at the species-level to explore whether relationships between traits differ in the two crops. All PCAs were run using the “princomp” function in the base R package.

To assess whether root functional trait expression in the intercrops diverged from expectations based on monocrop trait values, we measured the multidimensional distance between samples based on all five root functional traits and biomass. Specifically, we measured the distance between IWG and alfalfa samples in the full intercrop (Treatment 2), as well as the distance between the unfertilized monocropped IWG and monocropped alfalfa. This latter represents the “predicted” distance between IWG and alfalfa if root functional traits do not show plasticity when intercropped. Distance calculations were performed with the euclidean method using the “funct.dist” function from the *mFD* package in R.

Results

Species-level analysis of root traits and biomass across management systems

Our study provides valuable new documentation of root functional trait expression in intermediate wheatgrass (IWG) and alfalfa. Of the measured traits, most differed by species with the exception of root diameter (RD, in both 2-mm and 0.5-mm sieve samples) and root branching density (Tables 2 and 3). IWG had higher C:N and root biomass, and lower root N content (RN) and specific root length (SRL), than alfalfa.

Table 1. Mean, standard deviation, and coefficients of variation (in parenthesis) of root trait values and biomass of 2-mm sieve class for each species across all management systems.

	IWG (N = 16)	ALF (N = 12)
Specific root length, SRL (cm g ⁻¹)	10833.11 +/- 3469.25 (32.02)	19227.19 +/- 6955.69 (36.18)
Root diameter, RD (mm)	0.67 +/- 0.03 (5.11)	0.67 +/- 0.02 (3.44)
Root branching density, RBD (cm ⁻¹)	5.37 +/- 0.94 (17.63)	5.19 +/- 0.67 (12.95)
Root N content, RN (%)	1.17 +/- 0.10 (8.76)	1.67 +/- 0.14 (8.13)
Root C content, RC (%)	42.98 +/- 2.04 (4.76)	43.10 +/- 2.92 (6.76)
Root C:N, CN (%)	37.15 +/- 3.48 (9.37)	25.92 +/- 2.80 (10.79)
Biomass in trait core (kg ha ⁻¹)	17364.68 +/- 5260.69 (30.30)	6751.07 +/- 2593.38 (38.41)

Table 2. Mean, standard deviation, and coefficient of variation (in parenthesis) of root trait values and biomass of 0.5-mm sieve class for each species across all management systems.

	IWG (N = 16)	ALF (N = 12)
Specific root length, SRL (cm g ⁻¹)	17156.47 +/- 3647.33 (21.26)	18747.60 +/- 3144.96 (16.78)
Root diameter, RD (mm)	0.72 +/- 0.05 (6.94)	0.75 +/- 0.04 (5.17)
Root N content, RN (%)	1.83 +/- 0.10 (5.69)	2.10 +/- 0.09 (4.28)
Root C content, RC (%)	36.93 +/- 3.31 (8.97)	37.56 +/- 2.41 (6.41)
Root C:N, CN (%)	20.38 +/- 0.92 (4.52)	17.87 +/- 1.09 (6.09)

Biomass in trait core (kg ha ⁻¹)	5773.74 +/- 1428.33 (24.74)	4371.11 +/- 923.83 (21.13)
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Our analysis of IWG and alfalfa root functional trait values by cropping treatment revealed that, on the whole, root functional traits for these two crops vary little in response to intercropping. Indeed, there were no significant treatment effects on most of the traits measured with two notable exceptions: root N content and specific root length in the 0.5-mm sieve class. IWG in the full intercrop (IWG+Alf) had significantly higher root N (1.90% vs. 1.69%, $p = 0.005$, fig. 1) than the unfertilized IWG monocrop (IWG+Alf shoots, fig. 1). RN in the fertilized monocrop (IWG-mono, 1.90%) was also significantly higher than that in unfertilized monocrop ($p = 0.005$), and not significantly different from RN of IWG in either of the intercropped treatments (fig. 1). While RN of IWG in the dual-use intercrop (IWG+Alf roots, 1.83%) was not significantly different from T3, it tended to be higher and the range of observations does not overlap with that of IWG+Alf shoots samples. There were no significant, species-level treatment differences in mean RN values in the 2-mm sieve roots, though IWG+Alf tended to have higher RN than IWG+Alf shoots. RN of alfalfa samples was not significantly different across treatments in either the 2-mm or 0.5-mm sieve classes.

Meanwhile, IWG specific root length (SRL) was higher in the full intercrop, relative to the unfertilized monocrop (19944 vs. 13710 cm g⁻¹, $p = 0.0138$, fig. 1). This effect may have been driven by one exceptionally low value in the IWG+Alf shoots samples. Re-running the ANOVA test without this data point resulted in no significant difference between the two treatments ($p = 0.0599$, fig. 2).

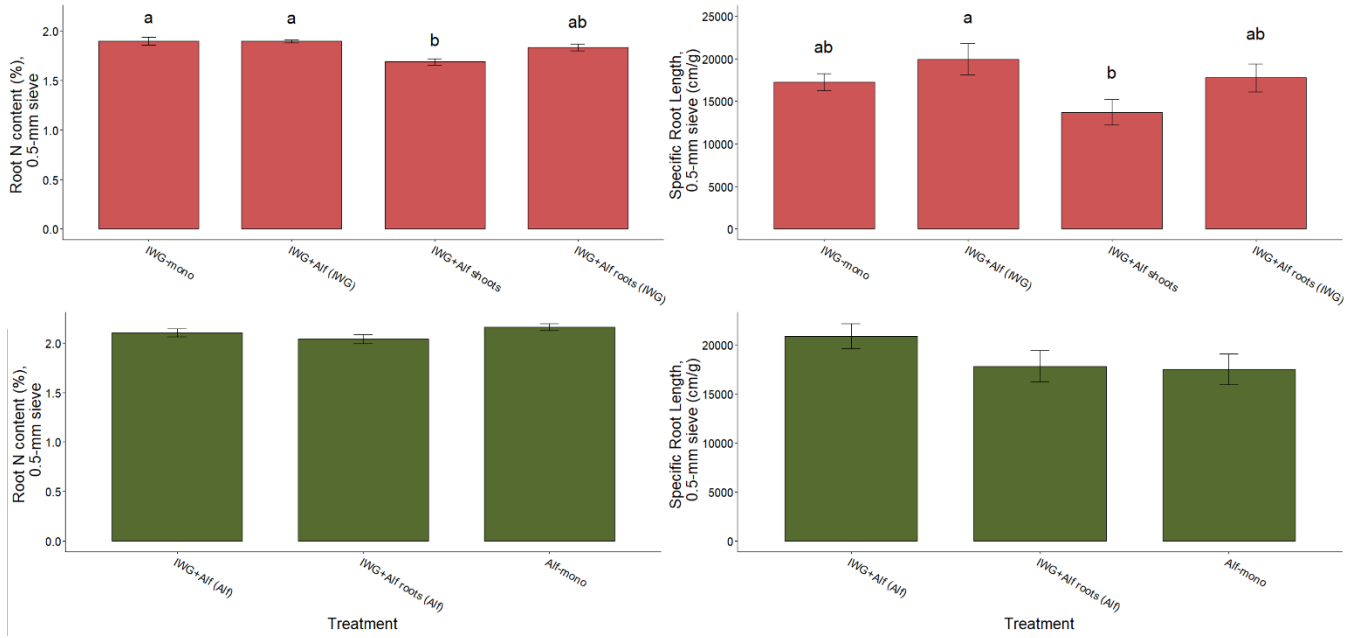


Figure 1. RN and SRL of 0.5-mm sieve roots in IWG (top) and alfalfa (bottom). Different letters indicate significant differences.

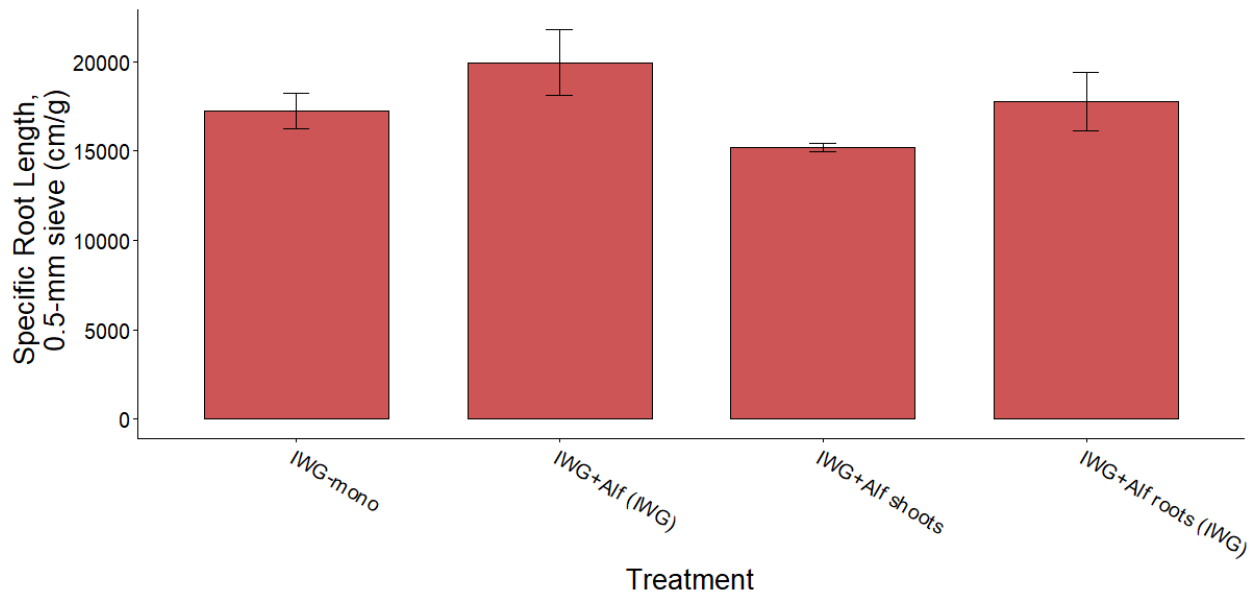


Figure 2. SRL of 0.5-mm sieve roots in IWG after removing lowest value in IWG + Alf shoots.

