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RESEARCH ARTICLE

Differential utilization of submerged leaf litter by microbial biofilms and macroinvertebrates in a large dryland river

Alexi C. Besser [1*,a], **Alana L. Robinson** [1], **Thomas F. Turner** [1,2], **Cristina D. Takacs-Vesbach** [1], **Seth D. Newsome** [1]

[1]Department of Biology, University of New Mexico, Albuquerque, New Mexico, USA; [2]Museum of Southwestern Biology, University of New Mexico, Albuquerque, New Mexico, USA

Abstract

In river food webs, the energetic coupling of nutrient-rich "fast" autochthonous (algal) and recalcitrant "slow" allochthonous (terrestrial) resources may confer stability. Additionally, microbial biofilms may enhance the nutritional quality of allochthonous resources for macroinvertebrate consumers, potentially facilitating trophic linkages within slow energy channels. We used 16S and 18S rRNA gene sequencing and essential amino acid (AA_{ESS}) carbon isotope ($\delta^{13}C$) analysis to characterize microbial biofilms and quantify proportional contributions of AA_{ESS} derived from algae, terrestrial plants, and microbes (archaea, bacteria, fungi) to macroinvertebrates across a series of mesocosm and field leaf pack experiments in the middle Rio Grande of central New Mexico, USA. In our 12-week mesocosm experiment, leaves of native Rio Grande Cottonwood trees (*Populus deltoides wislizeni*) contributed greater mean estimated proportions of AA_{ESS} (0.41–0.81) to Chironomidae larvae than those of nonnative Russian Olive trees (*Elaeagnus angustifolia*; 0.18–0.50). Microbes were notable sources of AA_{ESS} to macroinvertebrates inhabiting native C_4 grass (Spike Dropseed; *Sporobolus contractus*) leaf packs (0.08–0.26). Our field experiment demonstrated river sediment was the main source of microbes colonizing submerged Cottonwood leaves and that allochthonous resource use varied across macroinvertebrate taxa, such that Chironomidae assimilated the highest mean estimated proportions of AA_{ESS} from leaves (0.36–0.65), while Ephemeroptera and Trichoptera assimilated the highest mean estimated proportions of AA_{ESS} from algae (0.82–0.94). Our work indicates terrestrial tree leaves are important sources of AA_{ESS} to Chironomidae, while algae are the dominant source of AA_{ESS} to other macroinvertebrates in the middle Rio Grande.

The coupling of "fast" energy channels characterized by rapid rates of primary production and associated high rates of consumer turnover with "slow" energy channels characterized by lower rates of production and turnover has been shown to promote stability across a variety of ecosystems (Rooney et al. 2006). Consumers feeding in both "fast" autotrophic (green) energy channels and "slow" detrital (brown) energy channels across trophic levels can confer stability in terrestrial and aquatic food webs (Wolkovich et al. 2014). In freshwater ecology, nearly a century of research has focused on quantifying the relative importances of "fast" autochthonous (algal; green) channels and "slow" allochthonous (terrestrial; brown) channels across space and time (Brett et al. 2017). Despite recognizing the significance of allochthonous resources to river food webs (e.g., Wallace et al. 1997; Zeug and Winemiller 2008) and having several conceptual models of the mechanisms transporting them into rivers (e.g., Vannote et al. 1980; Junk et al. 1989; Ward and Stanford 1995), a comprehensive understanding of the mechanisms by which they are assimilated by aquatic animals is lacking.

Diverse communities of microbial decomposers (archaea, bacteria, fungi) that possess specialized enzymes capable of degrading the complex carbohydrates found in terrestrial organic matter play an important role in the decomposition process (Artigas et al. 2011; Marks 2019) and may aggregate to form biofilms (Battin et al. 2016). The "peanut butter on

*Correspondence: acbesser@asu.edu

[a]Present address: School of Earth and Space Exploration, Arizona State University, Tempe, Arizona, USA

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crackers" hypothesis (Cummins 1974) posits that aquatic macroinvertebrates feeding on terrestrial organic matter consume both the recalcitrant plant matter (cracker) and the associated nutritionally rich microbial biofilms (peanut butter). This hypothesis stresses the increased nutritive value of microbes growing on terrestrial organic matter. However, the roles of microbial decomposers can also vary from "priming" or "conditioning" terrestrial organic matter for macroinvertebrate grazing to competing with or deterring macroinvertebrate grazing (Bärlocher 1980; Marks 2019). Only a handful of studies to date have been able to quantify macroinvertebrate assimilation of nutrients synthesized by microbial biofilms growing on terrestrial organic matter using ^{14}C-labelling of aquatic hyphomycetes (Findlay et al. 1986; Chung and Suberkropp 2009) and whole-stream ^{13}C-enrichment (Hall 1995; Hall and Meyer 1998).

Essential amino acid (AA_{ESS}) carbon isotope ($\delta^{13}C$) "fingerprinting" is an emerging molecular technique for quantifying energy channel utilization due to its ability to reliably distinguish among AA_{ESS} sources without the need to account for trophic discrimination (Larsen et al. 2009; McMahon et al. 2010). Primary producers and other basal organisms capable of synthesizing AA_{ESS} de novo (e.g., algae, bacteria, fungi, plants) use unique biochemical pathways to do so, which imprints on the $\delta^{13}C$ values of AA_{ESS} to create distinct multivariate patterns or "fingerprints" (Besser et al. 2022). Animal consumers must directly route AA_{ESS} from their diet or symbiotic gut microbes because they are incapable of synthesizing AA_{ESS} de novo (Wu 2009; Bergen 2015). Thus, AA_{ESS} $\delta^{13}C$ fingerprinting can be used to trace dietary protein source(s) (Manlick and Newsome 2022). Pairing this compound-specific isotopic approach with 16S and 18S rRNA gene sequencing enables correlation of macroinvertebrate reliance on biofilm-derived protein with the microbial taxonomic composition of these biofilms, providing insights into the potential roles microbes play in allochthonous energy channels.

To examine how microbes influence the nutritional quality of submerged leaf litter for macroinvertebrates, we conducted a series of freshwater mesocosm and field leaf pack experiments in the middle Rio Grande, Albuquerque, New Mexico, USA. Specifically, we asked: (1) How do microbial assemblages of submerged leaf litter vary across leaf types and time? (2) How do submerged leaf litter and associated microbial biofilm AA_{ESS} $\delta^{13}C$ fingerprints change over time? (3) Are microbes important sources of protein for freshwater macroinvertebrates? (4) What are the origins of microbes colonizing submerged leaf litter? We first implemented a 12-week freshwater mesocosm experiment using leaf packs containing green leaves of two native riparian plant species—a C_3 tree, *Populus deltoides wislizeni* (Rio Grande Cottonwood; hereafter referred to as Cottonwood), and a C_4 grass, *Sporobolus contractus* (Spike Dropseed)—and one nonnative species—a C_3 tree, *Elaeagnus angustifolia* (Russian Olive). We then conducted a 28-day field experiment by placing senesced Cottonwood leaf packs in the middle Rio Grande. We predicted that microbial assemblages would differ across leaf types in the mesocosm experiment due to unique phytochemical compositions, and time in both the mesocosm and field experiments due to ecological succession. We hypothesized that leaf packs would develop increasingly microbial AA_{ESS} $\delta^{13}C$ fingerprints due to the replacement of plant protein by microbial protein over time. We also hypothesized that detritivorous macroinvertebrates colonizing leaf packs would assimilate AA_{ESS} from leaves and associated microbial biofilms. Lastly, we hypothesized that the microbial assemblages of senesced Cottonwood leaf litter would resemble those of underlying riparian topsoil and predicted the microbial assemblages of submerged Cottonwood leaf packs would converge with those of river sediment and water over time.

Materials and methods

Mesocosm experiment

Our mesocosm experiment consisted of three experimental treatments containing Cottonwood, Russian Olive, or Spike Dropseed leaf packs, and a control treatment containing terracotta tiles (21.5 cm^2). Leaf packs were constructed using coarse mesh (~ 10 mm pore size) produce bags and 20 g of oven-dried green leaves collected from the riparian forest adjacent to the middle Rio Grande in Albuquerque, New Mexico, in June 2018. Treatments contained three replicate mesocosms each for a total of 12 mesocosms, which were constructed in polyethylene stock tanks (180 cm × 60 cm) and placed outdoors at the Aquatic Conservation Facility of the Albuquerque BioPark on June 26, 2018 and June 27, 2018. River sediment and water were hauled from the middle Rio Grande and used to fill tanks to a sediment depth of ~ 7 cm and water depth of ~ 40 cm. A total of 18 leaf packs or terracotta tiles were placed in each tank. Macroinvertebrates colonized tanks via three sources: (1) the river sediment, (2) the river water, and (3) drilled logs that were placed in the middle Rio Grande for 16 d and then thoroughly rinsed in the tanks. Approximately 100 snails collected from the facility's fish-rearing tanks were also placed in each tank to help control algal growth. Tanks were covered with netting to prevent excessive mosquito colonization, although this likely reduced colonization by other aerial insects as well. Tanks were refilled with groundwater roughly every other week to maintain the desired water depth of ~ 40 cm. Hobo Pendant data loggers (UA-002-08; Onset) were positioned at the bottom center of each tank to record temperature and light conditions every 2 h, and a 556 handheld multiparameter instrument (YSI) was used to measure various water chemistry parameters weekly (Supporting Information Tables S1–S3). Filamentous green algae and particulate organic matter (> 0.7 μm; vacuum filtered onto pre-combusted 47 mm diameter, 0.7 μm pore size Whatman GF/F filters) were

sampled from tanks regularly to characterize an in situ algal isotopic endmember.

