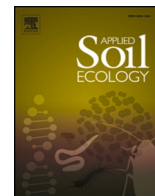




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Wetland soil microbial responses to upland agricultural intensification and snail invasion

Yuxi Guo^{a,*}, Chase M. O'Neil^a, Elizabeth Boughton^b, Willm Martens-Habbena^c, Jiangxiao Qiu^{a,d,*}

^a School of Forest, Fisheries, and Geomatics Sciences, Fort Lauderdale Research and Education Center, University of Florida, Davie, FL 33314, USA

^b Archbold Biological Station, Buck Island Ranch, 300 Buck Island Ranch Road, Lake Placid, FL 33852, USA

^c Department of Microbiology and Cell Science, Fort Lauderdale Research and Education Center, University of Florida, Davie, FL 33314, USA

^d School of Natural Resources and Environment, University of Florida, Gainesville, FL 32611, USA

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ABSTRACT

Wetland soils harbor diverse microorganisms including bacteria, archaea, and fungi, all of which play significant roles in maintaining soil health and ecosystem functions but are susceptible to influences from multiple environmental changes. Agricultural intensification and biological invasion are two fundamental drivers of environmental change for wetlands, but their interactive effects on soil microbial communities remain less well understood, particularly for seasonal subtropical wetlands. Yet such understanding is vital for wetland conservation and management. In this study, we conducted a 14-week field mesocosm experiment to investigate how agricultural intensification and invasive apple snail (*Pomacea maculata*) – an emerging and increasingly concerning threat to natural and agricultural systems in southeastern U.S. – altered wetland soil microbial communities and structures. We found that invasive *P. maculata* showed selective effects on relative abundance of specific microbial taxa (e.g., Proteobacteria, Nitrospirata), and interactive effects with upland intensification on Spirochaetota and Mortierellomycota. Upland agricultural intensification also exerted significant and consistent effects on microbial composition and diversity across microbial domains. Changes in microbial composition were partly manifested through modifications in water chemistry, such as dissolved oxygen, which acted as environmental regulators. In addition, upland intensification led to more complex but sparsely connected microbial networks, while invasive snail presence decreased network complexity and resulted in greater modularity, less edge density, and longer path length, indicative of lower molecular information exchange efficiency. Our research emphasizes the need for a comprehensive evaluation of microbial responses (i.e., composition, diversity, and co-occurrence patterns) to better understand multi-stressor impacts from human activities on wetland belowground microbial communities. By holistically characterizing microbial responses, our findings show how global change drivers may impact wetland microorganisms in subtropical biomes and infer their functional consequences. Our results have important implications for sustainable landscape management and conservation of wetlands that are experiencing escalating human-induced environmental changes.

1. Introduction

Bacteria, archaea, and fungi are dominant soil microorganisms that play fundamental roles in sustaining soil health and ecosystem functions, which are also shaped by interacting environmental factors (Paul, 2014). In particular, the structure of soil microbial community has been acknowledged to serve as a sensitive indicator of changes in soil fertility, quality, and stability (Hill et al., 2000; Sharma et al., 2011). A growing

body of research suggests that human-induced environmental changes affect taxonomic composition and diversity of soil microbial communities, with consequences for agricultural production and sustainability (Chen et al., 2020; Dobrovolskaya et al., 2015; Maron et al., 2018; Wagg et al., 2019; Yang et al., 2020). For example, soil microbial diversity loss due to climate warming or direct anthropogenic pressures can result in declining nutrient cycling efficiency (Luo et al., 2018) and a reduction of gross primary productivity (Wu et al., 2022). However,

* Corresponding authors at: 3205 College Ave, Davie, FL 33314, USA.

E-mail addresses: yuxigu@ufl.edu (Y. Guo), qiu@ufl.edu (J. Qiu).

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studies also found that relationships between microorganisms and ecosystem functioning under global changes could be decoupled across spatial scales (Hendershot et al., 2017), or when multiple drivers are imposed simultaneously (Yang et al., 2022). This is likely because differential abundances of certain taxonomic groups or microbial diversity alone cannot reveal the interactions among diverse microbial taxa that ultimately determine their metabolic rates and ecosystem functions (Ma et al., 2020). Therefore, a more comprehensive understanding of complexity and stability of microbial communities integrating interactions among present taxa is needed. Such understanding can be achieved via building co-occurrence networks then better inform environmental assessment and microbially-mediated function predictions (Berry and Widder, 2014; Coyte et al., 2015). Further, microbial co-occurrence patterns could also provide a unique lens through which influential taxa and the number of ecological niches in microbial communities can be revealed (Schmidt et al., 2019).

Human population growth and increasing demand for agricultural products are causing global agricultural intensification with serious disturbances to both in situ managed production systems and adjacent natural ecosystems (Didham et al., 2015; Stamenković et al., 2021; Tilman et al., 2011). Recent studies indicate that agricultural intensification could reduce the complexity and stability of local microbial networks (Banerjee et al., 2019; Xue et al., 2022), which could be a key mechanism for soil carbon loss (Wu et al., 2021), lower resistance to plant pathogens, and thus reduced primary production (Fan et al., 2020). However, the agricultural intensification potential for far-reaching impacts beyond production areas within the larger meta-ecosystem (i.e., a set of ecosystems connected by spatial flows of energy, materials, and organisms across ecosystem boundaries) remain less well understood (Gounand et al., 2018; Guo et al., 2023b; Jeanneret et al., 2021). In particular, few studies have investigated whether agricultural intensification effects on microbial networks cascade to adjacent natural systems, such as freshwater wetlands embedded within agroecosystems (Rocca et al., 2021). Such a holistic and across-scale understanding is crucial for the management of well-functioning agricultural landscapes and mitigation of unexpected negative consequences from land intensification.

Biological invasion is another key global change driver that alters ecosystem functions and services, decreases biodiversity and can even cause ecosystem destruction (Simberloff et al., 2013). Agricultural intensification can interact synergistically with biological invasion (e.g., through facilitating the spread, growth, and performance of invasive species), thereby amplifying impacts on biotic communities and ecosystems (Driscoll et al., 2014; Medan et al., 2011). While there is a large body of literature on invasion ecology, it remains elusive how upland agricultural intensification interacts with invasive species to affect soil microbial communities (Geisen et al., 2019). Furthermore, the existing literature on soil microbial responses to invasive species almost entirely focuses on invasive plants (Coats and Rumpho, 2014; Fahey et al., 2020; van der Putten et al., 2007; Zhang et al., 2019), whereas effects of other taxa, such as invertebrates that have direct and indirect interactions with soil microorganisms, have received much less attention (Lowe et al., 2000). To our knowledge, there is no research thus far demonstrating specific effects of invasive macroinvertebrates on wetland soil microbiomes yet.