Aggregating trait observations at the plot level by calculating community-weighted mean (CWM) traits revealed similar patterns. Unsurprisingly, RN of the 0.5-mm sieve class in the alfalfa monocrop was significantly higher than in any other treatment (fig.3), while C:N in the alfalfa monocrop was significantly lower than in any other treatment. Additionally, RN in the fertilized IWG monocrop and both intercrops was significantly higher than in the unfertilized IWG monocrop (p-values ranging from 0.0002 to 0.0051). Meanwhile, SRL in the 0.5-mm sieve class was significantly higher in the full intercrop relative to the unfertilized monocrop ($p = 0.0042$). In the 2-mm sieve class, however, only the alfalfa monocrop had higher SRL than the unfertilized IWG monocrop ($p = 0.0204$). RN and CN in the 2-mm sieve class followed similar patterns, with the only exception that, while higher, RN in the dual-use intercrop (IWG+Alf roots) was not significantly different from the unfertilized monocrop.

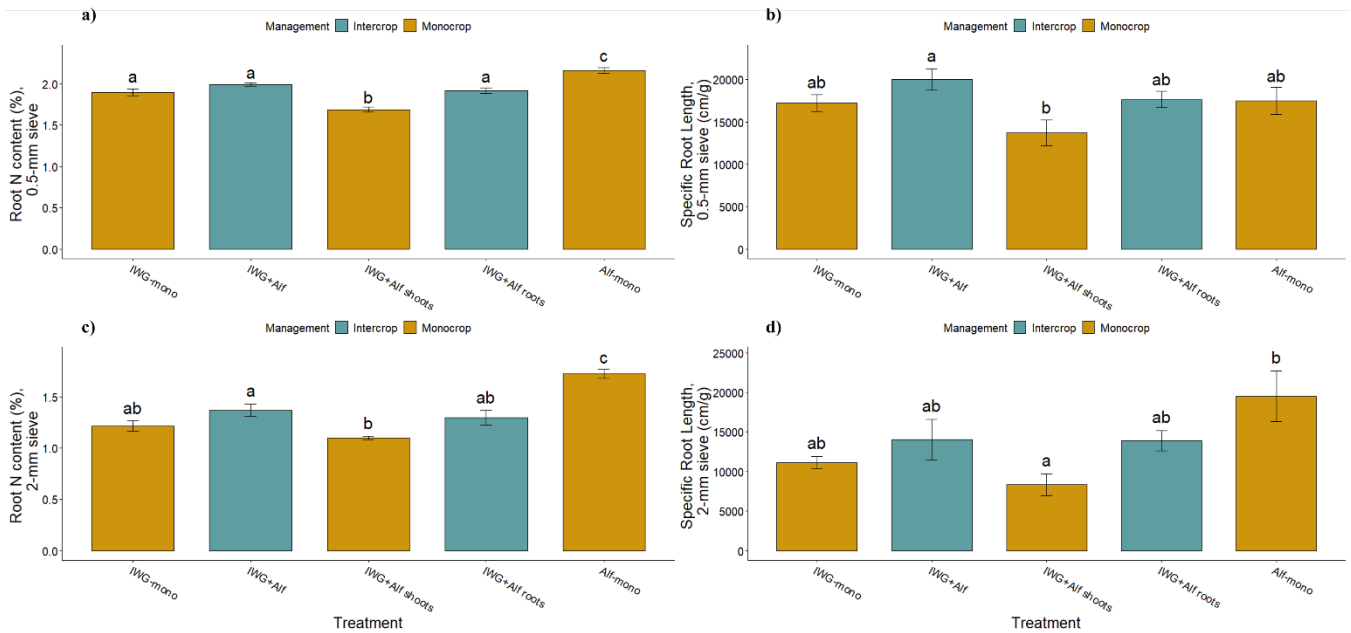


Figure 3. Plot-level RN and SRL of (a, b) 0.5-mm sieve roots and (c, d) 2-mm sieve roots. Different letters indicate significant differences.

Our analysis of root branching density and root diameter did not reveal any species- or plot-level differences among cropping treatments (fig. 4). There was a trend toward lower RBD for alfalfa in the dual-use intercrop (IWG+Alf roots) than in the full intercrop (IWG+Alf), while the monocrop (Alf-mono) showed the greatest variability among alfalfa samples.

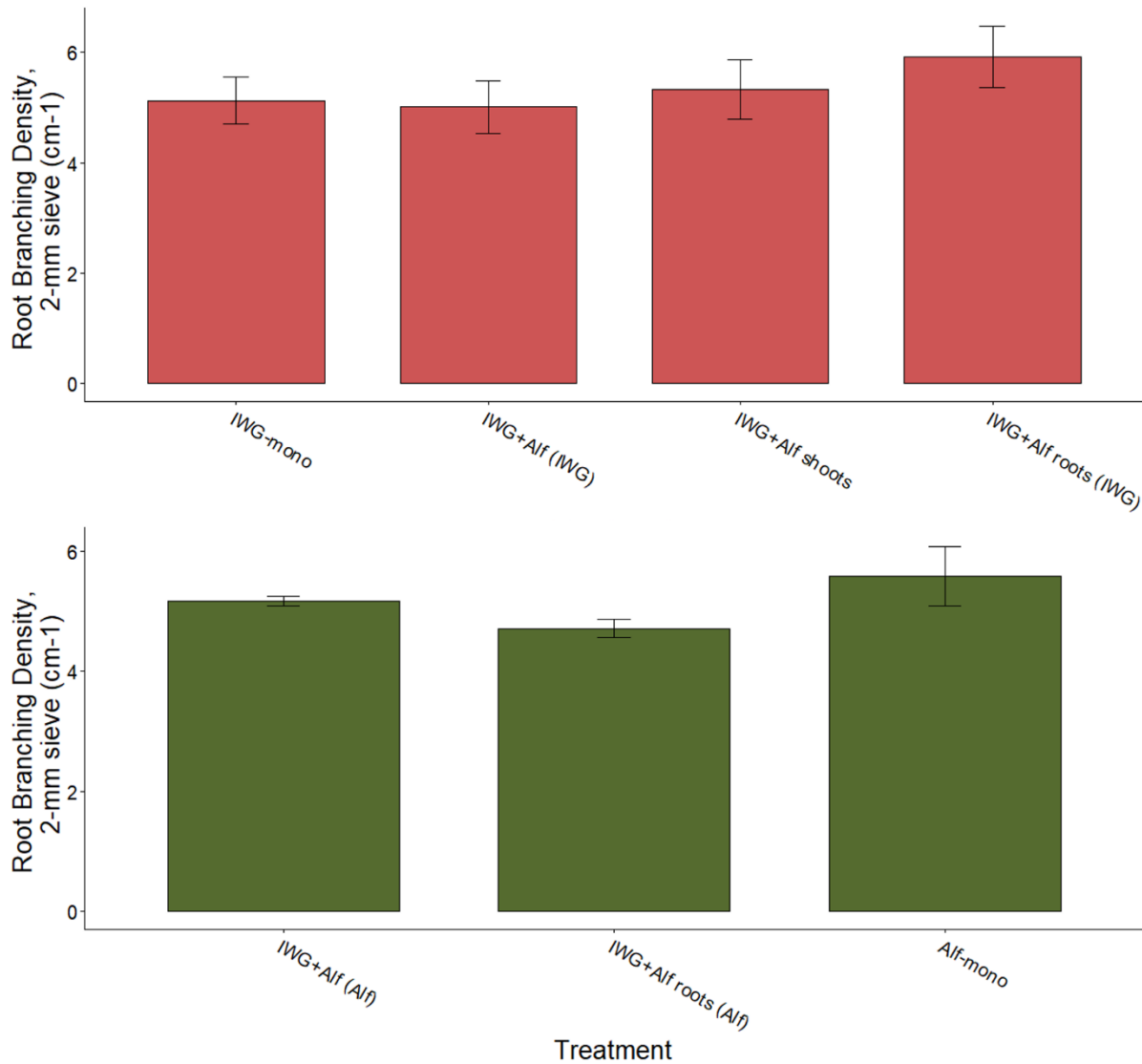


Figure 4. RBD in IWG (top) and alfalfa (bottom).

Considering both root size classes combined, cropping treatment had no significant effect on standing root biomass for IWG or alfalfa measured at the species level (fig. 4). Pairwise comparisons showed that the root biomass of IWG was significantly higher than that of alfalfa,

except when comparing IWG in the full intercrop (18740.60 +/- 8017.33 kg ha⁻¹) to any of the alfalfa treatments (e.g. alfalfa in IWG+Alf with 12822.72 +/- 3190.58). In the dual-use intercrop, IWG root biomass was significantly greater than alfalfa's (23820.47 +/- 4793.06, vs. 10147.12 +/- 2563.00, $p = 0.0028$).