Two leaf packs (or tiles) were removed from each tank at 1, 2, 3, 4, 5, 6, 8, 10, and 12 weeks submerged. During each sampling event, leaf packs or tiles were placed in one-gallon sealable plastic bags and transported back to the laboratory on ice for immediate processing. Leaf pack material was subsampled into sterile 2-mL cryogenic vials containing sucrose lysis buffer for marker gene sequencing and 50-mL conical tubes for isotopic analysis. Subsamples for marker gene sequencing were frozen at −70°C, while subsamples for isotopic analysis were frozen at −20°C and lyophilized. Macroinvertebrates were picked from leaf packs and kept in bottles containing deionized water in the refrigerator overnight to clear their guts. The next day, macroinvertebrates were identified to Order or Family (Merritt et al. 2008) and frozen at −20°C. Multiple individuals were combined for some samples to ensure sufficient mass for isotopic analysis. Macroinvertebrates were not found on any terracotta tiles collected from control tanks, so the control treatments are not considered further.

Field experiment

We focused our field study on Cottonwood leaves, which account for the vast majority (∼ 75%) of local annual litterfall biomass (Eichhorst 2021). Senesced Cottonwood leaves were collected from the uppermost layers of litterfall in Albuquerque, New Mexico in May 2021. A total of 25 leaf packs consisting of coarse mesh (∼ 10 mm pore size) produce bags and 8 g of dried senesced leaves were deployed in the middle Rio Grande upstream of the Central Avenue bridge in Albuquerque, New Mexico (35.1012°, −106.6934°) on May 11, 2021. Ten rebar stakes (∼ 1 cm diameter, 1.2 m length) were driven into sediment near the edge of the main channel along a ∼ 100 m reach on the eastern side of the river. Two or three leaf packs were fastened to each rebar with zip ties. River flow conditions at a nearby USGS gage (site 08330000) were monitored daily. To characterize an in situ algal isotopic endmember and potential origins of microbes colonizing leaf packs, we also collected benthic algae, riparian topsoil, river sediment, and river water (Supporting Information Protocol S1).

Five leaf packs were removed, placed on ice, and transported back to the laboratory for processing at 3, 7, 14, and 21 d submerged, while only four leaf packs were removed at 28 d submerged. Leaf pack material was subsampled by taking 25 random punches from leaves using flame-sterilized metal icing tips for isotopic analysis (8 mm diameter) and marker gene sequencing (6 mm diameter). Leaf punches for marker gene sequencing were placed in sterile 2-mL microcentrifuge tubes containing 1 mL of sucrose lysis buffer and six autoclaved glass beads each, bead beat for 1 min, and frozen at −20°C. Leaf punches for isotopic analysis were placed into 15-mL conical tubes, frozen, and lyophilized. Macroinvertebrates were picked from leaf packs, identified to Order or Family (Merritt et al. 2008), and frozen at −20°C.

Freshwater microbial cultures

To characterize a freshwater microbial isotopic endmember, pure bacterial and fungal isolates, as well as some mixed colonies, were cultured from river sediment, river water, and submerged leaf litter collected from the middle Rio Grande in Albuquerque, New Mexico in February to May 2021 (Supporting Information Protocol S2).

16S and 18S rRNA gene sequencing

We performed 16S and 18S rRNA gene sequencing on a subset of samples from the freshwater microbial cultures, mesocosm experiment (leaf packs), and field experiment (senesced leaves, topsoil, river sediment, river water, fine particulate organic matter filters, and leaf packs) at the University of New Mexico following protocols adapted from the Earth Microbiome Project (Thompson et al. 2017; Supporting Information Protocol S3).

Amino acid $\delta^{13}C$ analysis

We analyzed the AA_{ESS} $\delta^{13}C$ values of a subset of samples from the freshwater microbial cultures, mesocosm experiment (algae, C_3 trees, C_4 grasses, leaf packs, and macroinvertebrates), and field experiment (algae, leaf packs, and macroinvertebrates; Supporting Information Tables S4–S8; Supporting Information Fig. S1). Whole macroinvertebrates were lipid extracted via immersion in a 2 : 1 chloroform : methanol solvent solution for three 24-h periods, rinsed with deionized water, and lyophilized. All samples were hydrolyzed and derivatized to *N*-trifluoroacetic acid isopropyl esters (Silfer et al. 1991) alongside an in-house reference material containing a mixture of commercially available AA powders (Besser et al. 2022). AA $\delta^{13}C$ values were measured on a TRACE 1310 outfitted with a 60 m × 0.32 mm ID BPX5 × 1.0 μm column and GC IsoLink II coupled to a Delta V Plus isotope ratio mass spectrometer (Thermo Scientific) at the University of New Mexico Center for Stable Isotopes (Albuquerque, New Mexico) following established procedures (Besser et al. 2022; Supporting Information Protocol S4).

Linear discriminant analysis (LDA; R package *MASS* version 7.3.54) of isoleucine, leucine, phenylalanine, threonine, and valine $\delta^{13}C$ values was used to characterize the AA_{ESS} $\delta^{13}C$ fingerprints of potential AA_{ESS} sources, leaf packs, and macroinvertebrates. In addition to the source AA_{ESS} $\delta^{13}C$ data generated in the present study, we also included published data for aquatic filamentous green algae, C_3 plants, and C_4 plants collected in central New Mexico (Besser et al. 2022; Supporting Information Table S4). Proportional contributions of potential AA_{ESS} sources to leaf packs and macroinvertebrates were quantified via Bayesian mixing models (R package *MixSIAR* version 3.1.12; Stock

et al. 2018) on LDA coordinates (Supporting Information Protocol S5).

Results

Microbial assemblages differed across leaf types and time in mesocosms

Following our prediction, the compositions of bacterial and archaeal (Fig. 1) and eukaryotic (Fig. 2) assemblages varied among leaf types and over time in the mesocosm experiment. Shannon Diversity Indices of leaf pack bacterial and archaeal assemblages increased until three (Russian Olive and Spike Dropseed) or five (Cottonwood) weeks submerged then plateaued (Fig. 1; Supporting Information Tables S9, S10). In contrast, Shannon Diversity Indices of eukaryotic assemblages decreased after 1 week submerged for Spike Dropseed leaf packs, increased after 1 week submerged then generally