One such example of an invasive macroinvertebrate is the apple snail (*Pomacea* spp.), which was listed as one of the worst invasive species worldwide (Lowe et al., 2000). Apple snails, a family of South American tropical and subtropical aquatic snails, are extensive global invaders and have caused severe economic loss to agricultural wetlands by consuming substantial plant biomass (Carlsson et al., 2004; Horgan et al., 2014). Invasion by *Pomacea maculata* in the Southeast United States has heightened the need to understand ecological impacts beyond agricultural production, because this species has been found in both natural and managed freshwater and coastal wetlands (Pierre et al., 2017). As ecosystem engineers, invasive snails can be highly destructive due to

high fecundity, reproductive capacities, and plasticity in life history traits (Barnes et al., 2008). Indeed, recent research in central Florida has demonstrated that *P. maculata* substantially reduced wetland plant diversity and biomass, shifted plant community composition, and accelerated nutrient cycling by increasing carbon, nitrogen (N) and phosphorus (P) in the water column (O'Neil et al., 2023). As plants and environmental chemistry significantly interact with soil microbes (Fierer, 2017; Islam et al., 2020), *P. maculata* invasion can likely affect soil microbial communities and consequently the underpinning mechanisms for ecosystem functions.

To improve our understanding of how wetland soil microbiomes are affected by upland agricultural intensification and *P. maculata* invasion, we conducted a factorial mesocosm experiment in south-central Florida, USA. This region exemplifies intensive agricultural land uses (e.g., managed grasslands for livestock production) and apple snail's invasion, along with abundant wetlands (wetlands cover >31 % of Florida) that are susceptible to influences of both invasion and intensification. We analyzed microbial community composition using high-throughput sequencing of 16S ribosomal RNA (16S rRNA) genes and ribosomal internal transcribed spacer (ITS) genes (Schirmer et al., 2015). We specifically addressed the following questions: (1) How do taxonomic composition of wetland soil bacterial, archaeal, and fungal communities respond to upland agricultural intensification and *P. maculata* invasion? (2) How do variations in water chemistry and soil nutrients resulting from agricultural intensification and invasion alter the structure of microbial communities? and (3) How do upland intensification and *P. maculata* invasion interact to affect microbial co-occurrence patterns? Overall, we hypothesized that upland agricultural intensification and *P. maculata* invasion would interact to alter microbial taxonomic compositions, primarily through changes in water chemistry and soil nutrients. We also expected that upland agricultural intensification and apple snail invasion would reduce the complexities and stabilities of microbial networks (e.g., as reflected by less number of nodes, lower edge density, or reduced natural connectivity). Findings from this research will improve understanding of microbial responses to anthropogenic pressures from agricultural production and biological invasion in subtropical wetland ecosystems and inform conservation and sustainable management practices.

2. Material and methods

2.1. Study site

Our experiment was conducted at the Archbold Biological Station's Buck Island Ranch (BIR) in south-central Florida (27°09' N, 81°11' W), which is a full-scale commercial cattle ranch operation and is part of the Archbold-University of Florida Long-Term Agroecosystem Research (LTAR) site. The study region is in a humid subtropical climate with an average annual temperature of 19.8 °C and an average annual rainfall of 1360 mm, >70 % of which falls during the wet season (June to October). The BIR contains 4336 ha of pastures which have been managed under two land-use intensities originating from the 1960s: intensively managed (IM) and semi-natural (SN) pastures, which are two exemplary pasture types of regional grazing agroecosystems (Swain et al., 2013). The IM pastures were constructed with dense ditches for drainage during wet seasons, extensively planted with productive non-native forage grasses, regularly limed, fertilized with N (56 kg ha⁻¹ as NH₄SO₄ or NH₄NO₃) every 1–2 years, fertilized with P (34–90 kg ha⁻¹ of P₂O₅) and potassium (34–90 kg ha⁻¹ of K₂O) until 1987, and served as high-intensity cattle grazing with an average animal use days (AUD) of 391 per hectare (Boughton et al., 2010). In contrast, SN pastures were constructed with fewer ditches, only partially seeded with non-native forage grasses, never fertilized, and served as low-density cattle grazing with an average AUD of 178 per hectare (McClelland et al., 2023). On BIR pastures, there are numerous isolated seasonal wetlands embedded in grazing grasslands due to the humid climate and shallow groundwater

table, accounting for 12 % of the total BIR area (Swain et al., 2013). These wetlands were not directly managed for livestock production, but are highly influenced by upland management intensities with respect to soil nutrients (Ho et al., 2018), water quality (Jansen et al., 2019), plant traits and composition (Boughton et al., 2016; Sonnier et al., 2020), soil microbial communities (Guo et al., 2023b), and ecosystem functions (Guo et al., 2023a; Guo et al., 2021). These wetlands are also increasingly inhabited by the invasive apple snail (Pierre et al., 2017). For further convenience, we term wetlands embedded in IM or SN pastures as IM or SN wetlands in subsequent sections.

2.2. Mesocosm experimental design

To address our research questions, we designed a wetland mesocosm experiment with a 2×2 factorial design across levels of upland intensification (i.e., representative of SN and IM wetlands) and presence/absence of invasive apple snails (i.e., introducing 8 *P. maculata* individuals to invasion treatment) (Fig. S1). We had 8 replicates for each treatment and thus a total of 32 wetland mesocosms. Further details on the mesocosm experimental design can be found in O'Neil et al. (2023). Briefly, mesocosms were created in 32-gallon white trashcans using soils and dominant plant species collected from SN and IM wetlands, respectively. To capture the variations in soil texture and properties, we collected wetland soils from four SN and four IM wetlands individually. Dominant plant species in SN wetlands were *Amphicarpum muehlenbergianum*, *Bacopa caroliniana*, *Justicia angusta*, *Panicum hemitomon*, *Pontederia cordata*, and *Sagittaria lancifolia*, while in IM wetlands the dominant plant species were *Altermanthera philoxeroides*, *P. hemitomon*, and *P. cordata*. Plants were grown in a density and composition similar to field conditions (Boughton et al., 2016). We filled the mesocosms with soils, transplanted representative plant species, watered, and arranged them randomly in a 4×8 grid in April 2018. Then mesocosms were maintained for two months so that plants were established, dead plant litter and sprouted unwanted plants could be removed, and the surface water table was maintained at 25 cm above the soil. To simulate actual wetland water microbial communities, in situ water was collected from the 8 wetlands, and added into mesocosms with corresponding wetland soils collected when constructing the mesocosms. In June 2018, juvenile *P. maculata* were collected from a ditch at BIR. These snails were individually labelled using white nail polish and weighed. Snails were then sorted by their weight and evenly divided (based on the total weight per mesocosm) with 8 snails in each invasion treatment mesocosm, in alignment with the upper limit density observed in the field (Howells et al., 2006). After snails were deployed, all mesocosms were covered by a 6-mm wire mesh top so that snails would not escape or be predated on. The mesocosm experiment was conducted for 14-weeks from June 22 to September 27, 2018.

2.3. Environmental variables

After 14 weeks, we collected all water chemistry, soil nutrient, and soil microbial measurements. We used a calibrated YSI ProDss multi-parameter handheld probe to measure in situ water chlorophyll *a* (Chla), dissolved oxygen (DO), pH, oxidation reduction potential (ORP), temperature, and total dissolved solids (TDS). We collected soil from the top layer (0–5 cm) from each mesocosm to analyze total N, and plant available P and sulfur (S). Soil samples were collected by 5-cm depth \times 10-cm diameter soil cores, transported with ice packs, sieved (2-mm), and stored at 4 °C until laboratory analyses. Soil total N was determined via LECO CN628 C/N Determinator (LECO Corporation, MI). The plant-available P and S were extracted by Mehlich-3 solution (Mehlich, 1984) and then determined via Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES) on a Perkin Elmer Avio 200 (Perkin-Elmer, CT, USA). Soil subsamples for microbial analyses were prepared and immediately placed on dry ice for transportation, then stored at -80 °C freezer until DNA extraction.