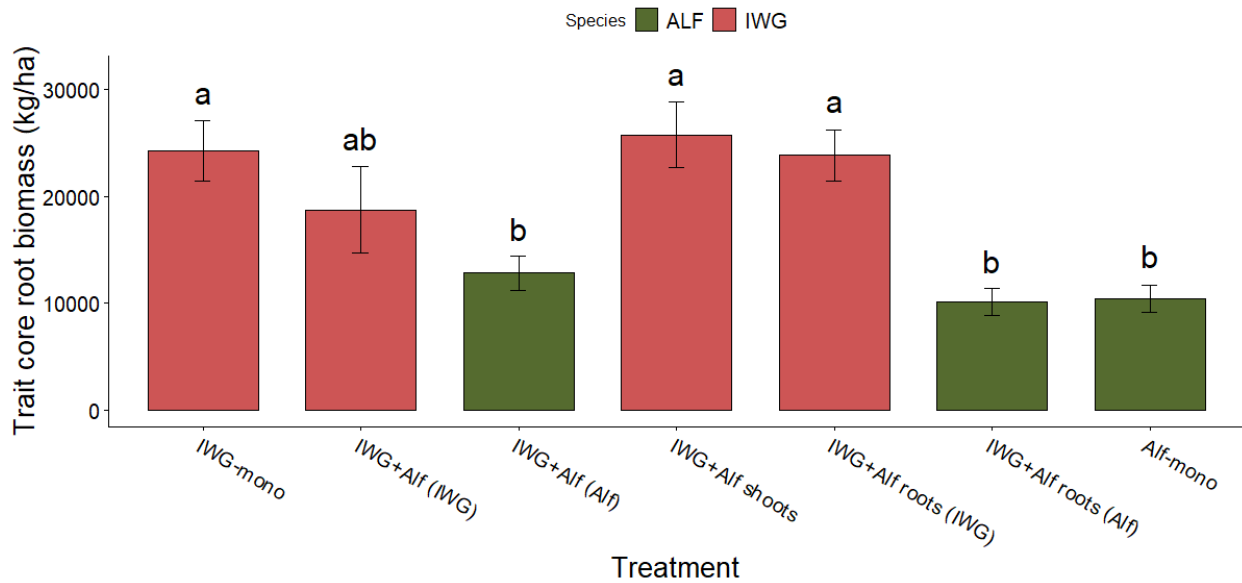


Figure 5. Biomass of root trait core samples. Different letters indicate significant differences.

Principal components analysis of root functional trait variation

Separate principal components analyses (PCAs) for each species revealed that RN and SRL are coordinated with each other in the case of IWG roots, but not alfalfa, where they are orthogonal (fig. 6). This pattern holds for both the 2-mm and 0.5-mm sieve classes; across these four PCAs, the first two principal components explained 65-77% of the variance in root traits. Samples do not cluster by treatment, with the exception of IWG+ALF shoots (T3), especially in the 0.5-mm sieve class analysis.

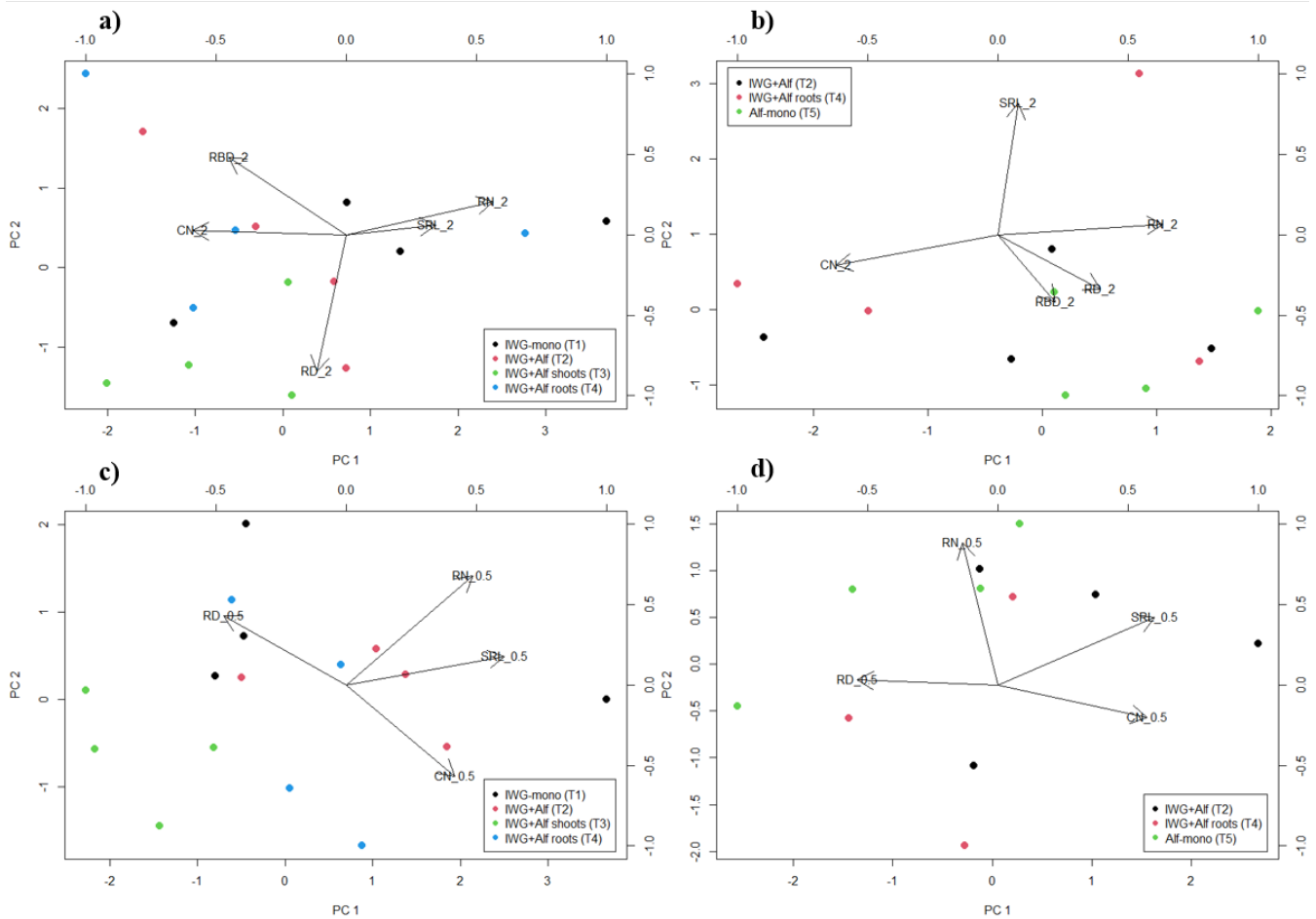


Figure 6. PCAs showing patterns of root trait covariation across cropping treatments within a species: (a) IWG 2-mm sieve roots, (b) alfalfa 2-mm sieve roots, (c) IWG 0.5-mm sieve roots, (d) alfalfa 0.5-mm sieve roots.

Analysis of rhizosphere microbial functions

We tested for treatment effects on the rhizosphere microbial community with mixed effects ANOVAs at the species- (i.e., comparing rhizosphere variables across management systems within a species) and plot-level (comparing rhizosphere variables across management systems, combining IWG and alfalfa samples in the intercrops).

Our analysis revealed no significant differences in microbial biomass C (MBC) and N (MBN) across cropping treatments at either the species- or plot-level (fig. 7). Similarly, the

activities of all three extracellular enzymes were not significantly different across treatments. The activity levels for BG were slightly higher in the rhizosphere of intercropped IWG, relative to the two monocrops (147.9 and 134.4 nmol h⁻¹ g⁻¹ dry soil in IWG+Alf and IWG+Alf roots, vs. 124.9 and 121.1 nmol h⁻¹ g⁻¹ dry soil in IWG-mono and IWG+Alf shoots). This trend remained when BG activity was normalized by microbial biomass C. The highest observed absolute activity was in the alfalfa monocrop (157.8 nmol h⁻¹ g⁻¹ dry soil) and highest relative activity in intercropped alfalfa (IWG+Alf, fig. 8). At the plot level, the full intercrop had the highest relative BG activity, followed by the alfalfa monocrop. NAG activities were slightly higher in the alfalfa rhizosphere than in the IWG rhizosphere across cropping treatments, with the highest mean activity observed again in the alfalfa monocrop (75.1 nmol h⁻¹ g⁻¹ dry soil). This pattern held when normalizing NAG activity by MBC, with the exception that the highest relative activity was observed in alfalfa in IWG+Alf, whereas the mean activity in the alfalfa rhizosphere in IWG+Alf roots was lower and more similar to that in IWG rhizosphere samples from the same cropping treatment (fig. 9). At the plot-level, relative NAG activity was highest in the alfalfa monocrop, followed by the full intercrop (IWG+Alf). Both absolute and relative LAP activities, meanwhile, were similar across cropping treatments (fig. 10).

We also calculated the ratio of extracellular enzyme activities, specifically, the ratio of BG to the combined NAG and LAP activities (i.e., BG:(NAG+LAP)). Generally, this ratio was invariant to cropping treatment at either the species- or plot-level, except when comparing the IWG rhizosphere in the full intercrop (IWG+Alf) and the unfertilized monocrop (IWG+Alf shoots, fig. 12). BG:(NAG+LAP) was nearly significantly lower in IWG+Alf shoots (pairwise comparison $p = 0.0594$; ANOVA treatment effect $p = 0.0448$).