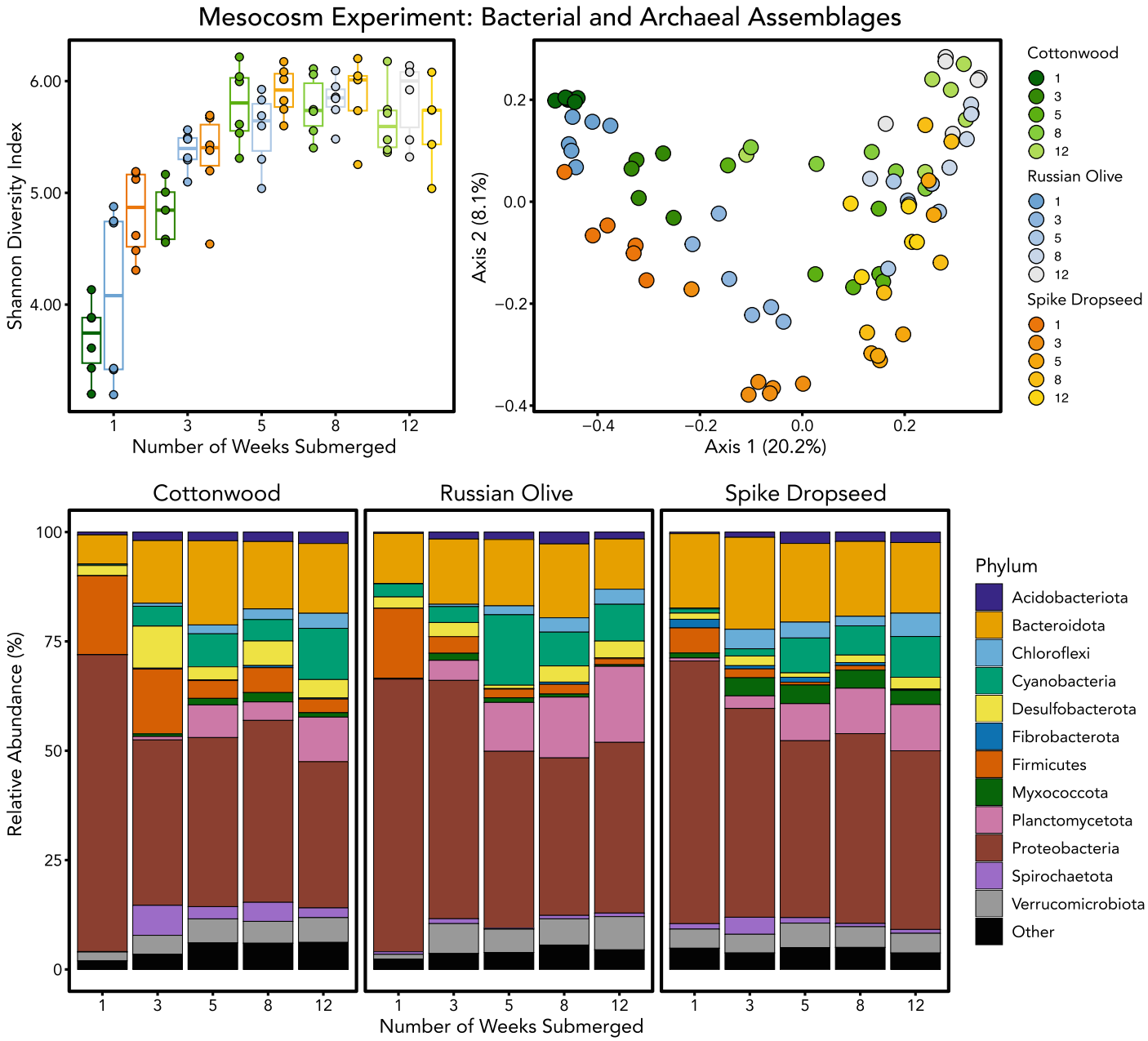

Fig. 1. Leaf pack bacterial and archaeal α- and β-diversity across leaf types and time in the mesocosm experiment. Shannon Diversity Indices (upper-left panel). Principal coordinate analysis of Bray–Curtis dissimilarities (upper-right panel). Leaf pack bacterial and archaeal assemblage composition differed across leaf types ($R^2 = 0.095$, $F = 6.063$, adjusted $p = 0.0001$) and over time ($R^2 = 0.277$, $F = 8.821$, adjusted $p = 0.0001$). Relative abundances of bacterial and archaeal phyla across leaf types and time (bottom panel). Phyla with maximum relative abundances < 5% across all samples are grouped together as *Other*.

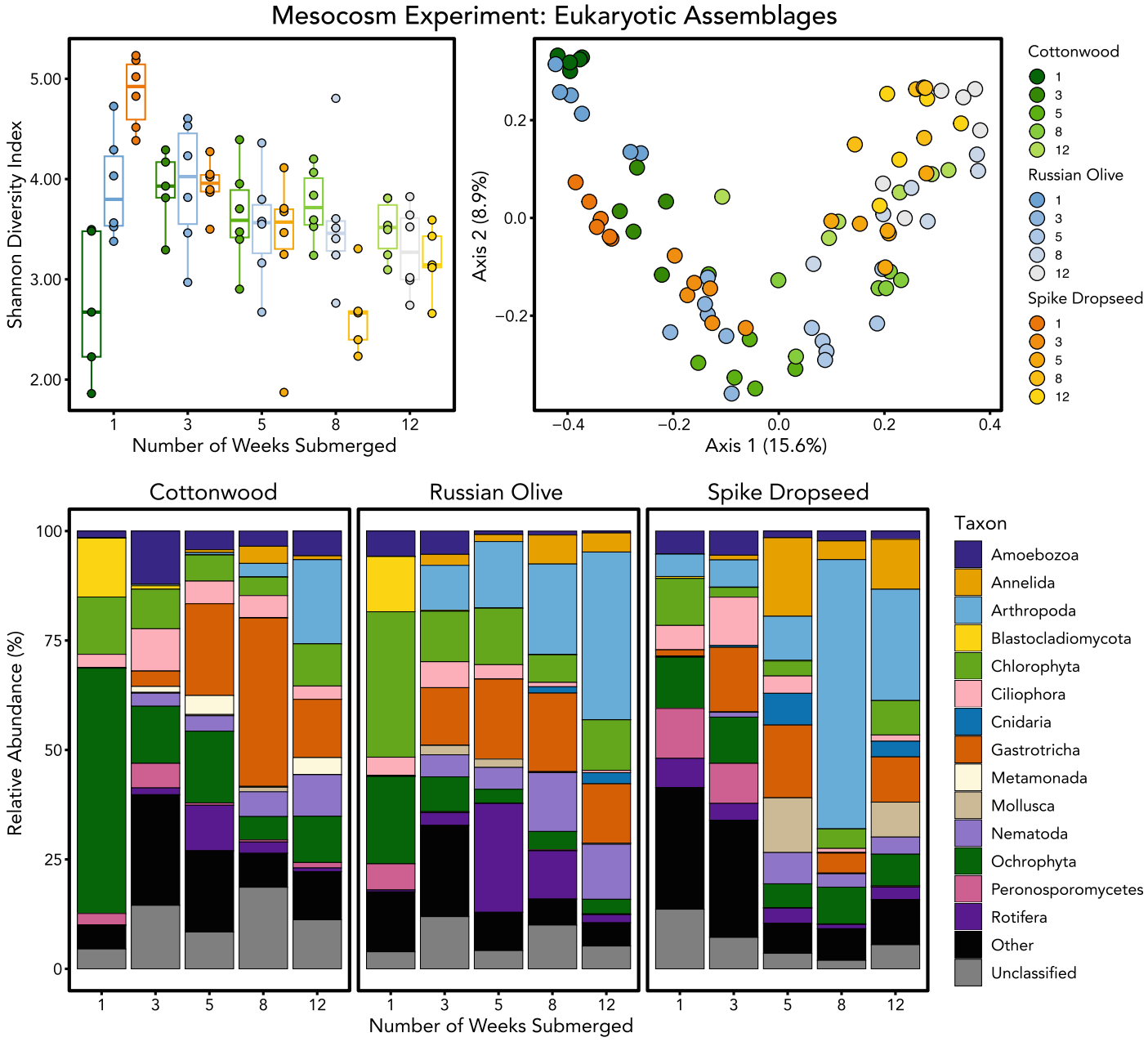

Fig. 2. Leaf pack eukaryotic α- and β-diversity across leaf types and time in the mesocosm experiment. Shannon Diversity Indices (upper-left panel). Principal coordinate analysis of Bray–Curtis dissimilarities (upper-right panel). Leaf pack eukaryotic assemblage composition differed across leaf types ($R^2 = 0.072$, $F = 4.123$, adjusted $p = 0.0001$) and over time ($R^2 = 0.242$, $F = 6.961$, adjusted $p = 0.0001$). Relative abundances of eukaryotic taxa across leaf types and time (bottom panel). Taxa with maximum relative abundances < 20% across all samples are grouped together as *Other*.

stabilized for Cottonwood leaf packs, and remained constant over time for Russian Olive leaf packs (Fig. 2; Supporting Information Tables S11, S12). The three most important 16S rRNA gene amplicon sequence variants (ASVs) for distinguishing bacterial and archaeal assemblages among leaf types belonged to the genera *Clostridium* sensu stricto *1* and *Lacunisphaera* (Supporting Information Tables S13, S14), and the three most important for distinguishing bacterial and archaeal assemblages across time belonged to the genera *Gemmobacter*, *Aeromonas*, and *Tolumonas* (Supporting Information Tables S15, S16). The three most important 18S rRNA gene ASVs for distinguishing eukaryotic assemblages among leaf types belonged to the phylum Amoebozoa and the family Sphaeropleales (Supporting Information Tables S17, S18), and the three most important for distinguishing eukaryotic assemblages across time belonged to the class Chromadorea, order

Rhizophydiales, and class Bacillariophyceae (Supporting Information Tables S19, S20).

Macroinvertebrate assimilation of AA_{ESS} differed across leaf types and time in mesocosms

The AA_{ESS} $\delta^{13}C$ fingerprints of sources were nearly perfectly separated in both LDA models (Supporting Information Fig. S2; Fig. 3). The LDA model including C_3 plants, algae, and microbes as sources yielded a 98.5% overall successful reclassification rate, while the LDA model including C_4 plants, algae, and microbes as sources yielded a 97.8% overall successful reclassification rate (Supporting Information Tables S21–S24). The AA_{ESS} $\delta^{13}C$ fingerprints of leaf packs and macroinvertebrates varied across leaf types and taxa, respectively (Supporting Information Figs. S2; Fig. 3). All Cottonwood leaf packs classified with C_3 plants (Supporting Information Table S25). The majority of macroinvertebrates collected from Cottonwood leaf packs classified with C_3 plants (71.4%) and the rest with algae (28.6%; Supporting Information Table S26). Most Russian Olive leaf packs classified with C_3 plants (85.2%; Supporting Information Table S25), while most macroinvertebrates collected from Russian Olive leaf packs classified with algae (75.6%; Supporting Information Table S26). Two-thirds of Spike Dropseed leaf packs classified with microbes (66.7%; Supporting Information Table S25); however, only one macroinvertebrate sample collected from Spike Dropseed leaf packs classified with microbes and the rest with algae (97.5%; Supporting Information Table S26).