2.4. Soil DNA extraction, amplicon sequencing, and taxonomic assignment

Genomic DNA of soil microbes was extracted from 0.25 g of soil (i.e., the subsamples from the -80 °C freezer) using a DNeasy PowerSoil extraction kit (Qiagen, Germantown, MD) following the manufacturer's protocol. Quality and quantity of extracted soil DNA quantified using a UV/VIS spectrophotometer (NanoDrop™, ThermoFisher Scientific, Waltham, MA, USA) and ranged between 16.0 and 66.2 ng/ μ l. PCR amplification of target genes, barcoded library preparation, and sequencing was done on an Illumina MiSeq instrument at the Argonne National Laboratory, Chicago, Illinois. Bacterial and archaeal 16S rRNA genes were amplified using the primer pairs 515F (5'- GTGY-CAGCMGCCGCGGTAA) (Parada et al., 2016) and 926R (5'- CCGY-CAATYMTTTRAGTTT) (Quince et al., 2011), which targets the V4–V5 regions. The fungal ITS region was amplified with the primer pair ITS1F (5'- TCCGTAGGTGAACCTGCGG) - ITS2R (5'- GCTGCGTCTTCATC-GATGC) (White et al., 1990). The resulting sequence data were deposited at the NCBI Sequence Read Archive (SRA) database under BioProject number PRJNA908233.

The amplicon sequencing data were processed using QIIME2 (version 2022.2). After denoising and quality filtering by DADA2 using standard settings (Callahan et al., 2016), high-quality reads (1,042,129 16S rRNA genes, and 1,868,038 ITS genes) were obtained. Amplicon Sequence Variants (ASVs) were determined at the 99 % similarity level of the nucleotide sequences. Quality-filtered and non-chimeric 16S and ITS sequences were aligned to the SILVA database version 138 (Quast et al., 2013) and UNITE version 8.0 databases (Koljalg et al., 2013), respectively, for taxonomic classification. Sequences not belonging to bacteria/archaea or fungi were removed from downstream analyses. For the compositional analysis presented, the bacterial/archaeal and fungal ASV tables were randomly rarefied to 3760 and 7000 reads per sample, respectively, according to the rarefaction curves (Fig. S2). Compositional analyses with higher cutoffs ($>10,000$) led to exclusion of some samples, but yielded identical results.

2.5. Statistical analyses

All statistical analyses were performed in R version 4.1.2 (Team R.C., 2021) using the RStudio interface. Non-metric multidimensional scaling (NMDS) was applied to visualize differences in microbial composition at the ASV level across mesocosm treatments (i.e., upland intensification, presence of invasive apple snail, and their interactions) based on the Bray-Curtis distance matrix. Significant environmental variables according to permuted data (Table S1) were selected and fitted onto the NMDS ordination using the 'envfit' function from the 'vegan' package (Oksanen et al., 2013). The significance of compositional differences was evaluated using permutational multivariate analysis of variance (PERMANOVA) with 999 permutations. We also tested treatment effects on microbial compositional differences based on weighted UniFrac distances, which showed similar results (Table 1).

Alpha diversity of microbial communities was characterized by ASV and genus richness, Shannon diversity index, and Simpson diversity index of bacterial/archaeal and fungal communities, respectively. We tested whether mesocosm treatments affected the alpha diversity metrics using two-way ANOVA tests. We checked linear regression assumptions by visually inspecting the distribution of model residuals, and applied log, square root, or cube root transformations to response variables when necessary. We used type III sums of squares (SS) to calculate *p* values to determine the significance of individual treatments and their interactions. Post-hoc pairwise comparisons were performed when significant interactions were detected, which were conducted using the 'lsmeans' package (Lenth and Lenth, 2018).

We examined the effects of mesocosm treatments on the relative abundance of microbial taxonomic groups via the composition of microbiomes (ANCOM) approach (Mandal et al., 2015). Microbial

Table 1

Results of PERMANOVA on treatment effects on bacterial/archaeal and fungal ASV composition with Bray-Curtis or weighted UniFrac distance matrix methods. Bold values indicate significant coefficients. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$.

Kingdom & distance matrix	Treatment	Sum of sq	Pseudo-F	<i>p</i>
Bacteria/archaea (Bray-Curtis)	Upland intensification (I)	1.331	5.02	0.001***
	Snail (S)	0.250	0.94	0.535
	I × S	0.226	0.85	0.686
Bacteria/archaea (weighted UniFrac)	Upland intensification (I)	0.0003	3.24	0.004**
	Snail (S)	0.0001	0.63	0.824
	I × S	0	0.39	0.996
Fungi (Bray-Curtis)	Upland intensification (I)	2.127	6.50	0.001***
	Snail (S)	0.278	0.85	0.645
	I × S	0.246	0.75	0.799
Fungi (weighted UniFrac)	Upland intensification (I)	0.0031	6.00	0.001***
	Snail (S)	0.0006	1.18	0.214
	I × S	0.0004	0.69	0.838

groups were filtered for abundance (account for at least 0.1 % of total reads) and prevalence (present in at least 10 % of samples). We performed ANCOM-II using the R implementation of Lin (2021) that was designed to process data with excess zeros while controlling false discovery rate, and selected differentially abundant groups whose W values surpassed the 0.7 threshold.

Co-occurrence networks for each mesocosm treatment of the cross-domain microbial communities were established individually at the genus level by Spearman's correlation analysis with significant coefficients ($p < 0.05$, $\rho > 0.8$). We constructed cross-domain microbial networks because bacteria, archaea and fungi can interact in several ways in the soil environment (Cheung et al., 2018). Data were transformed using modified centered log-ratio (mclr) to ensure compositional robustness (Aitchison, 1982). Meanwhile, zero counts were replaced using a multiplicative simple replacement algorithm to avoid the numerical problems with the mclr transformation (Peschel et al., 2021). We characterized the network using a set of topological features, including number of nodes, global clustering coefficient, modularity, positive edge percentage, edge density, natural connectivity, and average path length. Global clustering coefficient measures the ratio of triangles to the total number of connected triples (Junker and Schreiber, 2011), where a higher clustering coefficient indicates nodes are more likely to form clusters. Modularity measures the degree to which a network is divided into delimited communities (Peschel et al., 2021). Edge density measures the proportion of the actual number of edges with respect to the possible largest number of edges. Natural connectivity represents the stability of a network by measuring the average eigenvalue derived from the adjacency matrix. Network hub nodes (keystone taxa) were identified as the nodes within the top 2 % of degree and closeness centralities (Zhang et al., 2021). Degree centrality of a node refers to its total connections with adjacent nodes, while closeness centrality of a node refers to the sum of all its shortest paths to all other nodes. The network analyses were conducted using the *NetCoMi* version 1.0.3 (Peschel et al., 2021) and *igraph* version 1.3.2 (Csardi and Nepusz, 2006) packages.