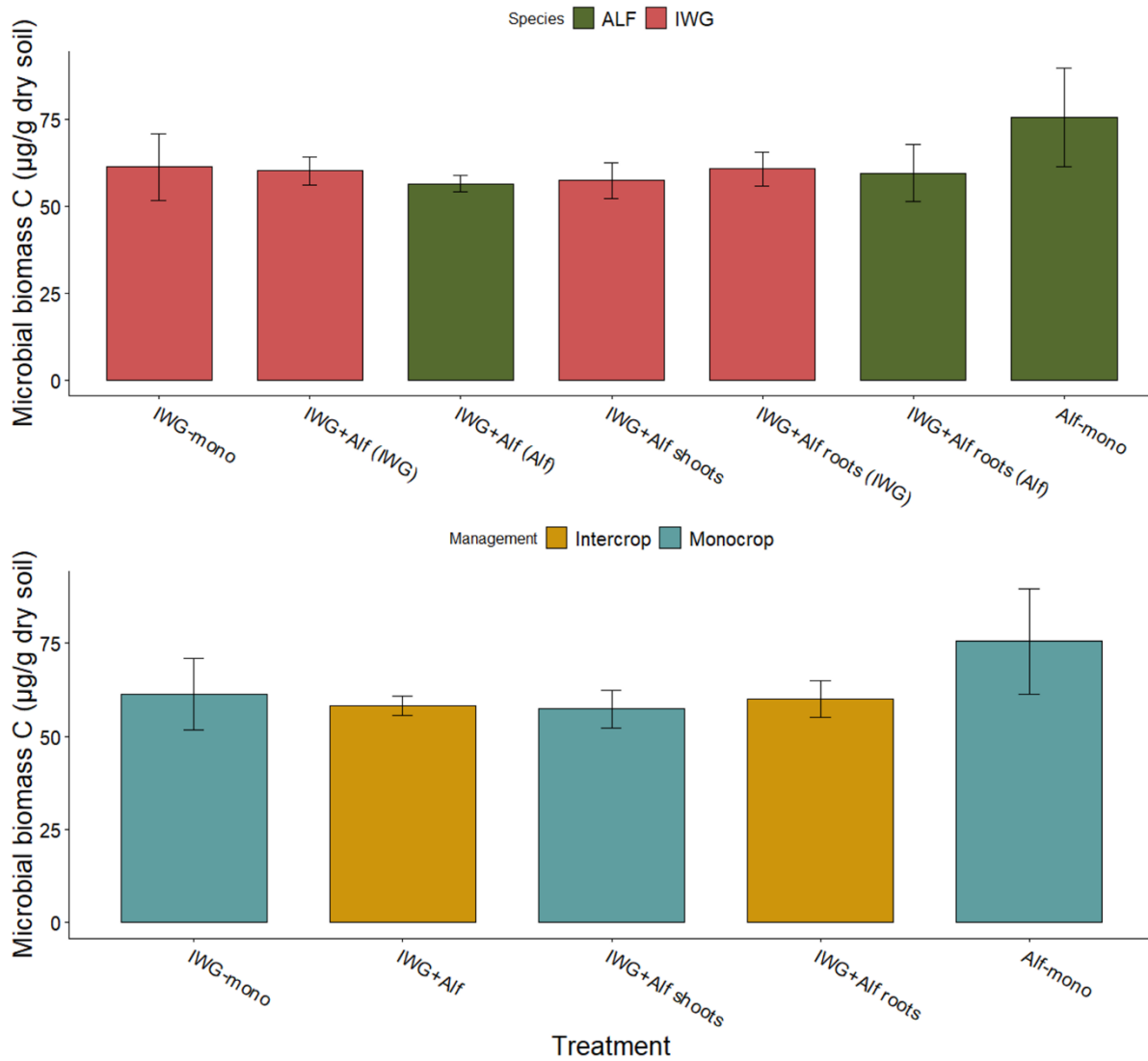


Figure 7. Microbial biomass C in rhizosphere soil of IWG and alfalfa at species-by-treatment level (top) and plot-level (bottom).

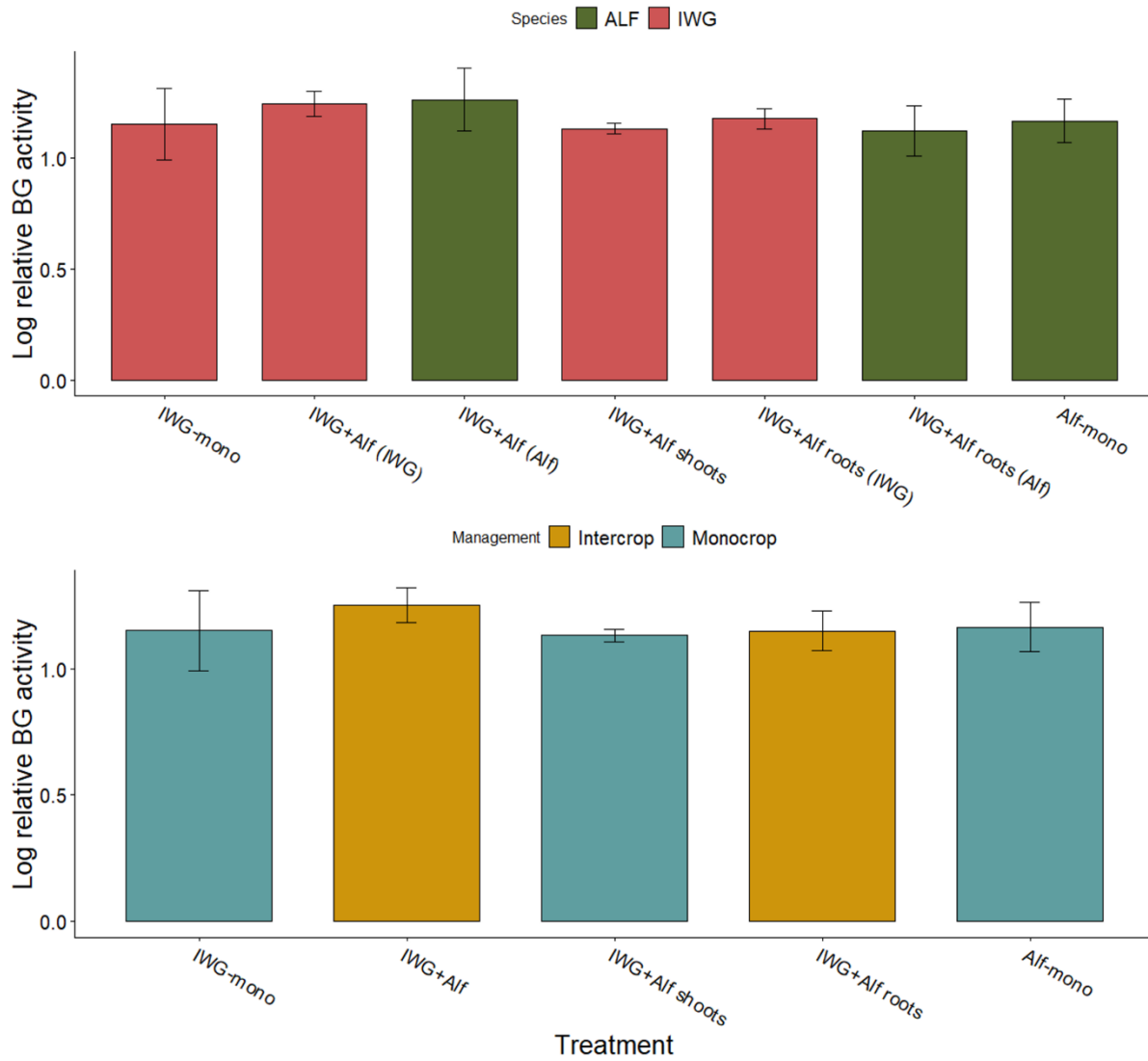


Figure 8. Log relative BG activity in rhizosphere soil of IWG and alfalfa at species-by-treatment level (top) and plot-level (bottom).

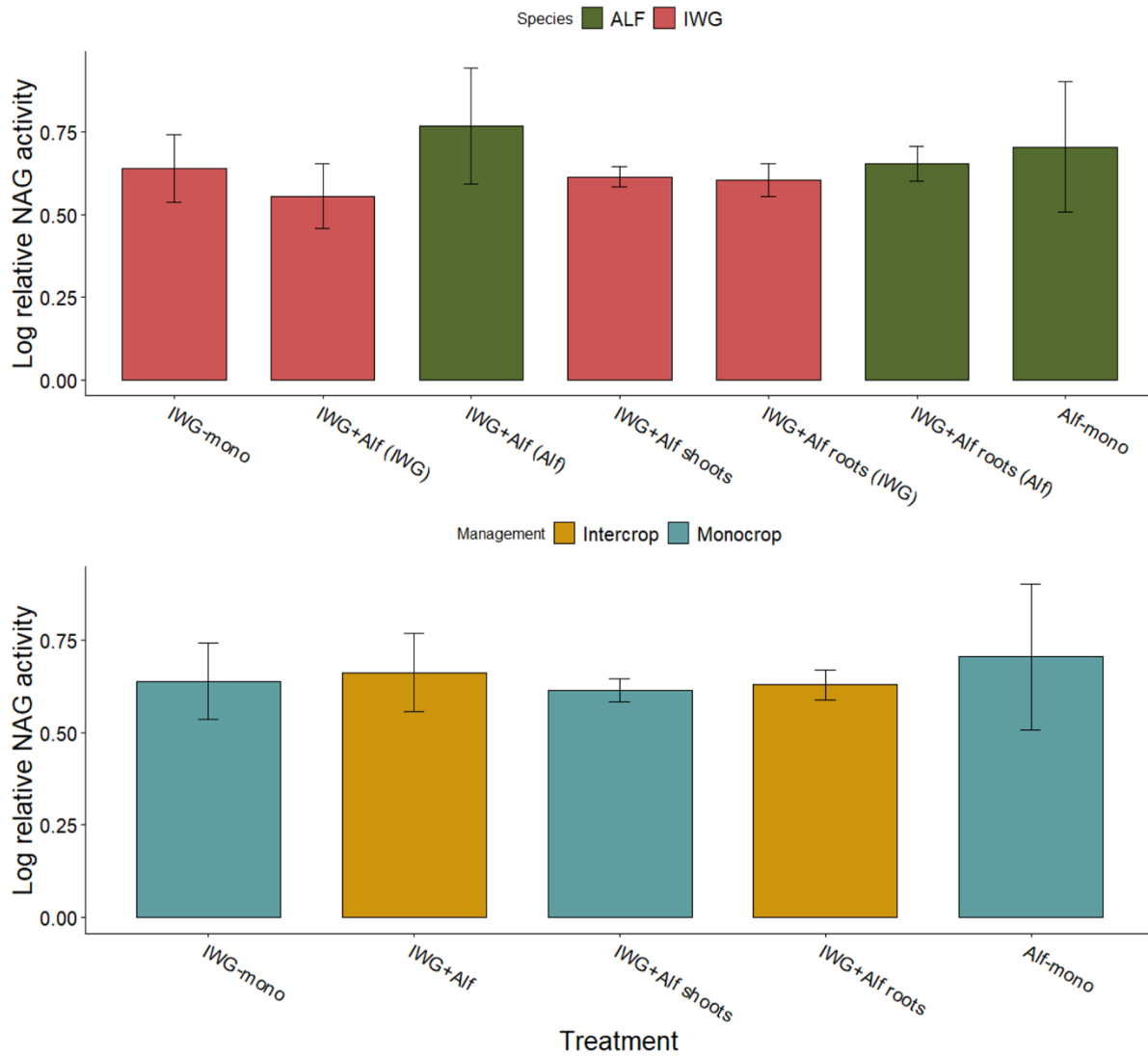


Figure 9. Log relative NAG activity in rhizosphere soil of IWG and alfalfa at species-by-treatment level (top) and plot-level (bottom).