MixSIAR models indicated shifts in the availability of different AA_{ESS} sources in leaf packs over time, with associated temporal shifts in the assimilation of AA_{ESS} from different sources by macroinvertebrates (Fig. 4; Supporting Information Figs. S3–S12). Cottonwood leaf packs contained minimal amounts of AA_{ESS} synthesized by algae or microbes until weeks 10–12 submerged, when the amount of microbial AA_{ESS} peaked at mean estimated proportions of 0.22–0.23 (Fig. 4; Supporting Information Fig. S3). In contrast, Russian Olive leaf packs contained higher mean estimated proportions of AA_{ESS} synthesized by microbes after 6–12 weeks submerged (0.24–0.47; Fig. 4; Supporting Information Fig. S4), while Spike Dropseed leaf packs contained the highest mean estimated proportions of AA_{ESS} synthesized by microbes (0.37–0.84) and algae (0.04–0.12; Fig. 4; Supporting Information Fig. S5). Chironomidae from Cottonwood and Russian Olive leaf packs assimilated the highest mean estimated proportions of AA_{ESS} synthesized by plants (0.41–0.81 and 0.18–0.50, respectively), peaking at 4–6 weeks submerged (Fig. 4; Supporting Information Figs. S7, S9), while macroinvertebrates from Spike Dropseed leaf packs assimilated negligible amounts of plant AA_{ESS} (0.01–0.03; Fig. 4; Supporting Information Figs. S10–S12). Macroinvertebrates from Spike Dropseed leaf packs assimilated the highest mean estimated proportions of AA_{ESS} synthesized by algae (0.72–0.91; Fig. 4; Supporting

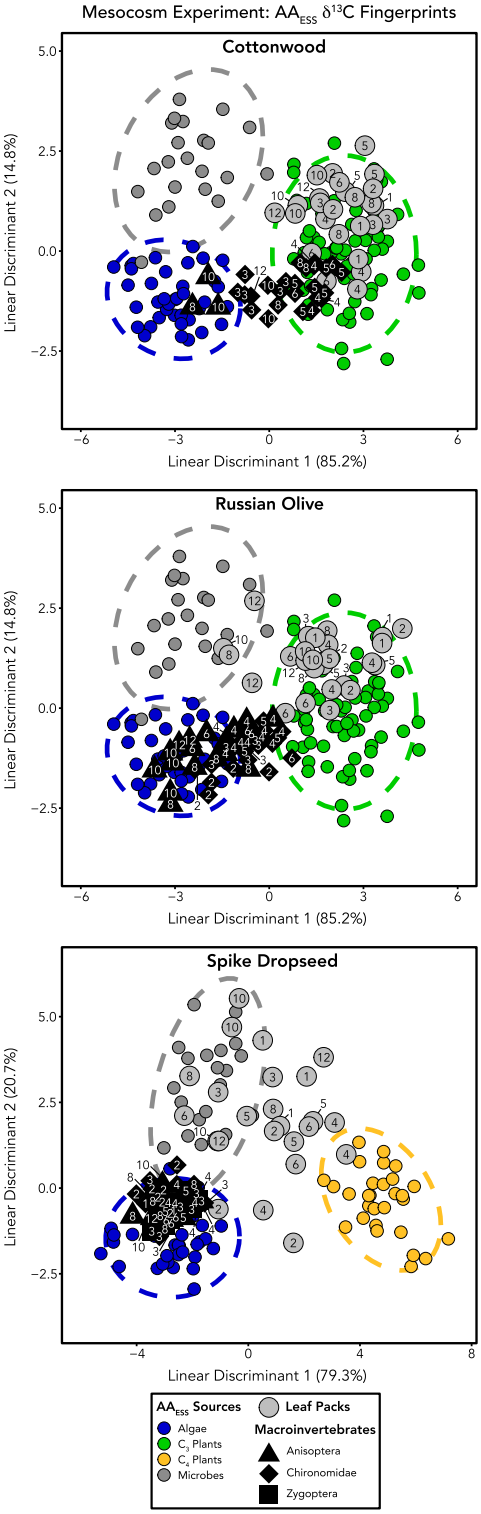

Fig. 3. AA_{ESS} $\delta^{13}C$ fingerprints of leaf packs and associated macroinvertebrates along with their potential AA_{ESS} sources for each mesocosm experimental treatment. Linear discriminant analysis was performed using the $\delta^{13}C$ values of five AA_{ESS} (isoleucine, leucine, phenylalanine, threonine, and valine). Dotted lines represent 95% confidence intervals. Numbers inside data points denote the number of weeks the leaf pack was submerged.

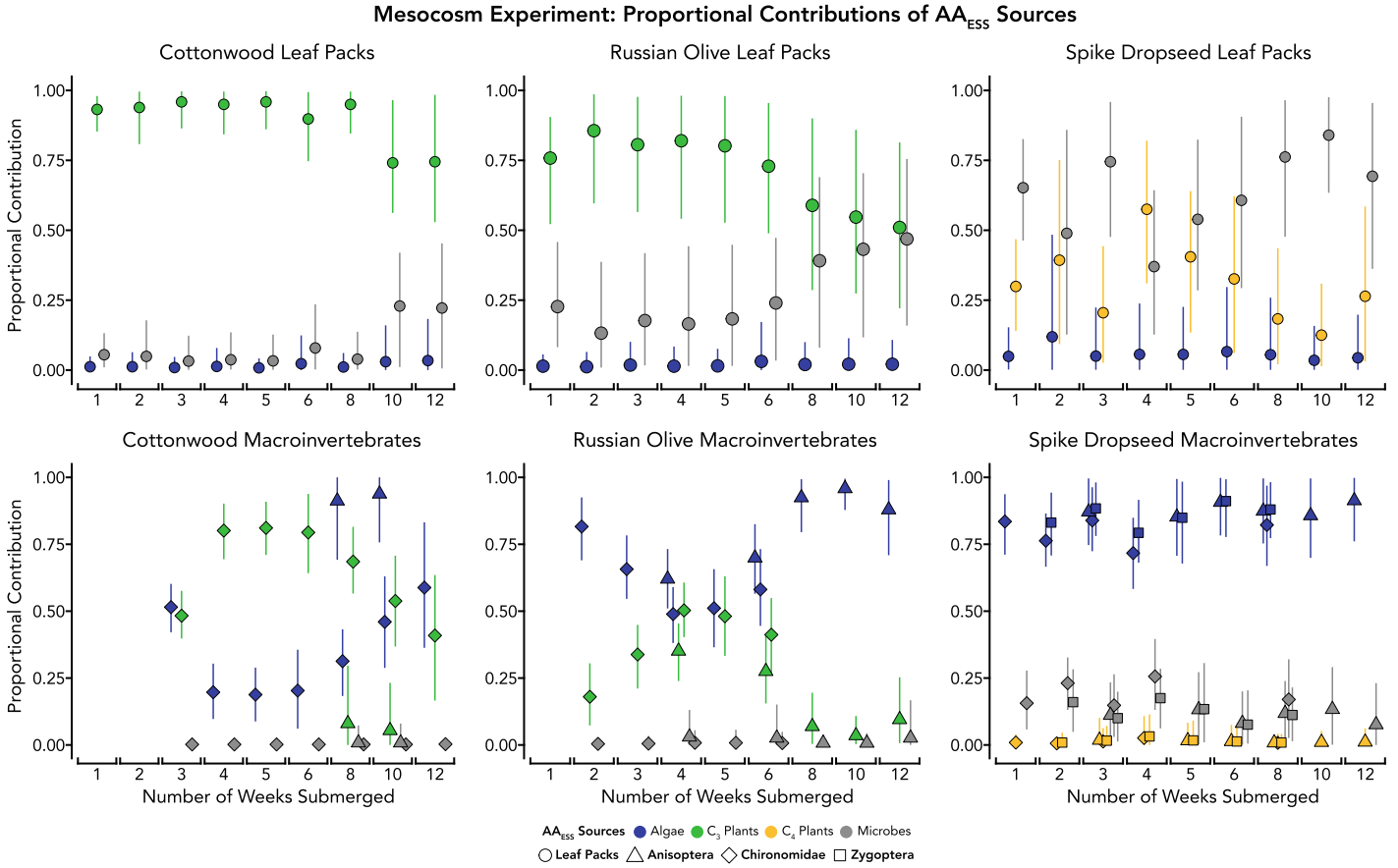

Fig. 4. Proportional contributions of AA_{ESS} sources to leaf packs and associated macroinvertebrates over time across mesocosm experimental treatments. Mean proportional contributions were estimated using MixSIAR models on the linear discriminant analysis coordinates shown in Fig. 3; error bars denote 95% confidence intervals.

Information Figs. S10–S12), followed by macroinvertebrates from Russian Olive (0.49–0.96; Fig. 4; Supporting Information Figs. S8, S9) and Cottonwood (0.19–0.94; Fig. 4; Supporting Information Figs. S6, S7) leaf packs. Macroinvertebrates from Spike Dropseed leaf packs assimilated the highest mean estimated proportions of AA_{ESS} synthesized by microbes (0.08–0.26; Fig. 4; Supporting Information Figs. S10–S12).