3. Results

3.1. Soil microbial taxonomic composition

In total, 59 bacterial/archaeal phyla (95.8 % of quality-filtered sequences), 154 classes (94.3 %), 312 orders (90.3 %), 438 families (81.8 %), and 578 genera (61.2 %) were identified across 32 mesocosm soil samples. The most abundant bacterial/archaeal phyla across all samples

were Acidobacteriota (31.4 ± 9.0 %), Proteobacteria (12.5 ± 2.0 %), Verrucomicrobia (8.6 ± 1.8 %), Chloroflexi (7.5 ± 3.6 %), Bacteroidota (7.3 ± 3.9 %), and Actinobacteriota (5.4 ± 1.5 %) (Fig. S3). At the class level, Acidobacteriae (29.3 ± 9.6 %), Alphaproteobacteria (9.6 ± 1.9 %), and Verrucomicrobiae (7.7 ± 1.6 %) were the dominant groups (Fig. S4).

For fungal communities, the numbers of identified phyla, classes, orders, families, and genera were 12 (33.1 % of quality-filtered sequences), 28 (19.1 %), 73 (17.4 %), 143 (14.9 %), and 193 (13.3 %), respectively. The major fungal phyla were Ascomycota (63.8 ± 17.9 %) and Basidiomycota (18.3 ± 13.9 %) (Fig. S5). At the class level, Sordariomycetes (37.2 ± 22.9 %), Agaricomycetes (10.2 ± 14.0 %), and Dothideomycetes (10.0 ± 17.9 %) were most abundant groups (Fig. S6).

3.2. Treatment effects on soil microbial composition in wetland mesocosms

Among wetland mesocosm treatments, upland agricultural intensification (i.e., IM vs SN wetland types) showed strong effects on soil microbial community composition (Table 1), such that all bacterial/archaeal and fungal ASV compositions were significantly different between SN and IM mesocosms (Fig. 1). We found that the most important environmental variables associated with bacterial/archaeal community composition were DO and ORP, while TDS and Chla concentrations were significantly correlated with fungal community composition (Fig. 1). Indeed, from our treatments, water DO and ORP were lower in IM than in SN mesocosms (Fig. S10), and in IM mesocosms, snail presence led to lower water Chla concentration but higher water TDS (Fig. S10).

The microbial compositional differences were not reflected by their alpha diversity metrics at the ASV level (Table S2, Fig. S8), but at the genus level (Table 2, Fig. 2). On average, upland intensification resulted in a 7.3 % increase in the Shannon diversity index and a 1.2 % increase in the Simpson diversity index of bacterial/archaeal genera (Fig. 2A), and a 38.3 % increase in the fungal genus richness (Fig. 2B).

Further, presence of invasive apple snail (*P. maculata*) and upland intensification showed individual and interactive effects on the relative abundances of certain microbial taxa (Fig. 3). For example, at the phylum level, soils in IM wetlands had greater relative abundance of Bacteroidota, Spirochaetota, Myxococcota, and Euryarchaeota, and less relative abundance of Nitrospirota, RCP2-54, and WPS-2 than in SN wetlands (Fig. 3). Invasive apple snail presence also significantly affected the relative abundance at the phylum level. After statistically accounting for effects of upland intensification, *P. maculata* invasion was associated with lower relative abundance of Proteobacteria and Nitrospirota (Fig. 3). The interaction between upland intensification and *P. maculata* invasion was also detected from bacterial phylum Spirochaetota and fungal phylum Mortierellomycota. Snail presence was associated with increased relative abundance of Spirochaetes only in SN wetlands, and IM wetlands only had greater relative abundance of Mortierellomycota than SN wetlands when the snail was present (Fig. 3).

3.3. Treatment effects on soil microbial co-occurrence network

To examine effects of upland intensification and presence of invasive apple snail on microbial interactions, we constructed bacterial-archaeal-fungal cross-domain networks at the genus level for the four types of wetland mesocosms (Fig. 4). We characterized the complexity, structure, and stability of microbial taxa co-occurrence patterns via a set of topological properties (Table 3). Higher number of nodes were identified in the IM than SN wetlands, while *P. maculata* invasion decreased the number of nodes in both types of wetlands (Fig. 4). However, specific effects of invasive snail were microbial taxa- or wetland type-dependent. For instance, snail presence led to negligible changes in IM but 10 % reduction in SN wetlands for fungal nodes. Bacterial taxa were the major components of all networks, accounting for over 70 % of the total number of nodes (Table 3, Fig. 4). Across the two upland land-use

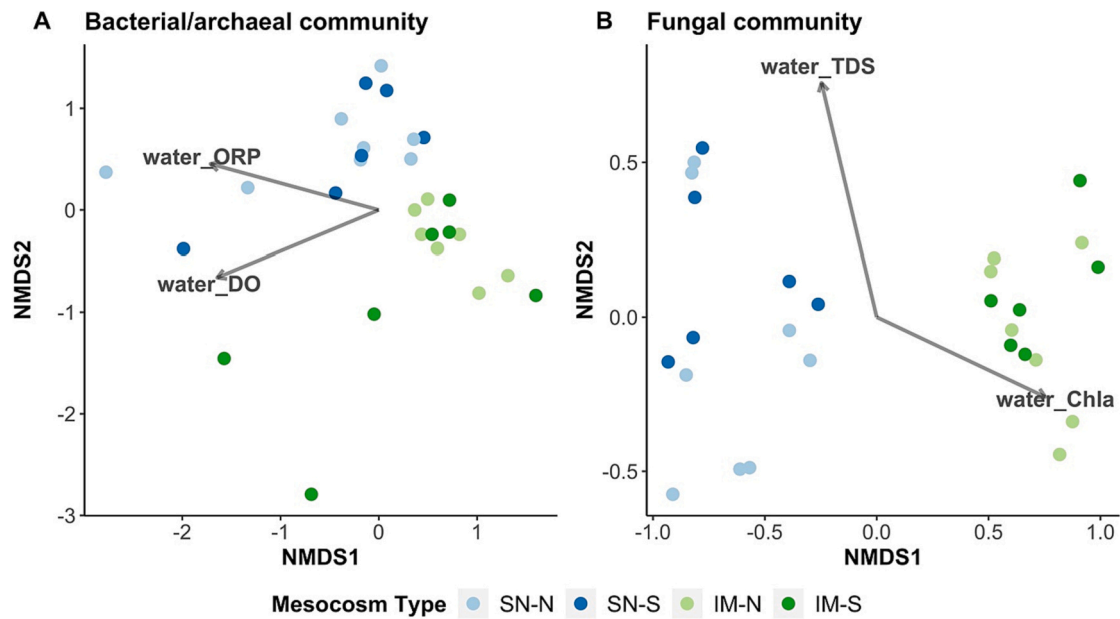


Fig. 1. Nonmetric multidimensional scaling based on Bray-Curtis distance matrix displaying wetland mesocosms and significant environmental factors of (A) bacterial/archaeal and (B) fungal community ASV composition (A: stress = 0.095, and B: stress = 0.086) among upland land-use intensities (semi-natural or SN and intensively managed or IM) and invasive apple snail treatments (No snail or N and with snail or S). Each point indicates one mesocosm soil sample.