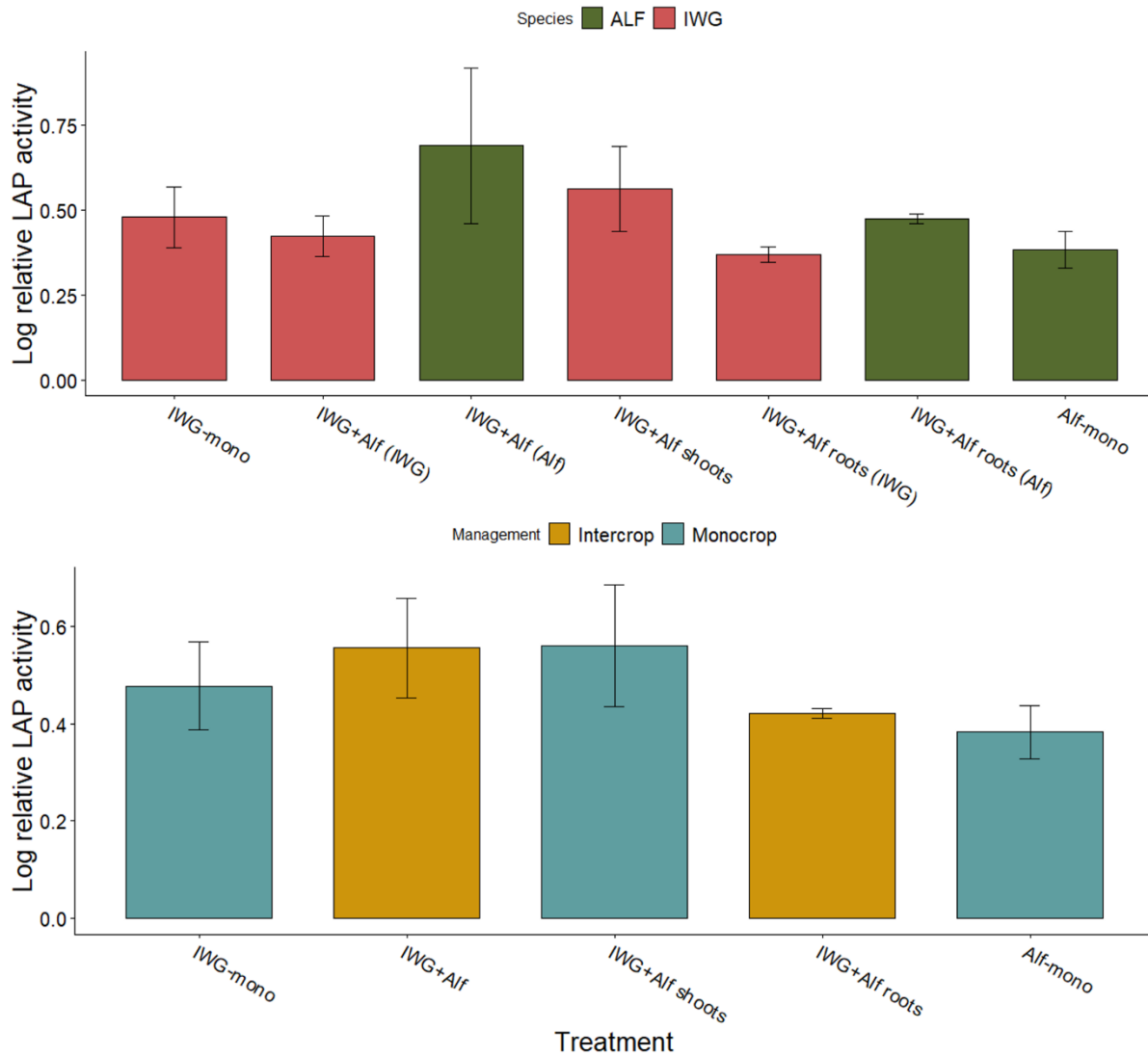


Figure 10. Log relative LAP activity in rhizosphere soil of IWG and alfalfa at species-by-treatment level (top) and plot-level (bottom).

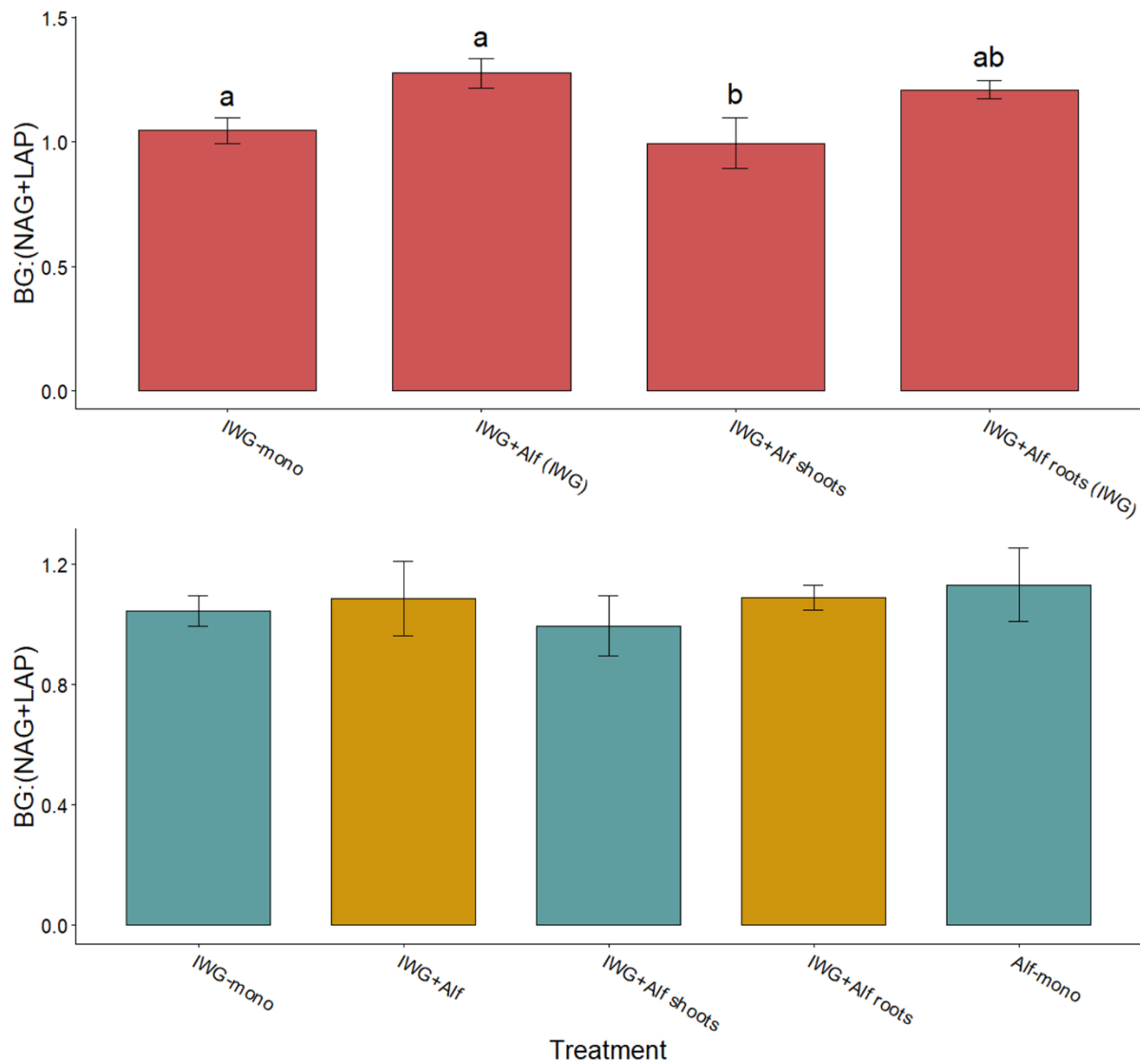


Figure 11. Ratio of BG to NAG and LAP in IWG rhizosphere samples (top) and at the plot-level (bottom). Different letters for IWG+Alf and IWG+Alf shoots sample indicate a nearly significant difference ($p = 0.0594$).

Discussion

We found that ecological nutrient management approaches that pair a perennial legume to a novel perennial grain crop had little effect on root functional trait expression and microbial functions relevant to soil nutrient cycling. Current knowledge of root functional trait expression and its ecological constraints is quite limited, in managed and unmanaged communities alike

(Freschet et al. 2021b, Freschet and Roumet 2017). For IWG and alfalfa, a search for the five traits studied in this project on the global TRY Plant Trait Database yielded observations from only three studies for alfalfa and none for IWG. This study helps fill this gap by documenting root functional trait expression in novel perennial grain-legume cropping systems and its response to species interactions and agricultural management. Our findings suggest that ecosystem function responses to ecological nutrient management in IWG-alfalfa cropping systems are not mediated by differences in root functional traits or soil microbial enzymatic activity at the period in the growing season sampled in this project.