Microbes originating from river sediment colonized Cottonwood leaf litter in the middle Rio Grande

Bacterial and archaeal (Fig. 5) and eukaryotic (Fig. 6) assemblages were distinct among substrates and over time (leaf packs only) in the field experiment. River sediment had the highest Shannon Diversity Indices of bacterial and archaeal assemblages (Fig. 5; Supporting Information Table S27), while river sediment and river water had equally high Shannon Diversity Indices of eukaryotic assemblages (Fig. 6; Supporting Information Table S28). Shannon Diversity Indices of bacterial and archaeal (Fig. 5; Supporting Information Table S29) and eukaryotic (Fig. 6; Supporting Information Table S30) assemblages of Cottonwood leaf packs submerged in the middle Rio Grande varied over time.

In partial agreement with our hypothesis, SourceTracker estimates indicated river sediment was the greatest source of bacteria, archaea, and eukaryota to Cottonwood leaf packs submerged in the middle Rio Grande (Supporting Information Figs. S13–S16). The 15 most important 16S rRNA gene ASVs for distinguishing bacterial and archaeal assemblages among substrates were most abundant in leaf packs (Supporting Information Tables S31, S32). A 16S rRNA gene ASV from the genus *Flectobacillus* was the most important for distinguishing leaf pack bacterial and archaeal assemblages over time (Supporting Information Tables S33, S34). The two most important 18S rRNA gene ASVs for distinguishing eukaryotic assemblages among substrates belonged to the family Peronosporomycetes and order Pleosporales and were highly abundant in leaf packs (Supporting Information Tables S35, S36). The three most important 18S rRNA ASVs for distinguishing leaf pack eukaryotic assemblages over time belonged to the genus

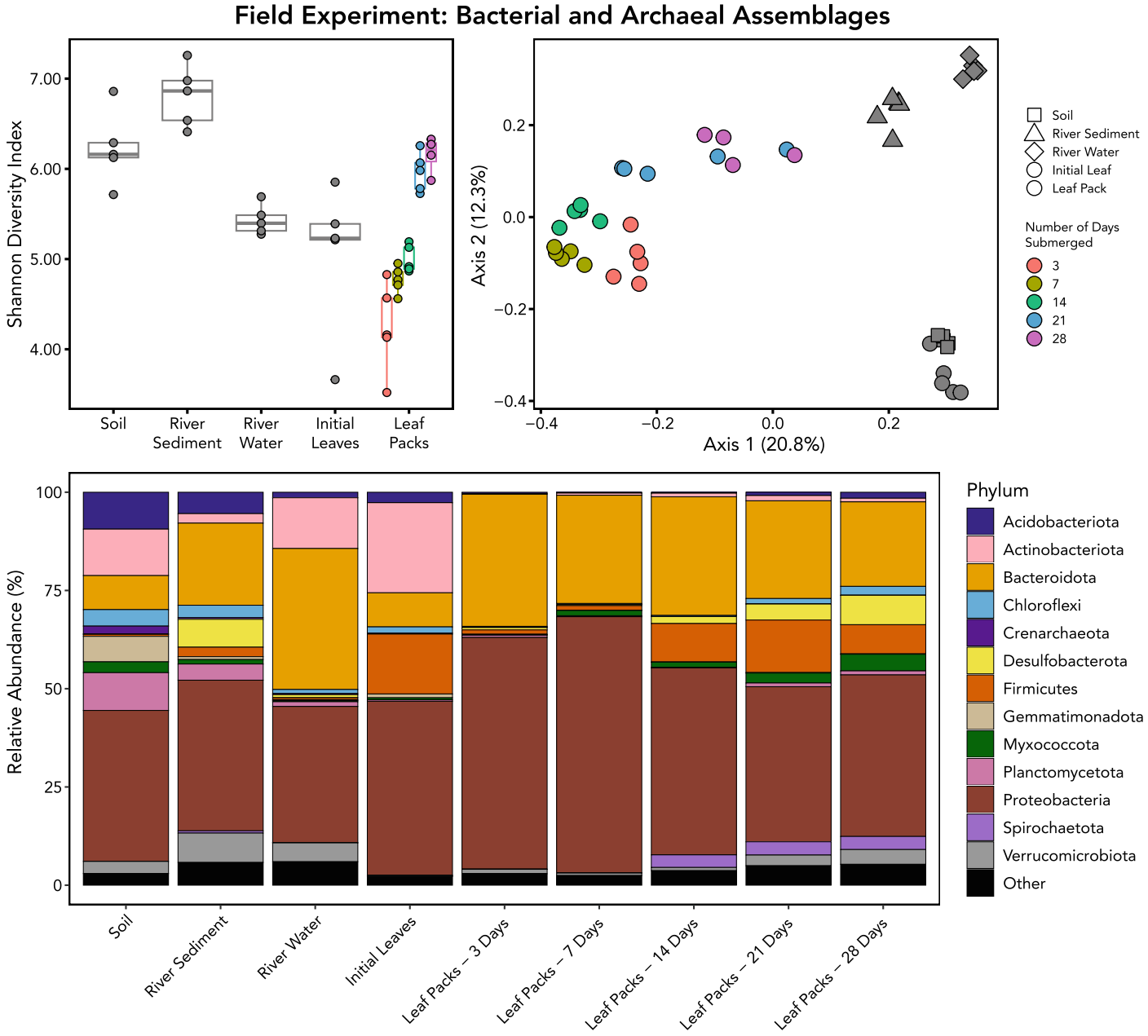

Fig. 5. Bacterial and archaeal α- and β-diversity across substrates and time [leaf packs only] in the field experiment. Shannon Diversity Indices (upper-left panel). Principal coordinate analysis of Bray–Curtis dissimilarities (upper-right panel). Bacterial and archaeal assemblage composition differed across substrates ($R^2 = 0.466$, $F = 8.501$, adjusted $p = 0.0001$) and over time for leaf packs ($R^2 = 0.589$, $F = 6.798$, adjusted $p = 0.0001$). Relative abundances of bacterial and archaeal phyla across substrates and time [leaf packs only] (bottom panel). Phyla with maximum relative abundances < 5% across all samples are grouped together as *Other*.

Pythium and phylum Ascomycota (Supporting Information Tables S37, S38).

Macroinvertebrates assimilated AA_{ESS} from algae and Cottonwood leaves in the middle Rio Grande

C_3 plants, algae, and microbes had distinct AA_{ESS} $\delta^{13}C$ fingerprints in our LDA model, with an overall successful reclassification rate of 98.7% (Supporting Information Fig. S17; Fig. 7; Supporting Information Tables S39, S40). The AA_{ESS} $\delta^{13}C$ fingerprints of leaf packs varied over time, and the AA_{ESS} $\delta^{13}C$ fingerprints of macroinvertebrates varied among taxa (Supporting Information Fig. S17; Fig. 7). All leaf packs classified with C_3 plants at 3, 7, and 14 d submerged, while two leaf packs at 21 d submerged and one leaf pack at