Table 2

Results of two-way ANOVA of upland intensification and presence of invasive apple snail effects on alpha diversity metrics (richness, Shannon diversity index, and Simpson diversity index) of bacterial/archaeal and fungal communities at the genus level. Bold values indicate significant coefficients.

Kingdom	Treatment	Estimated \pm SE		
		Richness	Shannon index	Simpson index
Bacteria/ archaea	Upland	18.81 \pm	0.14 \pm	0.006 \pm
	intensification (I)	17.08	0.06*	0.002*
	Snail (S)	-24.63 \pm	-0.08 \pm	-0.002 \pm
		17.08	0.06	0.002
	I \times S	3.69 \pm	0 \pm 0.06	-0.001 \pm
Fungi	Upland	8.92 \pm	0.14 \pm 0.11	0.001 \pm
	intensification (I)	3.86*		0.021
	Snail (S)	0.42 \pm 3.86	0.07 \pm 0.11	0.014 \pm
				0.021
	I \times S	-3.04 \pm	-0.01 \pm	-0.002 \pm
		3.86	0.11	0.021

intensities, *P. maculata* presence increased 20.8–37.5 % network clustering coefficient values, 18.8–57.4 % modularity, and 15.7–17.8 % natural connectivity. In addition, the invasion of apple snail decreased 11.9–15.4 % edge density and increased 44.6–104.9 % average path length.

Additionally, keystone taxa, which play disproportionately important roles in maintaining the overall structure and function of communities, were identified from the cross-domain microbial networks only in SN wetlands. In the SN-No snail wetland mesocosms, keystone taxa were assigned to bacterial phyla, Actinobacteriota and Proteobacteria (Table S3, Fig. 4). Under the SN-Snail treatment, the microbial network had keystone taxa that were from the bacterial phyla Bacteroidota and Chloroflexi and the archaeal phylum Nanoarchaeota (Table S3).

4. Discussion

4.1. Effects of upland agricultural intensification on soil microbial communities

Upland agricultural intensification showed significant impacts on wetland soil microbial composition (Fig. 1). In our study system, agricultural intensification embraces a range of practices, including introduction of forage grasses (i.e., alteration of plant communities), fertilization, lime application, irrigation or drainage, and intense livestock grazing, all of which have significant direct or indirect effects on not only the physical and chemical, but also the biological properties of embedded wetlands (Bansal et al., 2021; Boughton et al., 2016; Jansen et al., 2019). Specifically, in our experiment, we constructed IM and SN wetland mesocosms using three and six dominant and representative plant species, respectively, according to the field surveys of plant communities in each wetland type (Boughton et al., 2016). Multiple studies have found plant composition and diversity profoundly affected belowground microbial composition (Grüter et al., 2006; Laughlin et al., 2010; Liu et al., 2020; Schmid et al., 2021). For example, herbaceous plant species diversity has been found to be positively associated with the abundance of nitrifiers (Laughlin et al., 2010). Our results showed a consistent finding that SN wetlands, which have higher plant diversity than IM wetlands, showed greater relative abundance of Nitrospirota and Nitrososphaeria (Figs. 3 & S8). Furthermore, Schmid et al. (2021) suggested that plant communities with grasses tended to harbor more Bacteroidota and less Nitrospirota in soils than plant communities without grasses. Our results to a large extent aligned with these findings, as IM wetland mesocosms, which contained higher grass coverage (O'Neil et al., 2023), also showed greater relative abundance of Bacteroidota and lower Nitrospirota than SN wetlands (Fig. 3). While this may be due to varying root exudates and litter quantity and quality from different plant functional groups (Steinauer et al., 2016), future studies are needed to further validate the mechanisms behind such effects of different plant species (e.g., resulting from agricultural intensification) on soil microbial compositions. Additionally, previous studies conducted in this region showed wetland vertebrate and plant richness decreased with upland intensification, while macroinvertebrate richness had no response (Medley et al., 2015). Nonetheless, our results showed