Trait variation for IWG and alfalfa

We predicted that root N content would be higher in IWG roots in the intercrop than in an unfertilized monocrop, as species interactions in the intercrop would induce greater biological N fixation in the legume, increasing total N inputs to soil. Our finding that IWG root N content in the 0.5-mm sieve class was significantly higher in the full intercrop relative to the unfertilized monocrop, but indistinct from the fertilized monocrop, aligns with our expectation and suggests that intercropping alfalfa and IWG supports N acquisition levels that are comparable to moderate fertilization in established IWG stands (2-3 years old). RN is commonly understood as an acquisitive trait indicative of root protein content and nutrient uptake capacity per unit gram of root (Bukovsky-Reyes et al. 2019, Bergmann et al. 2020). Plant resource uptake is reflective of both uptake per unit of root biomass, as well as the size of the entire root system. Given that there were no differences in root biomass, our observations suggest that, at the flowering stage, IWG in the fertilized monocrop and full intercrop may have been taking up soil N at a greater rate than in the unfertilized monocrop. Confirming this, however, would require measuring whole-plant N content. RN of IWG in the dual-use intercrops was not significantly different from the other

cropping treatments, suggesting that this management option—which is currently more economically feasible for farmers—may supply adequate N inputs. However, N pools and fluxes should be measured to corroborate this. That IWG RN tended to be higher in the dual-use intercrop relative to the monocrop with alfalfa shoot additions, which at the time of sampling had last received alfalfa shoot amendments in September 2021, suggests that the presence of alfalfa roots may support more consistent levels of N uptake by IWG than the decomposition of alfalfa shoots transferred to the plot during the growing season. This could result from continuous root litter and exudate inputs into soil food webs, or the tendency for root-derived soil organic matter (SOM) to be physically protected within aggregates (and decompose more slowly than shoot-derived SOM) (Puget and Drinkwater 2001). Measuring RN at various plant growth stages could give us greater insight into the temporal dynamics of N acquisition in these perennial cropping systems and help illuminate the mechanisms behind the well-documented pattern of IWG yield declines around three years following stand establishment (Tautges et al. 2018, Dobbratz et al. 2023).

Like RN, SRL in IWG was lowest in the unfertilized monocrop (0.5-mm sieve class) and significantly different from that in the full intercrop, though this difference was smaller ($p = 0.0599$) when a particularly low SRL observation was removed from the unfertilized monocrop sample. These two traits were positively correlated for IWG in the 0.5-mm sieve roots (Pearson's $r = 0.64$), but not in the 2-mm sieve roots or for alfalfa in either root class. This partly aligns with previous studies in natural populations that have found a positive correlation between RN and SRL, which posit that high RN and SRL constitutes an acquisitive resource allocation strategy (Bukovsky-Reyes et al. 2019, Isaac et al. 2017). Whether the observed differences in SRL and RN actually result in higher levels of resource acquisition, however, depends on the size of the root

system and its distribution in three-dimensional space (i.e., the distribution of root zones of depletion) (Brady and Weil 2002, de Parseval et al. 2017).

Together, our RN and SRL observations indicate that root trait expression in IWG responds to nutrient availability, and further supports our conclusion that the 0.5-mm sieve samples captured a functionally distinct class of fine roots. Interestingly, our observations also align with a previous study of root trait variation in cover crops which found that RN and SRL in cereal rye (a grass) were positively correlated and coordinated with each other in a principal components analysis, but not in hairy vetch (a legume) (Bukovsky-Reyes et al. 2019). Indeed, our principal components analysis on IWG and alfalfa roots of the 0.5-mm sieve class revealed that SRL and RN coordinate with each other for IWG, but are orthogonal to each other in alfalfa. The legumes' ability to fix atmospheric N and the optimization of this trait in crop breeding programs likely produce different relationships between soil resource acquisition, root N content, and specific root length.

Plot-level trait variation

We calculated community-weighted mean trait values in the intercropped treatments to describe root functional trait variation at the plot-level, which could be indicative of ecosystem function at a level that is relevant for agricultural management. Our analysis revealed that fine roots in alfalfa monocrop plots have higher RN and lower CN than those of any other treatment, whereas fertilized IWG monocrops and the two intercrops had higher RN than the unfertilized IWG monocrop. This suggests that intercropping IWG with alfalfa, both as a dual-use intercrop where alfalfa shoots are harvested and a full intercrop where legume shoots remain on the plot, supports similar levels of N uptake as organic fertilizer amendments in an IWG monocrop. Given that, in general, N content promotes faster decomposition of plant litter, this indicates that

perennial grain-legume intercrops, like organic fertilization treatments, may support higher rates of N cycling at the field-level (Zak and Pregitzer 1990, Vitousek 1982, Kaye et al. 2019).

Together with the RN observations, plot-level differences in SRL of the 0.5-mm sieve class suggest that the full intercrop may follow a more acquisitive resource allocation strategy (Bukovsky-Reyes et al. 2019, Isaac et al. 2017) than an unfertilized IWG monocrop, though what magnitude of difference in this trait is actually functionally relevant for the plant and the broader plot ecosystem is unclear. Moreover, as noted above, whether these trait differences result in greater soil exploration and N acquisition also depends on belowground biomass allocation and whole-root system architecture (Duchene et al. 2020, de Parseval et al. 2017), which we did not measure in this study. Finally, it was surprising that SRL of the 2-mm sieve class was significantly higher in the alfalfa monocrop plots relative to unfertilized IWG, as grasses tend to have higher SRL. We reasoned this observation could have resulted from a difference in soil nutrient availability driving a more conservative resource allocation strategy in the unfertilized IWG monocrops. Indeed, IWG has been shown to shift from a more resource-acquisitive to resource-conservative strategy with stand age (Duchene et al. 2020) and this may be accentuated in the IWG+Alf shoots treatment as a result of presumed temporal dynamics of N availability in those plots. Nevertheless, it is important to note that perennial grains associate with arbuscular mycorrhizae (AMF) and that this interaction can trade-off with SRL (Duchene et al. 2020). We did not measure AMF abundance or colonization in our study, and we may therefore have a partial picture of soil exploration function in these perennial grain cropping systems. It should also be noted that the alfalfa monocrop plots sustained considerable pressure from a grass weed during the 2022 growing season, which may have biased the SRL measurements from those plots.

Fine root biomass

Across species, IWG consistently had greater root biomass than alfalfa, except in the full intercrop. Indeed, IWG in T2 was not significantly different from alfalfa in the same treatment, nor alfalfa in any other cropping treatment. This suggests that intercropping with alfalfa, and retaining alfalfa shoots in the plot as a mulch between rows, may harm IWG root production early in the growing season. Meanwhile, alfalfa tended to produce lower root biomass in the dual-use intercrop, where alfalfa shoots were harvested 3 times during the previous growing season and removed from the plot. While this difference was not significant, it nevertheless suggests that a dual-use management strategy, which both promotes aboveground production and removes N-rich aboveground biomass from the plots, may potentially suppress root production in subsequent seasons.

Sampling methods to study root functional traits

In herbaceous species, functional classes of fine roots (e.g., absorptive, transport, storage) are ill-defined, resulting in the popular use of diameter-based cut-offs—most commonly ≤ 2 mm—to delineate the pool of fine roots involved in nutrient scavenging and absorption (Freschet and Roumet 2017). In this study, we sampled roots with 2 different diameter-based cutoffs: on a 2-mm diameter sieve (effectively capturing the entire root system except for tap roots, rhizomes, and lignified roots ≥ 5 mm, which were removed from the samples) and on a 0.5-mm sieve. Our findings of cropping treatment effects on RN and SRL only in the 0.5-mm sieve samples suggest that this group constitutes a functionally distinct class of fine roots with a common response to environmental conditions. The 2-mm sieve samples, on the other hand, may have comprised different functional classes, which is further suggested by the presence of roots across the full range of observed diameters (i.e., very small to very large) in the 2-mm sieve samples' WinRhizo scans. This interpretation is also supported by the higher coefficients of variation for SRL, RN,

and C:N in the 2-mm sieve class relative to the 0.5-mm sieve class. Nevertheless, trait measurements made on entire root systems of herbaceous species can give an aggregate estimation of plant investment in soil exploration (Freschet and Roumet 2017). Our observations of SRL in the 2-mm sieve class therefore suggest that soil exploration was higher in the alfalfa monocrop than in the unfertilized IWG monocrop at this point in the growing season; how this pattern shifts through time remains unclear (Duchene et al. 2020).

Rhizosphere microbial functions

Soil microbial enzymatic activity is closely linked to the decomposition of plant litter, which in turn drives biogeochemical cycling in terrestrial ecosystems (Sinsabaugh et al. 2002). We measured the activities of three hydrolytic enzymes (BG, NAG, and LAP) involved in the degradation and acquisition of cellulose, protein, and chitin to assess whether agricultural management of perennial grain-legume cropping systems affects decomposition dynamics via changes in these microbial functions. The cropping and fertility management practices tested in this study produced no significant difference in extracellular enzyme activity (EEA) in the rhizosphere of IWG and alfalfa, suggesting that soil physical and chemical characteristics may play a greater role in structuring soil microbial functions in these perennial grain-legume cropping systems (McDaniel et al. 2013). At the same time, data on ecosystem processes collected for the broader experiment showed that there were no differences in N mineralization at the time of sampling, or in PMC the summer prior, which suggests that all treatments in this study produced substrates of similar quality and quantity for microbial metabolism. This may be reflective of the fact that the cropping treatments compared in this study incorporate two perennial herbaceous crops that—though they crucially differ in the ability to perform biological N fixation—both maintain extensive root systems and high belowground C inputs with coordinated effects on soil

microbial communities when compared to annual crops (Louarn et al. 2015, Rakkar et al. 2023). Nevertheless, it is important to note that EEA indicates a maximum potential level of enzymatic activity and does not reflect actual metabolic activity in soil, which is affected not only by substrate concentration, but also by environmental conditions and microbial community composition (Sinsabaugh et al. 2002, Burns et al. 2013).