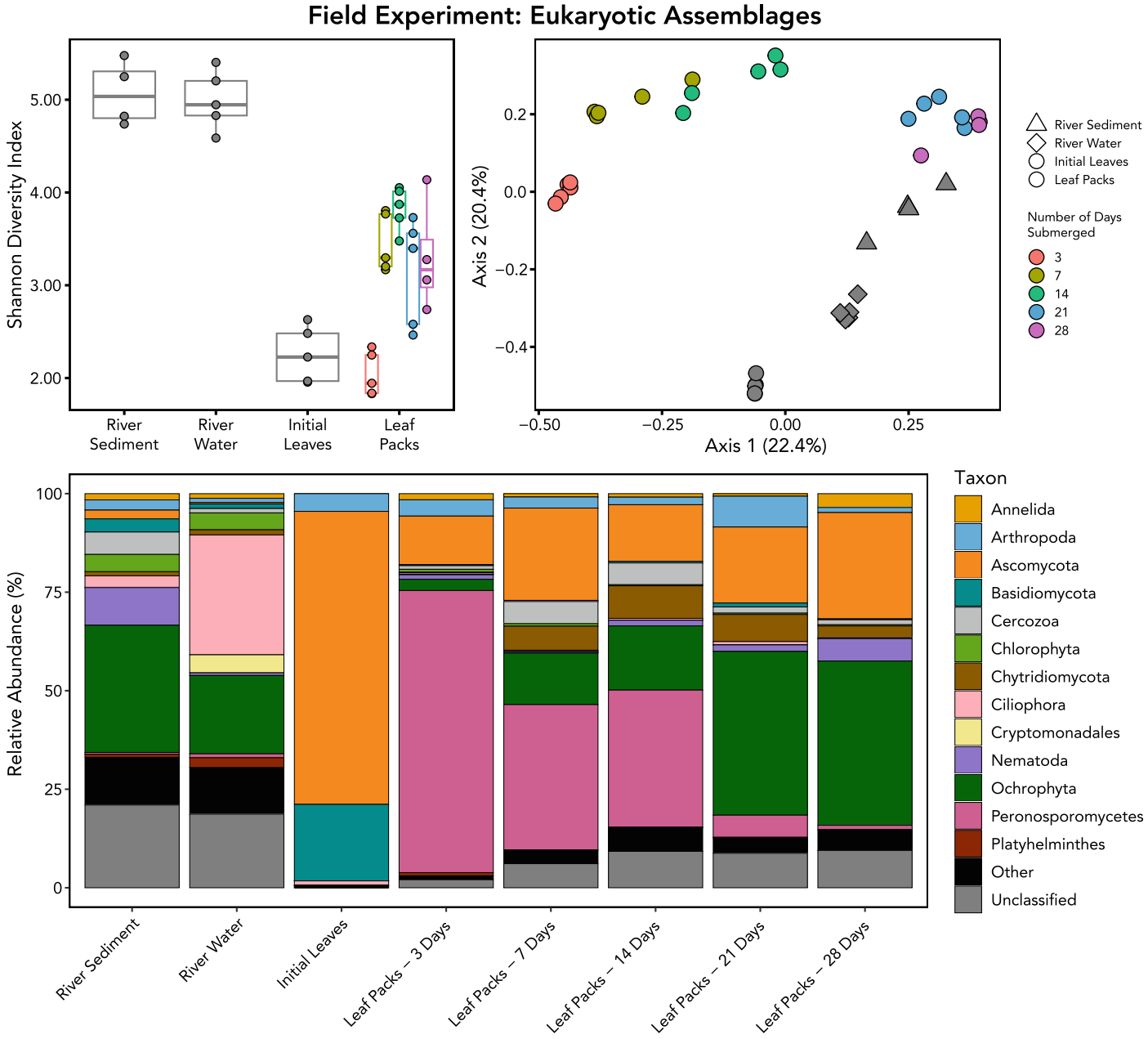

Fig. 6. Eukaryotic α- and β-diversity across substrates and time [leaf packs only] in the field experiment. Shannon Diversity Indices (upper-left panel). Principal coordinate analysis of Bray–Curtis dissimilarities (upper-right panel). Eukaryotic assemblage composition differed across substrates ($R^2 = 0.410$, $F = 7.873$, adjusted $p = 0.0001$) and over time for leaf packs ($R^2 = 0.689$, $F = 10.514$, adjusted $p = 0.0001$). Relative abundances of eukaryotic taxa across substrates and time [leaf packs only] (bottom panel). Taxa with maximum relative abundances < 5% across all samples are grouped together as *Other*.

28 d submerged classified with microbes (Supporting Information Table S41). Chironomidae were the only macroinvertebrate taxon to classify with C_3 plants (representing 22.0% of macroinvertebrate samples overall); all other macroinvertebrate samples classified with algae (Supporting Information Table S42).

The majority of the AA_{ESS} available in leaf packs were synthesized by C_3 plants and microbes throughout the 28-d experiment (mean estimated proportions of 0.60–0.78 and 0.20–0.38, respectively; Fig. 8; Supporting Information Fig. S18). However, Ephemeroptera and Trichoptera assimilated most of their AA_{ESS} from algae (0.82–0.94) followed by C_3 plants (0.04–0.16; Fig. 8; Supporting Information Figs. S19, S20). Mean estimated proportional contributions of C_3 plant AA_{ESS} to Ephemeroptera and Trichoptera peaked at 21 d submerged (0.12 and 0.16, respectively; Fig. 8; Supporting

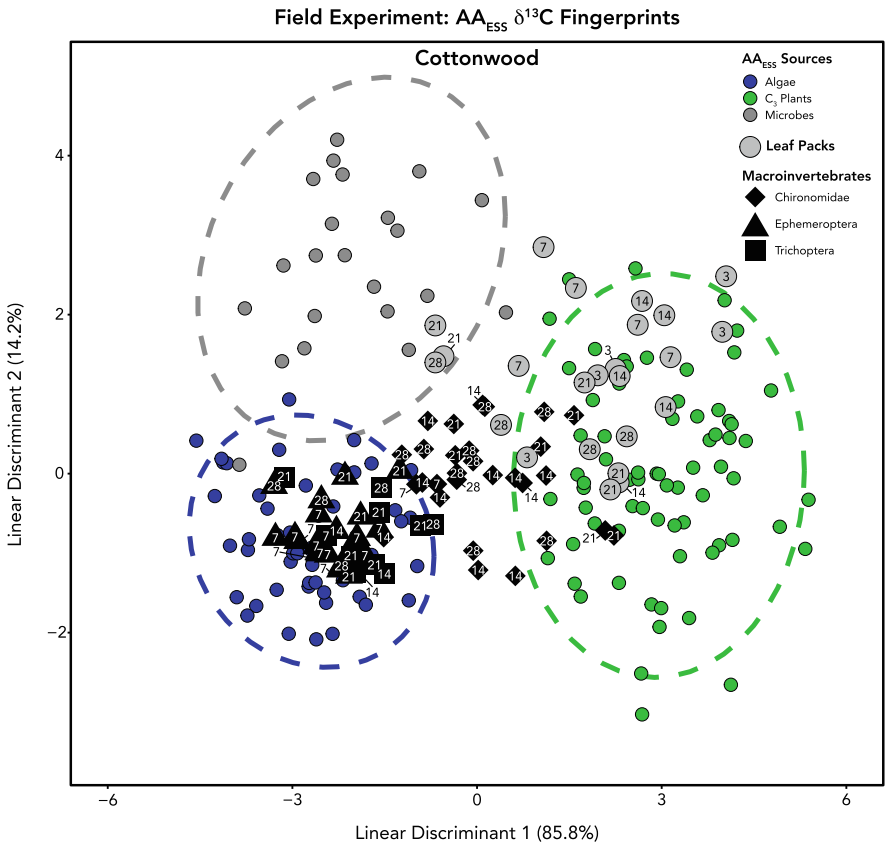

Fig. 7. AA$_{ESS}$ δ^{13}C fingerprints of Cottonwood leaf packs and associated macroinvertebrates along with their potential AA$_{ESS}$ sources for the field experiment. Linear discriminant analysis was performed using the δ^{13}C values of five AA$_{ESS}$ (isoleucine, leucine, phenylalanine, threonine, and valine). Dotted lines represent 95% confidence intervals. Numbers inside data points denote the number of days the leaf pack was submerged.

Information Figs. S19, S20). Chironomidae displayed the highest mean estimated proportional assimilation of AA$_{ESS}$ synthesized by C_3 plants at 21 d submerged (0.65; Fig. 8; Supporting Information Fig. S21). Assimilation of algal-derived AA$_{ESS}$ by Chironomidae was highest at 7 d submerged (0.55) and lowest at 21 d submerged (0.29; Fig. 8; Supporting Information Fig. S21). Microbes contributed minimally to macroinvertebrate AA$_{ESS}$ budgets, reaching maximum mean estimated proportional contributions for Chironomidae at 28 d submerged (0.11; Fig. 8; Supporting Information Fig. S21).

Discussion

By combining 16S and 18S rRNA gene sequencing with AA$_{ESS}$ δ^{13}C fingerprinting across a series of mesocosm and field leaf pack experiments, we connect microbial biofilm community dynamics with estimates of the basal AA$_{ESS}$ sources supporting macroinvertebrates. Our hypothesis that plant protein would be replaced by microbial protein as the decomposition of submerged leaves progressed and that detritivores would indiscriminately consume all available protein was partially supported. Although heterotrophic microbes originating from river sediment likely facilitated the initial breakdown of submerged leaves and the proportion of microbially derived AA$_{ESS}$ in leaf packs generally increased over time, microbes were significant sources of AA$_{ESS}$ to macroinvertebrates only in Spike Dropseed (C_4 grass) leaf packs in the mesocosm experiment. Chironomidae assimilated the highest mean estimated proportions of AA$_{ESS}$ from leaves, while all other macroinvertebrate taxa assimilated the highest mean estimated proportions of AA$_{ESS}$ from algae. Overall, our work provides a robust analytical framework for examining microbial roles in submerged leaf litter decomposition and allochthonous resource use in freshwater food webs.