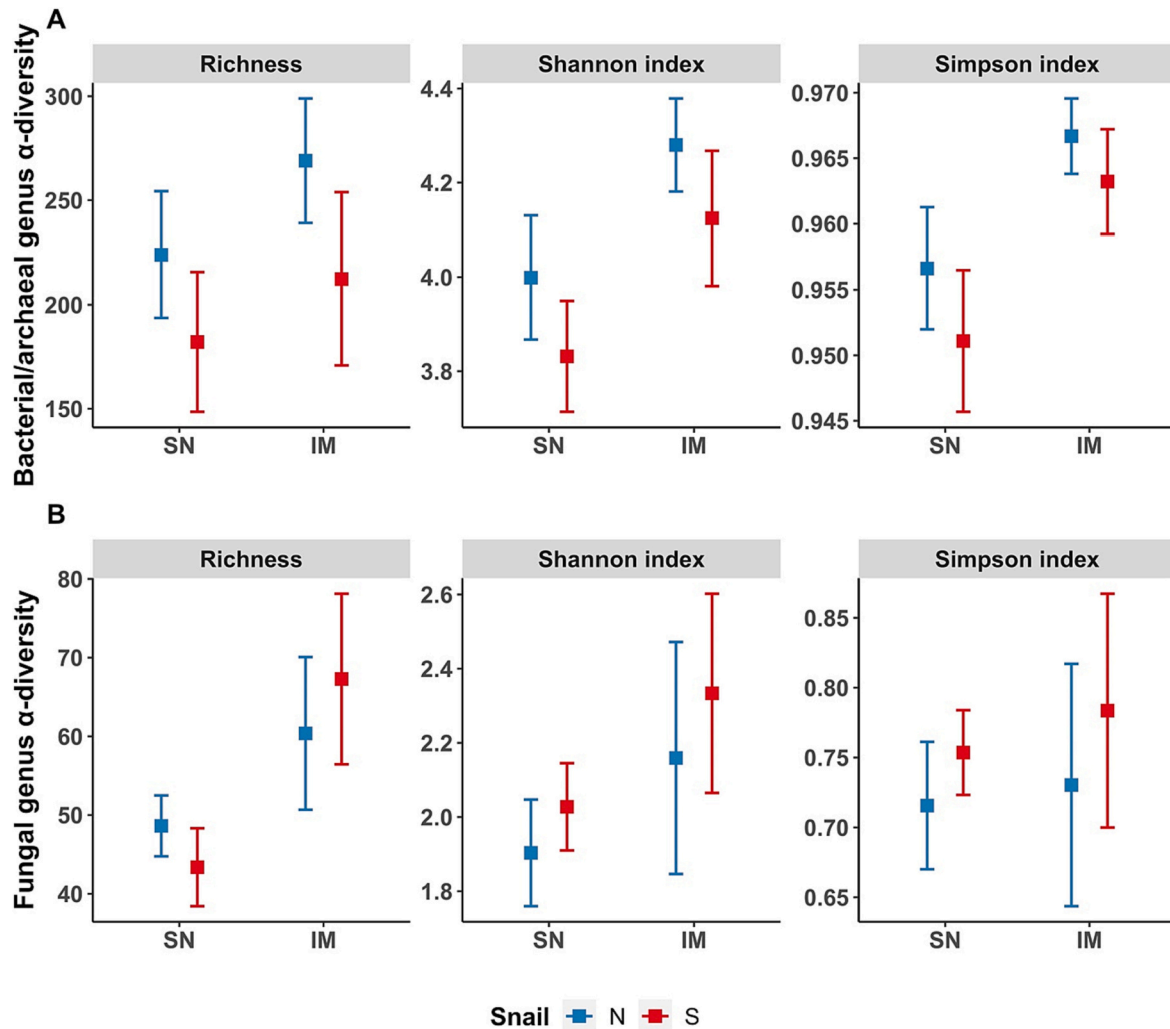


Fig. 2. Effects of upland land-use intensity and presence of the invasive apple snail on (A) bacterial/archaeal and (B) fungal genus richness, Shannon diversity index, and Simpson diversity index (mean \pm SE; N = 8). Mesocosm type: SN-semi-natural wetland, IM-intensively managed wetland, N-no snail, S-with snail.

upland intensification increased soil microbial diversity, indicating the importance of understanding responses of different trophic levels to land management. However, microbial taxonomic diversity alone may not reliably predict soil health and function, given the presence of functional redundancy and selective gene expressions (Louca et al., 2018). Hence, future work employing shotgun metagenomic and metatranscriptomic sequencing of soil microbes could more effectively unveil both potential and actual metabolic functions conducted by microbial communities, offering improved insights into the soil's health condition in response to external environmental stressors.

On the other hand, agricultural intensification may promote copiotrophic microbes, such as Bacteroidota (Fierer et al., 2007), that are able to grow and reproduce rapidly in nutrient-rich environments because of increasing nutrient availability from fertilization (Fierer et al., 2012; Xue et al., 2022). Our results confirmed that IM wetlands had higher relative abundances of Bacteroidota than SN wetlands (Fig. 3). As well-known carbohydrate decomposers, increasing Bacteroidota can lead to soil organic matter depletion (Leng et al., 2022), which would be consistent with our observations in O'Neil et al. (2023). However, at the end of this mesocosm experiment, labile nutrients in the soil and water column did not differ between IM and SN wetlands (Table S4; O'Neil et al., 2023). The major reason is that excessive labile nutrients are unlikely to persist and will be readily taken up by plants, as evidenced by the higher primary production found in IM wetlands compared to SN wetlands (O'Neil et al., 2023). Thus, increased Bacteroidota in IM

wetlands may be a temporary effect following the fertilization practice. Regardless, these results suggest that there upland agricultural intensification (or IM pasture management) had a consistent cascading effect on soil microbes in spatially connected natural ecosystems (i.e., embedded wetlands; Guo et al., 2023b).

4.2. Effects of invasive apple snail and its interaction with upland intensification on soil microbial communities

Our results revealed that several key microbial taxa significantly responded to invasive *P. maculata* and its interaction with upland intensification (Fig. 3). Specifically, invasive snail presence led to lower relative abundance of Nitrospirota (Daims and Wagner, 2018), and greater relative abundances of anaerobic metabolism microbes, such as Spirochaetota (Paster, 2010), particularly within SN wetlands (Fig. 3). Water DO level created bacterial/archaeal compositional differences across mesocosm treatments (Fig. 1A), which also corresponded to water DO variations across treatments (Fig. S10). This is likely because the invasive apple snail appeared to reduce water DO levels in SN wetlands (Fig. S10; Lucero and Wilson, 2023), which can be attributed to snail-induced increases in wetland temperature, TDS (Fig. S10) and associated microbial respiration, thus promoting oxygen depletion and rise of anaerobic microorganisms in the submerged soil. Firstly, *P. maculata* presence caused >60 % reductions in plant cover (O'Neil et al., 2023), thus exposing the water column to more solar radiation and leading to

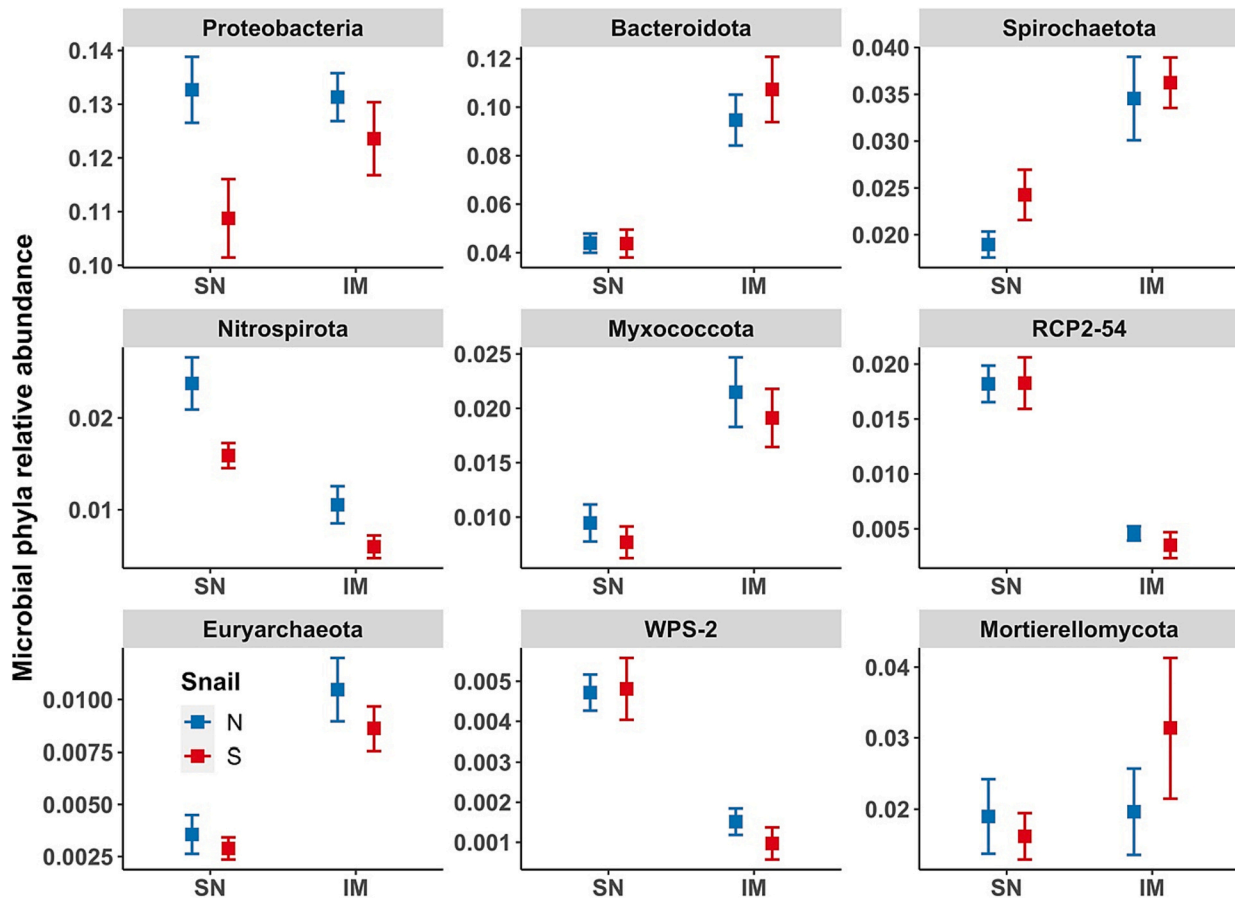


Fig. 3. Relative abundance of bacterial/archaeal and fungal phyla that showed significant responses (from ANCOM test with W-values higher than 0.7) to the upland land-use intensities and/or invasive apple snail treatments (mean \pm SE; N = 8). Mesocosm type: SN-semi-natural wetland, IM-intensively managed wetland, N-no snail, S-with snail.