Conclusion

Overall, we found that cropping treatment had little effect on root functional trait expression. This suggests that crop life history and genetic controls over belowground form and function were more influential than agricultural management in an IWG-alfalfa cropping system. Similarly, we found no effect of management system on soil extracellular enzyme activity, suggesting that the cropping systems tested provided similar substrates for microbial growth. In general, at peak flowering, IWG fine roots from a monocrop that last received organic N amendment as alfalfa shoots the previous summer, had lower SRL and RN than fine roots from IWG-alfalfa intercrops, suggesting a more conservative resource allocation strategy and lower N availability in the monocrop. These effects remained when considering community-weighted mean traits at the plot-level, which provide a more synthetic view of ecosystem functioning in a field. These results suggest that intercropping IWG with alfalfa, even in dual-use systems where legume shoots are periodically harvested for forage, can sustain adequate N acquisition in the grass. This is of practical import to farmers who are interested in trialing IWG but daunted by its low grain yields, as it suggests an option that is both economically feasible and advantageous from a soil health management perspective.

Literature Cited

- Allison, S. D., & Vitousek, P. M. (2005). Responses of extracellular enzymes to simple and complex nutrient inputs. *Soil Biology and Biochemistry*, 37(5), 937–944.
<https://doi.org/10.1016/j.soilbio.2004.09.014>
- Bargaz, A., Noyce, G. L., Fulthorpe, R., Carlsson, G., Furze, J. R., Jensen, E. S., Dhiba, D., & Isaac, M. E. (2017). Species interactions enhance root allocation, microbial diversity and P acquisition in intercropped wheat and soybean under P deficiency. *Applied Soil Ecology*, 120, 179–188.
<https://doi.org/10.1016/j.apsoil.2017.08.011>
- Bergmann, J., Weigelt, A., Van Der Plas, F., Laughlin, D. C., Kuyper, T. W., Guerrero-Ramirez, N., Valverde-Barrantes, O. J., Bruelheide, H., Freschet, G. T., Iversen, C. M., Kattge, J., McCormack, M. L., Meier, I. C., Rillig, M. C., Roumet, C., Semchenko, M., Sweeney, C. J., Van Ruijven, J., York, L. M., & Mommer, L. (2020). The fungal collaboration gradient dominates the root economics space in plants. *Science Advances*, 6(27), eaba3756.
<https://doi.org/10.1126/sciadv.aba3756>
- Blesh, J. (2018). Functional traits in cover crop mixtures: Biological nitrogen fixation and multifunctionality. *Journal of Applied Ecology*, 55(1), 38–48. <https://doi.org/10.1111/1365-2664.13011>
- Blesh, J. (2019). Feedbacks between nitrogen fixation and soil organic matter increase ecosystem functions in diversified agroecosystems. *Ecological Applications*, 29(8).
<https://doi.org/10.1002/eap.1986>
- Blesh, J., & Ying, T. (2020). Soil fertility status controls the decomposition of litter mixture residues. *Ecosphere*, 11(8). <https://doi.org/10.1002/ecs2.3237>

- Bowles, T. M., Jilling, A., Morán-Rivera, K., Schnecker, J., & Grandy, A. S. (2022). Crop rotational complexity affects plant-soil nitrogen cycling during water deficit. *Soil Biology and Biochemistry*, *166*, 108552. <https://doi.org/10.1016/j.soilbio.2022.108552>
- Breza, L. C., Mooshammer, M., Bowles, T. M., Jin, V. L., Schmer, M. R., Thompson, B., & Grandy, A. S. (2023). Complex crop rotations improve organic nitrogen cycling. *Soil Biology and Biochemistry*, *177*, 108911. <https://doi.org/10.1016/j.soilbio.2022.108911>
- Bukovsky-Reyes, S., Isaac, M. E., & Blesh, J. (2019). Effects of intercropping and soil properties on root functional traits of cover crops. *Agriculture, Ecosystems & Environment*, *285*, 106614. <https://doi.org/10.1016/j.agee.2019.106614>
- Burns, R. G., DeForest, J. L., Marxsen, J., Sinsabaugh, R. L., Stromberger, M. E., Wallenstein, M. D., Weintraub, M. N., & Zoppini, A. (2013). Soil enzymes in a changing environment: Current knowledge and future directions. *Soil Biology and Biochemistry*, *58*, 216–234. <https://doi.org/10.1016/j.soilbio.2012.11.009>
- Clément, C., Sleiderink, J., Svane, S. F., Smith, A. G., Diamantopoulos, E., Desbrøll, D. B., & Thorup-Kristensen, K. (2022). Comparing the deep root growth and water uptake of intermediate wheatgrass (Kernza®) to alfalfa. *Plant and Soil*, *472*(1), 369–390. <https://doi.org/10.1007/s11104-021-05248-6>
- Crews, T. E., Blesh, J., Culman, S. W., Hayes, R. C., Jensen, E. S., Mack, M. C., Peoples, M. B., & Schipanski, M. E. (2016). Going where no grains have gone before: From early to mid-succession. *Agriculture, Ecosystems & Environment*, *223*, 223–238. <https://doi.org/10.1016/j.agee.2016.03.012>

- Crews, T. E., Kemp, L., Bowden, J. H., & Murrell, E. G. (2022). How the nitrogen economy of a perennial cereal-legume intercrop affects productivity: Can synchrony be achieved? *Frontiers in Sustainable Food Systems*, 6, 755548. <https://doi.org/10.3389/fsufs.2022.755548>
- de Parseval, H., Barot, S., Gignoux, J., Lata, J.-C., & Raynaud, X. (2017). Modelling facilitation or competition within a root system: Importance of the overlap of root depletion and accumulation zones. *Plant and Soil*, 419(1), 97–111. <https://doi.org/10.1007/s11104-017-3321-y>.
- Dobbratz, M., Jungers, J. M., & Gutknecht, J. L. M. (2023). Seasonal plant nitrogen use and soil nitrogen pools in intermediate wheatgrass (*Thinopyrum intermedium*). *Agriculture*, 13(2), 468. <https://doi.org/10.3390/agriculture13020468>
- Drinkwater, L. E., & Snapp, S. S. (2022). Advancing the science and practice of ecological nutrient management for smallholder farmers. *Frontiers in Sustainable Food Systems*, 6, 921216. <https://doi.org/10.3389/fsufs.2022.921216>
- Duchene, O., Celette, F., Barreiro, A., Dimitrova Mårtensson, L.-M., Freschet, G. T., & David, C. (2020). Introducing perennial grain in grain crops rotation: The role of rooting pattern in soil quality management. *Agronomy*, 10(9), 1254. <https://doi.org/10.3390/agronomy10091254>
- Emmett, B. D., Buckley, D. H., & Drinkwater, L. E. (2020). Plant growth rate and nitrogen uptake shape rhizosphere bacterial community composition and activity in an agricultural field. *New Phytologist*, 225(2), 960–973. <https://doi.org/10.1111/nph.16171>
- Finzi, A. C., Abramoff, R. Z., Spiller, K. S., Brzostek, E. R., Darby, B. A., Kramer, M. A., & Phillips, R. P. (2015). Rhizosphere processes are quantitatively important components of terrestrial carbon and nutrient cycles. *Global Change Biology*, 21(5), 2082–2094. <https://doi.org/10.1111/gcb.12816>

- Freschet, G. T., Pagès, L., Iversen, C. M., Comas, L. H., Rewald, B., Roumet, C., Klimešová, J., Zadworny, M., Poorter, H., Postma, J. A., Adams, T. S., Bagniewska-Zadworna, A., Bengough, A. G., Blancaflor, E. B., Brunner, I., Cornelissen, J. H. C., Garnier, E., Gessler, A., Hobbie, S. E., ... McCormack, M. L. (2021). A starting guide to root ecology: Strengthening ecological concepts and standardising root classification, sampling, processing and trait measurements. *New Phytologist*, 232(3), 973–1122. <https://doi.org/10.1111/nph.17572>
- Freschet, G. T., & Roumet, C. (2017). Sampling roots to capture plant and soil functions. *Functional Ecology*, 31(8), 1506–1518. <https://doi.org/10.1111/1365-2435.12883>
- Freschet, G. T., Roumet, C., Comas, L. H., Weemstra, M., Bengough, A. G., Rewald, B., Bardgett, R. D., De Deyn, G. B., Johnson, D., Klimešová, J., Lukac, M., McCormack, M. L., Meier, I. C., Pagès, L., Poorter, H., Prieto, I., Wurzbürger, N., Zadworny, M., Bagniewska-Zadworna, A., ... Stokes, A. (2021). Root traits as drivers of plant and ecosystem functioning: Current understanding, pitfalls and future research needs. *New Phytologist*, 232(3), 1123–1158. <https://doi.org/10.1111/nph.17072>
- Gregorich, E. G., Wen, G., Voroney, R. P., & Kachanoski, R. G. (1990). Calibration of a rapid direct chloroform extraction method for measuring soil microbial biomass C. *Soil Biology and Biochemistry*, 22(7), 1009–1011. [https://doi.org/10.1016/0038-0717\(90\)90148-S](https://doi.org/10.1016/0038-0717(90)90148-S)
- Isaac, M. E., Martin, A. R., de Melo Virginio Filho, E., Rapidel, B., Rouspard, O., & Van den Meersche, K. (2017). Intraspecific trait variation and coordination: Root and leaf economics spectra in coffee across environmental gradients. *Frontiers in Plant Science*, 8. <https://www.frontiersin.org/articles/10.3389/fpls.2017.01196>