Microbial assemblages have diverse origins, compositions, and roles in leaf packs

The majority of archaea, bacteria, and eukaryota in Cottonwood leaf packs submerged in the middle Rio Grande originated from river sediment. Previous studies have shown most fungal decomposers colonize leaf litter prior to submersion, while bacterial decomposers colonize submerged leaf litter

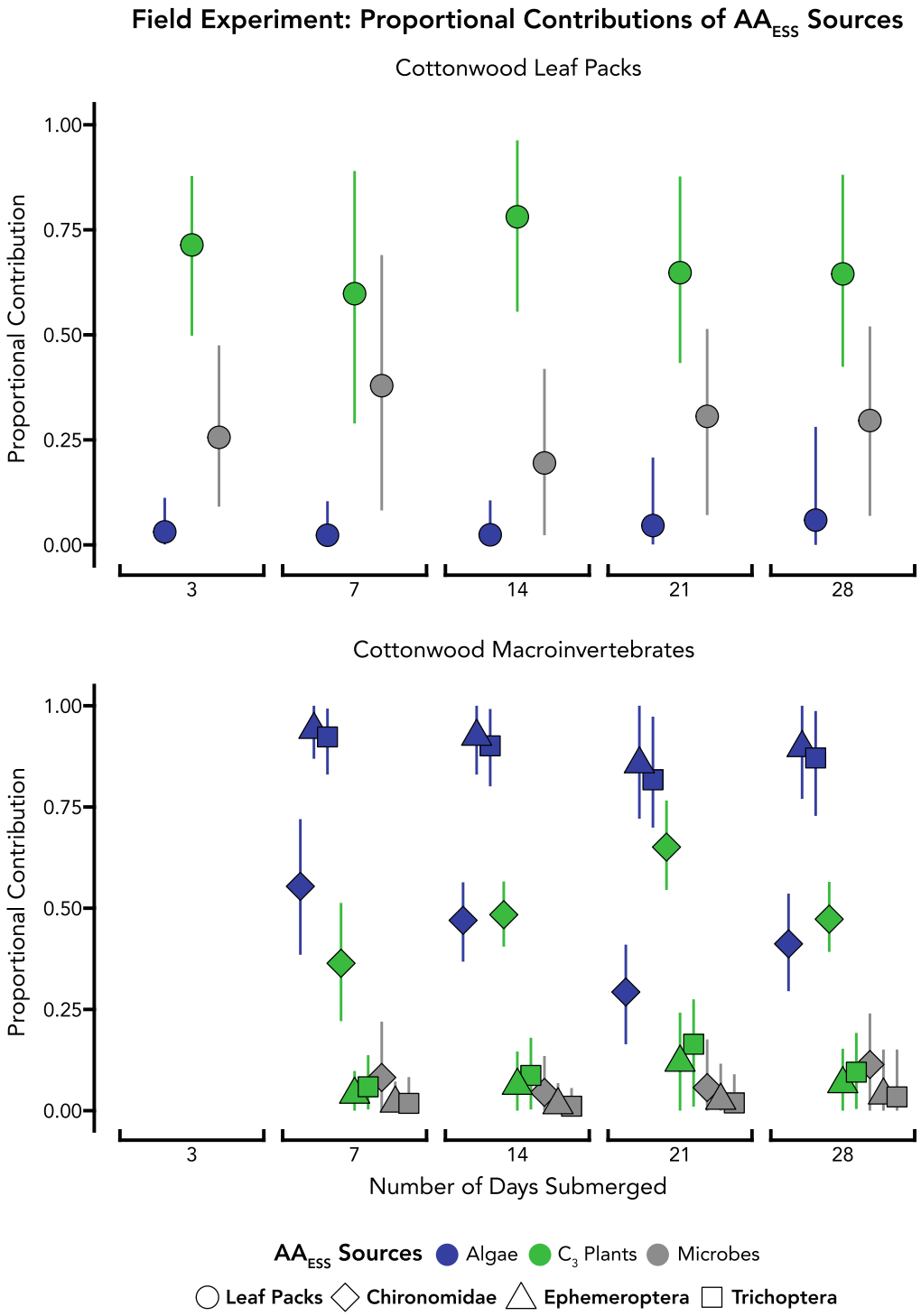

Fig. 8. Proportional contributions of AA_{ESS} sources to Cottonwood leaf packs and associated macroinvertebrates over time for the field experiment. Mean proportional contributions were estimated using MixSIAR models on the linear discriminant analysis coordinates shown in Fig. 7; error bars denote 95% confidence intervals.

from sediments or the overlying water column (Marks 2019; Koivusaari et al. 2019; Hayer et al. 2022). However, in our field experiment, the relative abundance of fungi in leaf packs rapidly decreased following submersion and recovered only slightly over time (Fig. 6). In fact, submersion caused almost complete turnover of the eukaryotic community and substantial decreases in the relative abundances of the bacterial phyla Actinobacteroita and Firmicutes (Figs. 5, 6).

Leaf pack microbial assemblages varied across leaf types and time in our mesocosm experiment (Figs. 1, 2), a finding supported by work showing that the species, genetics, and duration of submersion of leaf litter in streams impact microbial composition and diversity (e.g., Marks et al. 2009; Wymore et al. 2016). A driving factor of these patterns is differences in phytochemical compositions (e.g., tannin and lignin concentrations), which can vary both within and among species (Wymore et al. 2016). Environmental heterogeneity can also strongly influence microbial biofilm diversity and function in streams (Battin et al. 2016). The stacking of Spike Dropseed blades may have provided more structural complexity for the initial establishment of biofilms, while phenolic compounds and other secondary metabolites in Cottonwood and Russian Olive leaves may have initially inhibited microbial growth (Newman et al. 2015), leading to higher microbial Shannon Diversity Indices for Spike Dropseed leaf packs at 1 week submerged (Figs. 1, 2). However, in agreement with previous studies (e.g., Newman et al. 2015; Wymore et al. 2016), time exerted a stronger influence on leaf pack microbial community composition than leaf type (Figs. 1, 2). Leaching of water-soluble compounds over hours to days following submersion (Gessner et al. 1999) likely dampened the effects of variation in phytochemical compositions, resulting in the convergence of microbial assemblage composition and diversity across leaf types (Newman et al. 2015; Figs. 1, 2).

Amplicon sequence variants belonging to the fermentative bacterial genera *Gemmobacter*, *Aeromonas*, and *Tolumonas* (Dueholm et al. 2024) were particularly abundant in all leaf packs near the beginning of the mesocosm experiment (Supporting Information Tables S15, S16), suggesting fermentation may have been a key process in early decomposition. Similarly, ASVs belonging to the parasitic oomycete genus *Pythium*, which contains species known to produce pectinases and cellulases capable of degrading plant cell walls (Zerillo et al. 2013), rapidly established and dominated Cottonwood leaf packs in the field experiment (Supporting Information Tables S35-S38 S31–S38). Given their early dominance and rapid decline, *Pythium* and other Peronosporomycetes taxa potentially played an important role in the initial conditioning of submerged Cottonwood leaves (Gessner et al. 1999). This likely liberated labile forms of carbon and nitrogen, facilitating macroinvertebrate grazing by increasing access to digestible nutrients (Findlay et al. 1986; Marks 2019). Microbes can also enhance macroinvertebrate consumption of submerged leaves by synthesizing and supplementing essential nutrients (Marks 2019), while algae can increase the digestibility and nutritional quality of submerged leaves because they have higher nitrogen and lower structural carbon contents than terrestrial plants (Cross et al. 2005). Green algal (Chlorophyta) ASVs were among the most important for distinguishing eukaryotic assemblages across leaf types and time in the mesocosm experiment (Supporting Information Tables S17–S20; S9, S10), while several brown algal (Ochrophyta) ASVs were important for distinguishing eukaryotic assemblages among substrates and time in the field experiment (Supporting Information Tables S35–S38).

Relative abundances of ASVs belonging to autotrophic taxa, including the nitrogen-fixing cyanobacterial genus *JSC-12*, increased across all leaf types during the mesocosm experiment (Supporting Information Table S16), potentially indicating a shift from net heterotrophy to autotrophy as organic resources were depleted (Veach et al. 2016). In particular, microbes likely predominantly used leaf-derived nitrogen during the first hours to days following submersion but increasingly used water-derived nitrogen as decomposition progressed (Cheever et al. 2013). Likewise, relative abundances of several ASVs belonging to the autotrophic phylum Ochrophyta (brown algae) substantially increased over time in the field experiment (Fig. 6). Temporal increases in the relative abundances of algal taxa likely corresponded with increases in the availability of algal AA_{ESS} to macroinvertebrates.

Macroinvertebrate assimilation of AA_{ESS} varied across leaf types, taxa, and time

Our hypothesis that plant protein would be replaced by microbial protein as decomposition progressed was supported by AA_{ESS} $\delta^{13}C$ fingerprinting results for Cottonwood and Russian Olive leaf packs in the mesocosm experiment (Figs. 3, 4). These findings agree with previous studies that found C : N ratios decrease as the decomposition of submerged leaf litter progresses (e.g., Siders et al. 2018, 2021), likely reflecting increases in microbial growth supplemented by inorganic nitrogen from the water column (Pastor et al. 2014). In contrast, Spike Dropseed leaves contained low proportions of plant protein, as indicated by high initial C : N ratios (Supporting Information Table S8), allowing microbial protein to comprise a greater proportion of the total protein in leaf packs (Fig. 4). Cottonwood leaf packs in the field experiment also maintained consistently high mean estimated proportions of microbial AA_{ESS} over time (Fig. 8), potentially due to lower protein contents of senesced leaves compared to green leaves. Given that the relative abundances of green and brown algae in leaf packs increased over time in both experiments, we expected the proportion of algal AA_{ESS} to also increase over time. However, mean estimated proportions of algal AA_{ESS} were negligible in all leaf packs except Spike Dropseed leaf packs in the mesocosm experiment (Fig. 4).