elevated temperatures (Fig. S10). These warmer conditions can restrict the solubility of oxygen in water (Aldridge, 1983). Secondly, snails can lead to increased organic waste (e.g., through egestion), which resulted in >30 % increase of water TDS (O'Neil et al., 2023), contributing to DO depletion via stimulating organoheterotrophs' metabolisms (Ryan, 1991).

In addition, invasive *P. maculata* affected certain microbial groups' relative abundance to a greater magnitude in SN versus IM wetlands, such as for Proteobacteria, Spirochaetota, and Nitrospirota (Fig. 3). This finding was rather counter-intuitive that microbial communities with less disturbance and more diverse plant assemblages (i.e., SN wetlands) were more sensitive to external pressure from a macroinvertebrate invader than those with heavier disturbance and lower plant diversity (i.e., IM wetlands). This might be due to that microbial communities in IM wetlands were already adapted to low levels of dissolved oxygen as a result of long legacies of management (e.g., fertilization, cattle activities) (Fig. S11) and thus to be more tolerant to the disturbances caused by the invader, whereas communities in SN wetlands were assembled with less filtering for stress tolerance (Sonnier et al., 2022). Nevertheless, this phenomenon was consistent with Guo et al. (2023b), where the structure of soil microbial communities was more responsive to intensive ranching practices (i.e., livestock grazing and prescribed fire) in SN than IM wetlands. A deeper understanding of why freshwater ecosystems subject to more intensive management contained less affected microbial communities would provide valuable insight for wetland conservation in agriculture-dominated landscapes.

4.3. Effects of wetland mesocosm treatments on soil microbial networks

Microbial co-occurrence patterns provide a holistic perspective on the effects of upland agricultural land uses and *P. maculata* invasion on the interactions among bacterial, archaeal, and fungal taxa in wetland soils. Our network analysis primarily suggested that IM wetlands contained more complex (i.e., greater number of nodes) but less stable (i.e., lower natural connectivity) and sparsely connected (i.e., lower edge density and longer average path length) microbial networks than SN wetlands despite *P. maculata* invasion (Table 3, Fig. 4). This finding was partially different from previous studies in terrestrial croplands, where agricultural intensification simplified soil microbial networks (Banerjee et al., 2019; Xue et al., 2022). However, it was consistent with studies conducted in wetland ecosystems (Mu et al., 2021; Sun et al., 2023), where substrates enrichment and water disturbance increased the complexity of microbial networks. These contrasting observations might result from the breadth and context-specifics of the 'agricultural intensification' concept and associated discrepancy in the hydrothermal conditions, oxygen availability, and pH between terrestrial upland and inundated wetland soils (Mu et al., 2021; Zhang et al., 2022). For instance, neutral environmental pH is favorable for maintaining high complexity of soil microbial communities (Zhang et al., 2022). Wetland soils are typically more acidic than cropland soils. In our study area, upland intensification includes lime application which results in runoff inputs to surrounding wetlands thus neutralizing soil pH (Guo et al., 2023a). Yet in terrestrial croplands, intensification practices either caused soil acidification or alkalization (Xue et al., 2022; Zhou et al., 2021), therefore suggesting the 'seemingly' divergent results on the overall impacts of intensification.