- Kaye, J., Finney, D., White, C., Bradley, B., Schipanski, M., Alonso-Ayuso, M., Hunter, M., Burgess, M., & Mejia, C. (2019). Managing nitrogen through cover crop species selection in the U.S. mid-Atlantic. *PLOS ONE*, *14*(4), e0215448. <https://doi.org/10.1371/journal.pone.0215448>
- Kumordzi, B. B., Aubin, I., Cardou, F., Shipley, B., Violle, C., Johnstone, J., Anand, M., Arsenault, A., Bell, F. W., Bergeron, Y., Boulangeat, I., Brousseau, M., De Grandpré, L., Delagrangé, S., Fenton, N. J., Gravel, D., Macdonald, S. E., Hamel, B., Higelin, M., ... Munson, A. D. (2019). Geographic scale and disturbance influence intraspecific trait variability in leaves and roots of North American understorey plants. *Functional Ecology*, *33*(9), 1771–1784. <https://doi.org/10.1111/1365-2435.13402>
- Louarn, G., Pereira-Lopès, E., Fustec, J., Mary, B., Voisin, A.-S., de Faccio Carvalho, P. C., & Gastal, F. (2015). The amounts and dynamics of nitrogen transfer to grasses differ in alfalfa and white clover-based grass-legume mixtures as a result of rooting strategies and rhizodeposit quality. *Plant and Soil*, *389*(1), 289–305. <https://doi.org/10.1007/s11104-014-2354-8>
- Martin, A. R., & Isaac, M. E. (2018). Functional traits in agroecology: Advancing description and prediction in agroecosystems. *Journal of Applied Ecology*, *55*(1), 5–11. <https://doi.org/10.1111/1365-2664.13039>
- McCormack, M. L., Dickie, I. A., Eissenstat, D. M., Fahey, T. J., Fernandez, C. W., Guo, D., Helmisaari, H., Hobbie, E. A., Iversen, C. M., Jackson, R. B., Leppälammil-Kujansuu, J., Norby, R. J., Phillips, R. P., Pregitzer, K. S., Pritchard, S. G., Rewald, B., & Zadworny, M. (2015). Redefining fine roots improves understanding of below-ground contributions to terrestrial biosphere processes. *New Phytologist*, *207*(3), 505–518. <https://doi.org/10.1111/nph.13363>

- McDaniel, M. D., Kaye, J. P., & Kaye, M. W. (2013). Increased temperature and precipitation had limited effects on soil extracellular enzyme activities in a post-harvest forest. *Soil Biology and Biochemistry*, *56*, 90–98. <https://doi.org/10.1016/j.soilbio.2012.02.026>
- Philippot, L., Raaijmakers, J. M., Lemanceau, P., & Van Der Putten, W. H. (2013). Going back to the roots: The microbial ecology of the rhizosphere. *Nature Reviews Microbiology*, *11*(11), 789–799. <https://doi.org/10.1038/nrmicro3109>
- Pugliese, J. Y., Culman, S. W., & Sprunger, C. D. (2019). Harvesting forage of the perennial grain crop kernza (*Thinopyrum intermedium*) increases root biomass and soil nitrogen cycling. *Plant and Soil*, *437*(1–2), 241–254. <https://doi.org/10.1007/s11104-019-03974-6>
- Rakkar, M., Jungers, J. M., Sheaffer, C., Bergquist, G., Grossman, J., Li, F., & Gutknecht, J. L. (2023). Soil health improvements from using a novel perennial grain during the transition to organic production. *Agriculture, Ecosystems & Environment*, *341*, 108164. <https://doi.org/10.1016/j.agee.2022.108164>
- Rasmussen, J., Bodin Dresbøll, D., Lønne Enggrob, K., & Peixoto, L. (2021). A novel ¹⁵N vertical split-root method for in situ estimation of N rhizodeposition. *Geoderma*, *383*, 114782. <https://doi.org/10.1016/j.geoderma.2020.114782>
- Ryan, M. R., Crews, T. E., Culman, S. W., DeHaan, L. R., Hayes, R. C., Jungers, J. M., & Bakker, M. G. (2018). Managing for multifunctionality in perennial grain crops. *BioScience*, *68*(4), 294–304. <https://doi.org/10.1093/biosci/biy014>
- Schipanski, M. E., & Drinkwater, L. E. (2011). Nitrogen fixation of red clover interseeded with winter cereals across a management-induced fertility gradient. *Nutrient Cycling in Agroecosystems*, *90*(1), 105–119. c

- Schipanski, M. E., Barbercheck, M., Douglas, M. R., Finney, D. M., Haider, K., Kaye, J. P., Kemanian, A. R., Mortensen, D. A., Ryan, M. R., Tooker, J., & White, C. (2014). A framework for evaluating ecosystem services provided by cover crops in agroecosystems. *Agricultural Systems*, *125*, 12–22. <https://doi.org/10.1016/j.agsy.2013.11.004>
- Sinsabaugh, R. L., Carreiro, M. M., & Repert, D. A. (2002). Allocation of extracellular enzymatic activity in relation to litter composition, N deposition, and mass loss. *Biogeochemistry*, *60*(1), 1–24. <https://doi.org/10.1023/A:1016541114786>
- Sprunger, C. D., Culman, S. W., Robertson, G. P., & Snapp, S. S. (2018). How does nitrogen and perenniality influence belowground biomass and nitrogen use efficiency in small grain cereals? *Crop Science*, *58*(5), 2110–2120. <https://doi.org/10.2135/cropsci2018.02.0123>
- Stratton, A. E., Comin, J. J., Siddique, I., Zak, D. R., Dambroz Filipini, L., Rodrigues Lucas, R., & Blesh, J. (2022). Assessing cover crop and intercrop performance along a farm management gradient. *Agriculture, Ecosystems & Environment*, *332*, 107925. <https://doi.org/10.1016/j.agee.2022.107925>
- Streit, R. P., & Bellwood, D. R. (2022). To harness traits for ecology, let's abandon 'functionality.' *Trends in Ecology & Evolution*, *0*(0). <https://doi.org/10.1016/j.tree.2022.11.009>
- Tautges, N. E., Jungers, J. M., DeHaan, L. R., Wyse, D. L., & Sheaffer, C. C. (2018). Maintaining grain yields of the perennial cereal intermediate wheatgrass in monoculture v. bi-culture with alfalfa in the Upper Midwestern USA. *The Journal of Agricultural Science*, *156*(6), 758–773. <https://doi.org/10.1017/S0021859618000680>
- Vitousek, P. (1982). Nutrient cycling and nutrient use efficiency. *The American Naturalist*, *119*(4), 553–572. <https://doi.org/10.1086/283931>

Zak, D. R., Host, G. E., & Pregitzer, K. S. (1989). Regional variability in nitrogen mineralization, nitrification, and overstory biomass in northern Lower Michigan. *Canadian Journal of Forest Research*, *19*(12), 1521–1526. <https://doi.org/10.1139/x89-231>

Zak, D.R. & Pregitzer, K.S. (1990). Spatial and Temporal Variability of Nitrogen Cycling in Northern Lower Michigan. *Forest Science*, *36*(2), 367–380. <https://doi.org/10.1093/forestscience/36.2.367>

Zobel, R. W. (2016). Fine roots – functional definition expanded to crop species? *New Phytologist*, *212*(2), 310–312. <https://doi.org/10.1111/nph.14099>

Appendix

Table A1. Summary of data collected in the context of the broader experiment within which the present study was nested. Values in parentheses are standard deviations.

Treatment	Year	Aboveground Biomass		Total Root Biomass (to 40 cm depth)	IWG Grain Yield	Cumulative N Mineralization Rate	PMC	fPOM Total N	oPOM Total N
		Alfalfa (kg/ha)	IWG (kg/ha)	--kg/ha--	--kg/ha--	--(mg N / kg soil/ day)--	--(μ g CO ₂ -C/g soil/day)--	--g/kg--	--g/kg--
1	2022 (sampling year)	----	6567 (697)	5574 (592)	567 (83)	0.965 (0.165)	----	----	----
2	2022 (sampling year)	921 (250)	4952 (1705)	6553 (962)	457 (198)	0.687 (0.238)	----	----	----
3	2022 (sampling year)	----	7364 (1250)	5099 (975)	635 (234)	0.515 (0.061)	----	----	----
4	2022 (sampling year)	846 (275)	5493 (743)	7369 (1778)	547 (101)	0.613 (0.070)	----	----	----
5	2022 (sampling year)	3750 (658)	----	4804 (870)	----	0.711 (0.036)	----	----	----
1	2021	----	4166 (1676)	6985 (1834)	58 (28)	0.605 (0.108)	89.4 (16.6)	0.061 (0.021)	0.1 (0.029)
2	2021	2933 (639)	2000 (554)	8411 (2165)	58 (17)	0.675 (0.111)	85.4 (21.3)	0.033 (0.008)	0.063 (0.028)
3	2021	----	2068 (349)	7844 (2221)	31 (8.5)	0.554 (0.114)	71.3 (6.6)	0.046 (0.011)	0.083 (0.035)
4	2021	2455 (625)	1977 (1032)	7045 (2195)	39 (22)	0.617 (0.132)	63.0 (19.8)	0.038 (0.015)	0.059 (0.029)
5	2021	5833 (725)	----	6820 (1062)	----	0.658 (0.144)	78.6 (17.6)	0.049 (0.005)	0.084 (0.017)

1	2019 (baseline)	----	----	----	----	----	----	0.076 (0.016)	0.062 (0.013)
2	2019 (baseline)	----	----	----	----	----	----	0.068 (0.011)	0.072 (0.015)
3	2019 (baseline)	----	----	----	----	----	----	0.082 (0.01)	0.075 (0.005)
4	2019 (baseline)	----	----	----	----	----	----	0.072 (0.007)	0.072 (0.029)
5	2019 (baseline)	----	----	----	----	----	----	0.071 (0.022)	0.074 (0.021)