Patterns in AA_{ESS} assimilation by macroinvertebrates varied across taxa but generally aligned with patterns in AA_{ESS} availability. In both the mesocosm and field experiments, Chironomidae assimilated higher proportions of AA_{ESS} from C_3 tree leaves than any other macroinvertebrates (Figs. 4, 8). Chironomidae exhibit a wide variety of functional feeding habits, but generally consume fine to coarse detrital particles or algae as filterers, gatherers, scrapers, and shredders (Merritt et al. 2008). In the mesocosm experiment, Cottonwood leaves

supplied ~1.5–2.0× more AA_{ESS} to Chironomidae than Russian Olive leaves (Fig. 4), despite containing ~2.5× less nitrogen (Supporting Information Table S8). In a previous study, the decomposition rates of senesced Cottonwood and Russian Olive leaves were similar and positively correlated with macroinvertebrate (predominantly Chironomidae) density in the middle Rio Grande, while Russian Olive leaves decomposed significantly faster than Cottonwood leaves in adjacent floodplain soils due to higher initial nitrogen contents, extracellular enzyme activities, and fungal biomass (Harner et al. 2009). However, our results suggest submerged green Russian Olive leaves may have decomposed too quickly to support macroinvertebrates, which likely assimilate more nutrients from leaf litter that decomposes slowly (Siders et al. 2018, 2021). Russian Olive leaf packs also had higher mean estimated proportions of microbial AA_{ESS} than Cottonwood leaf packs over time (Figs. 3, 4), potentially indicating higher rates of microbial growth that could have competed with or deterred macroinvertebrate grazing (Marks 2019). High concentrations of structural carbon (57% of total carbon) in Russian Olive leaves compared with Cottonwood leaves (35% structural carbon) likely also prevented extensive grazing by macroinvertebrates (Moline and Poff 2008). Lastly, macroinvertebrates may be better adapted to assimilate nutrients from native than nonnative riparian plant species. For example, although *Tipula* (Diptera) larvae gained more mass when fed preconditioned nonnative Russian Olive and Salt Cedar (*Tamarix* spp.) leaves than when fed preconditioned native Cottonwood leaves, presumably due to the higher nitrogen contents of these nonnative leaves, they exhibited higher survival rates when fed Cottonwood leaves (90%) than when fed Salt Cedar (*Tamarix* spp.; 70%) and Russian Olive (55%) leaves (Moline and Poff 2008). Mean estimated proportional contributions of AA_{ESS} from leaves to macroinvertebrates peaked at 4–6 weeks submerged for Cottonwood and Russian Olive leaf packs in the mesocosm experiment and 21 d (3 weeks) submerged for Cottonwood leaf packs in the field experiment, suggesting some form of microbial conditioning needed to occur to facilitate macroinvertebrate feeding (Gessner et al. 1999; Marks 2019).

Despite their importance in the initial conditioning of submerged leaf litter, microbes contributed little to the AA_{ESS} budgets of most macroinvertebrates in both experiments (Figs. 4, 8). Although microbes are often more nutritious than the leaf litter they inhabit (Cummins 1974; Cross et al. 2005; Marks 2019), assimilation of bacterial carbon, whether derived from whole cells or extracellular exudates, can vary greatly across macroinvertebrate taxa, functional feeding groups, and life stages (Hall 1995; Hall and Meyer 1998). Mean estimated assimilation of microbial AA_{ESS} was highest for Chironomidae in Spike Dropseed leaf packs in the mesocosm experiment (Fig. 4; Supporting Information Fig. S11). Given the minimal assimilation of Spike Dropseed AA_{ESS}, microbial biomass assimilated by Chironomidae in these leaf packs was probably associated with attached algae that were either directly or indirectly consumed. Chironomidae in Cottonwood leaf packs in the field experiment assimilated significant mean estimated proportions of both Cottonwood and microbial AA_{ESS} (Fig. 8), indicating consumption of microbial biofilms (the "peanut butter") associated with decomposing Cottonwood leaf litter (the "crackers"; Cummins 1974). Similarly, Hall and Meyer (1998) reported a positive relationship between the fraction of amorphous detritus found in gut contents and the fraction of bacterial carbon incorporated by macroinvertebrates. It is worth noting that the lower mean estimated proportional contributions of microbial AA_{ESS} compared to Cottonwood AA_{ESS} may be attributed to low microbial biomass relative to that of Cottonwood leaf litter (Baldy et al. 2002). For example, Findlay et al. (1986) noted that even 100% assimilation of microbial biomass would contribute little to the metabolism of the stonefly *Peltoperla* spp. because microbial carbon constituted < 2.5% of leaf carbon. The taxonomic composition of microbial biomass also influences its utilization by macroinvertebrates, with previous work indicating fungal biomass associated with submerged leaves may account for up to 100% of the daily growth rate of shredder larvae (Chung and Suberkropp 2009).

The majority of macroinvertebrates in both experiments relied heavily on algal AA_{ESS} (Fig. 4), following predictions from the Riverine Productivity Model (Thorp and Delong 1994). Recent AA_{ESS} $\delta^{13}C$ fingerprinting studies on lotic (Thorp and Bowes 2017; Liew et al. 2019; Arsenault et al. 2022) and lentic (Saboret et al. 2023; Shipley et al. 2023) fish occupying primary to tertiary consumer trophic levels also indicated predominant reliance on algal energy channels. The assimilation of algal AA_{ESS} by macroinvertebrates was higher than expected considering mean estimated proportions of algal AA_{ESS} in leaf packs were low, probably because algal production can be much higher than its standing stock due to heavy grazing (Vadeboncoeur and Power 2017). Attached algae is recognized as the "cryptic base of inverted trophic pyramids" in freshwater ecosystems due to its low biomass yet potentially widespread importance as a food resource (Vadeboncoeur and Power 2017). Our results indicate algae supports consumers across functional feeding groups and trophic levels. In the field experiment, Ephemeroptera and some Trichoptera taxa were likely scrapers feeding on algae adhered to leaf packs, while other Trichoptera taxa may have been predators of algivorous prey (Merritt et al. 2008). In the mesocosm experiment, the generalist predators Anisoptera and Zygoptera assimilated algal AA_{ESS} indirectly, potentially using their high mobility to hunt prey both inside and outside of leaf packs (Merritt et al. 2008). Algae may even supplement the diets of detritivorous macroinvertebrates during the initial conditioning and later decay stages of decomposition, as indicated by high mean estimated proportional contributions of algal AA_{ESS} to

Chironomidae near the beginning and end of each experiment (Figs. 4, 8).

Ecosystem-level implications and future directions

We build on previous work demonstrating macroinvertebrate community composition varies between reaches bordered by native vs. nonnative riparian plants (Seeney et al. 2019; Little et al. 2021) by showing Chironomidae may assimilate higher mean estimated proportions of protein from native than nonnative riparian tree leaves. Chironomidae larvae dominate macroinvertebrate assemblages in many freshwater ecosystems, including the middle Rio Grande (Kennedy and Turner 2011), and thus serve as important conduits of allochthonous energy to higher trophic level aquatic (Muth and Snyder 1995) and terrestrial animals (Génier et al. 2022). Future work investigating the influences of other riparian plant species and additional factors, including intraspecific variation in leaf litter chemical composition due to genotypic (Compson et al. 2018) and environmental conditions (Tibbets and Molles 2005), on AA_{ESS} assimilation across a wider range of macroinvertebrate taxa and functional feeding groups will improve our understanding of the drivers of allochthonous resource use.

Our study also exemplifies how advances in molecular and isotopic techniques provide novel opportunities to investigate microbial roles in freshwater food webs with high resolution. Additional work quantifying variability in AA_{ESS} $\delta^{13}C$ fingerprints among microbial taxa that utilize different metabolisms is needed to fully explore the utility of this promising approach. Future work pairing -omics data (Gubelit and Grossart 2020) with leaf litter chemistry data could elucidate the specific metabolic activities of microbes growing in submerged leaf litter and enhance our understanding of the roles they play in liberating allochthonous resources for other consumers.

Author Contributions

Alexi C. Besser, Thomas F. Turner, Cristina D. Takacs-Vesbach, and Seth D. Newsome designed the study. Thomas F. Turner, Cristina D. Takacs-Vesbach, and Seth D. Newsome provided field and laboratory equipment. Alexi C. Besser and Alana L. Robinson collected the data. Alexi C. Besser analyzed the data. Alexi C. Besser wrote the manuscript. Alana L. Robinson, Thomas F. Turner, Cristina D. Takacs-Vesbach, and Seth D. Newsome edited the manuscript.

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Conflicts of Interest

None declared.

Data Availability Statement

Amino acid $\delta^{13}C$ data are archived in the Dryad Digital Repository: https://datadryad.org/share/nyouvKDksrrKq1SqFm8cGCDlTd7oUPGsPkZsw2o6jM8. 16S and 18S rRNA gene sequence data are archived in the NCBI Sequence Read Archive (SRA): https://dataview.ncbi.nlm.nih.gov/object/PRJNA1289908?reviewer=7h9ma2bes2cls6bl61flsoivde.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

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