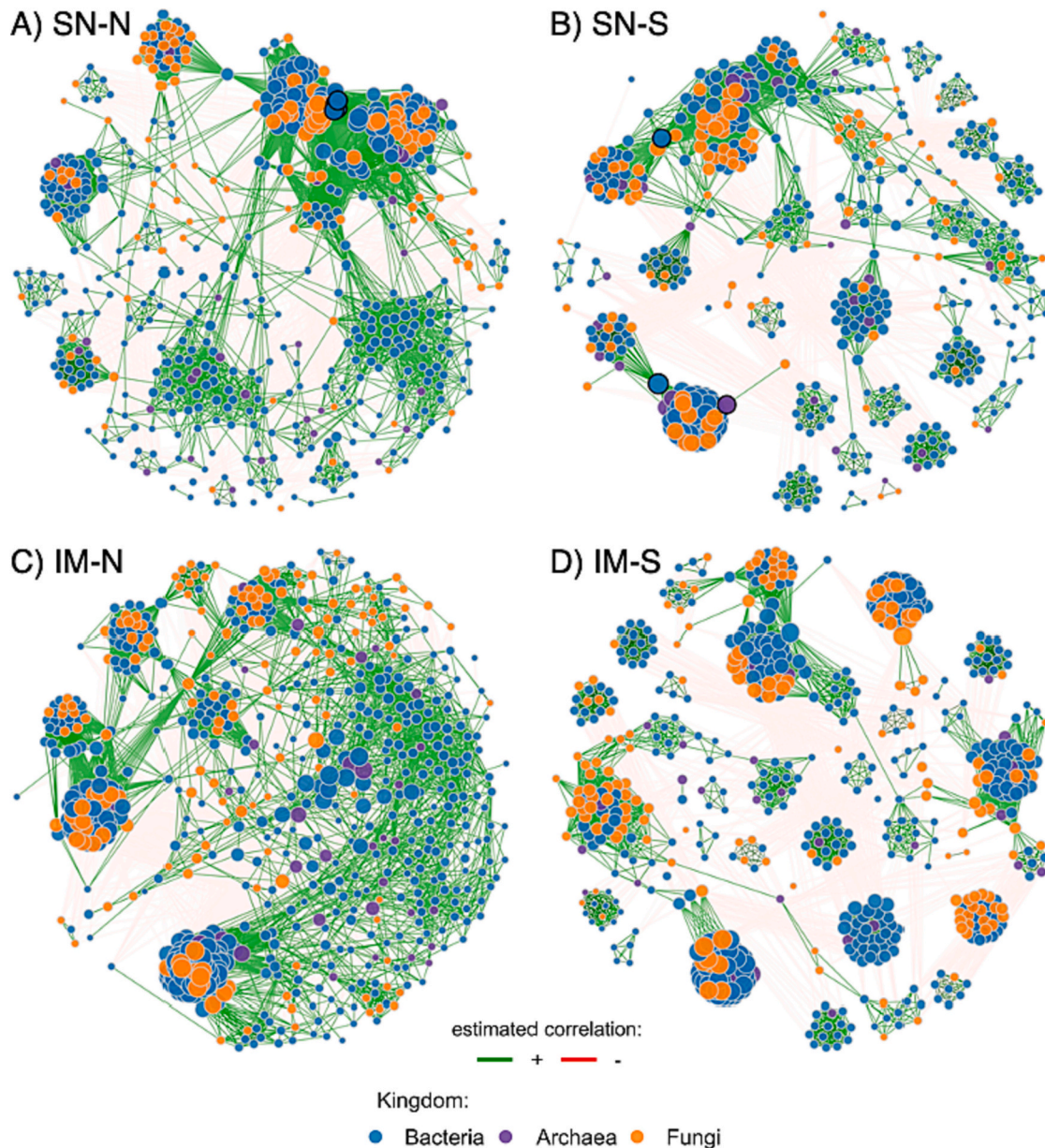


Fig. 4. Network visualization of bacterial-archaeal-fungal cross-domain co-occurrence patterns at A) semi-natural, no snail (SN-N), B) semi-natural, with snails (SN-S), C) intensively managed, no snail (IM-N), and D) intensively managed, with snails (IM-S) wetland mesocosms. Nodes indicate individual genera, while edges represent significant Spearman correlations ($\rho > 0.8$, $p < 0.05$) between genera. Nodes are colored by domain-level taxonomy and edges are colored by positive or negative correlation. Node size is proportional to its degree centrality and node is colored by the kingdom to which it belongs. Hub nodes (i.e., keystone taxa) were identified as having >98 % quantile of the normalized degree and closeness centralities and are highlighted with a black outline.

Furthermore, although compositional analysis and diversity metrics did not reflect any *P. maculata* impacts on soil microorganisms, the microbial co-occurrence patterns showed that *P. maculata* invasion resulted in highly clustered microbial networks with greater modularity across upland land-use intensities. This suggested that microorganisms were aggregating into more functional groups and ecological niches to deal with the rising stress (e.g., higher TDS and solar exposures) from this invasive invertebrate (Gao et al., 2022; Zhao et al., 2022). We also found that snail presence led to more positive interactions among microbial taxa in IM wetlands, which could be due to higher soluble substrates in IM-snail mesocosms (Fig. S10) that provide sufficient substrates for microbial metabolism and growth, thus eventually forming a less competitive co-occurrence network pattern (Xue et al., 2022). Meanwhile, a contrasting pattern was found on the average path length where invasive snail presence extended average distance among

microbial nodes (Table 3). Longer average path lengths imply more spread-out connections between network components and it would take more steps for nodes to have molecular communications (Junker and Schreiber, 2011). Thus, this indicates that *P. maculata* invasion might lead to networks with lower information transport and communication efficiency (Kuntal et al., 2019). A highly clustered microbial network with a long average path length caused by invasive snails may be less functionally redundant because of fewer connections between different clusters of microorganisms, and therefore the community could be more vulnerable to additional disruptions, and less able to adapt and maintain its stability (Junker and Schreiber, 2011).

4.4. Management implications

Wetlands are important ecosystems that deliver an array of

Table 3

Topological properties of microbial co-occurrence networks in wetland soil with and without invasive apple snail presence under two upland land-use intensities (semi-natural or SN and intensively managed or IM).

	SN wetlands		IM wetlands	
	No snail	With snail	No snail	With snail
Number of nodes	578	523	690	638
-Bacterial nodes	424	383	497	449
-Archaeal nodes	31	29	32	29
-Fungal nodes	123	111	161	160
Clustering coefficient	0.715	0.864	0.648	0.891
Modularity	0.602	0.715	0.516	0.812
Positive edges %	92.30	91.45	73.99	92.20
Edge density	0.065	0.055	0.059	0.052
Natural connectivity	0.102	0.118	0.090	0.106
Average path length	1.382	1.999	1.274	2.610
Number of keystone	4	3	0	0

ecosystem functions services (e.g., nutrient retention, carbon sequestration, water purification, and habitat provision) (Zedler and Kercher, 2005), but have been undergoing substantial loss and degradation at local to global scales (Fluet-Chouinard et al., 2023). Previous research has increasingly indicated that microbial communities and structures could be used as effective indicators for monitoring and managing the health and functioning of ecosystems (Lau et al., 2015; Urakawa and Bernhard, 2017). Indeed, our mesocosm experiment has demonstrated that soil microbial communities (including bacteria, archaea, and fungi) and their co-occurrence patterns can be highly responsive to and well suited for detecting individual and interactive effects of multiple stressors for wetlands. It is important to note that, due to logistic constraints, our results reflect relatively short-term microbial responses (i. e., 14-week); it is possible that microbial communities could slowly adapt and evolve in response to external disturbances as the incubation time increased, which remains to be investigated. Given the development of high-throughput sequencing techniques, it is thus critical to take advantage of and incorporate microbial indicators in wetlands conservation and management. Therefore, inclusion of microbial responses that accompany with shifting communities and structures as revealed here can empower us to timely detect and infer early signals of biogeochemical and biophysical consequences for wetlands from human-induced environmental changes.

Invasive *P. maculata* exerted strong individual and interactive impacts on certain microbial taxa, and upland agricultural intensification showed more consistent effects on microbial compositions across domains. Further, both snail invasion and agricultural intensification altered the complexity, structure, and stability of microbial networks. Collectively, these results highlight the importance of (1) understanding and considering multiple stressors and their interactive effects on soil microorganism in wetland conservation and management; and (2) assessing microbial responses comprehensively by including network analysis to better understand the impacts of agricultural land use and agriculture-induced biological invasion on wetland belowground biological communities. Results from our network analysis (in addition to diversity metrics and compositional changes) offer a more holistic understanding of the complexity, stability, and keystone taxa of microbial communities in response to environmental changes. Such understanding can improve prediction of microbially-mediated functions that often depend on interaction among different present taxa (Berry and Widder, 2014; Coyte et al., 2015), thus better informing environmental assessment and wetland management.

5. Conclusions

Corroborating with our hypotheses, we found that invasive *P. maculata* exerted more pronounced impacts on certain microbial taxa in SN wetlands compared to IM wetlands, but upland agricultural

intensification showed more pronounced and consistent effects on overall microbial compositions across domains. Microbial compositional shifts due to treatment effects were partially manifested through alterations in water chemistry that serve as environmental controls. Further, both snail invasion and agricultural intensification treatments altered the complexity, structure, and stability of microbial networks. Upland intensification resulted in more complex but sparsely connected microbial networks, while snail presence led to more clustered and modularized microbial networks with longer path length. Alterations in microbial composition and network structure due to upland intensification and invasive apple snails could further exert important consequences for wetland soil health and functions. Our study highlights the importance of assessing microbial responses comprehensively to better understand the impacts of agricultural land use and agriculture-induced biological invasion on wetland belowground microbial communities. Findings from this work can help predict and understand potential ecological impacts on wetlands from surrounding agricultural landscape management. Given the national and global concern for invasion by apple snails as well as their northward expansions due to climate warming, our results are timely and relevant for conserve wetland microbiomes, ecosystems, and their supported functions in an era of increasing anthropogenic environmental changes.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be deposited into the Dryad Digital Repository and be made publicly available should the manuscript be accepted for publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.apsoil.2023.105212>.

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