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An inquiline mosquito modulates microbial diversity and function in an aquatic microecosystem

Aldo A. Arellano[1,2] | Erica B. Young[3,4] | Kerri L. Coon[2]

[1]Microbiology Doctoral Training Program, University of Wisconsin-Madison, Madison, Wisconsin, USA

[2]Department of Bacteriology, University of Wisconsin-Madison, Madison, Wisconsin, USA

[3]Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin, USA

[4]School of Freshwater Sciences, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin, USA

Correspondence
Kerri L. Coon, Department of Bacteriology, University of Wisconsin-Madison, Madison, WI, USA.
Email: kerri.coon@wisc.edu

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Abstract

Understanding microbial roles in ecosystem function requires integrating microscopic processes into food webs. The carnivorous pitcher plant, *Sarracenia purpurea*, offers a tractable study system where diverse food webs of macroinvertebrates and microbes facilitate digestion of captured insect prey, releasing nutrients supporting the food web and host plant. However, how interactions between these macroinvertebrate and microbial communities contribute to ecosystem functions remains unclear. We examined the role of the pitcher plant mosquito, *Wyeomyia smithii*, in top-down control of the composition and function of pitcher plant microbial communities. Mosquito larval abundance was enriched or depleted across a natural population of *S. purpurea* pitchers over a 74-day field experiment. Bacterial community composition and microbial community function were characterized by 16S rRNA amplicon sequencing and profiling of carbon substrate use, bulk metabolic rate, hydrolytic enzyme activity, and macronutrient pools. Bacterial communities changed from pitcher opening to maturation, but larvae exerted minor effects on high-level taxonomic composition. Higher larval abundance was associated with lower diversity communities with distinct functions and elevated nitrogen availability. Treatment-independent clustering also supported roles for larvae in curating pitcher microbial communities through shifts in community diversity and function. These results demonstrate top-down control of microbial functions in an aquatic microecosystem.

KEYWORDS
carnivorous plant, microbiota, nitrogen, nutrient degradation/cycling, symbiosis, top-down control

1 | INTRODUCTION

Microbes carry out essential ecosystem functions across aquatic and terrestrial environments, mediating primary productivity, decomposition and nutrient cycling (Das et al., 2006; Delgado-Baquerizo et al., 2016). However, while the importance of microbe-mediated processes in ecosystem function is well-understood, integration of top-down trophic processes into models of microbial assembly and function is needed to expand our understanding of feedbacks between top-down and bottom-up controls (Mikola & Sktälä, 1998). The tripartite symbiosis of the purple pitcher plant, *Sarracenia purpurea*, the pitcher plant mosquito, *Wyeomyia smithii*, and their associated microbiota provides an ideal microecosystem to explore cross-trophic impacts on microbial functions (Bier et al., 2015). Purple pitcher plants are carnivorous and reside in nutrient-depleted wetlands, where they produce water-filled leaves (pitchers) that act as pitfall traps for insect

prey that supplement their nutrient requirements, particularly for nitrogen (Ellison & Gotelli, 2002; Gotelli & Ellison, 2002). However, *S. purpurea* do not produce several classes of enzymes required to digest prey and instead rely on inquiline macroinvertebrate and microbial communities present in pitcher fluid for the mechanical and hydrolytic enzymatic breakdown of captured insects, respectively (Adlassnig et al., 2011; Bittleston et al., 2018; Gallie & Chang, 1997; Luciano & Newell, 2017; Prankevicius & Cameron, 1991; Young et al., 2018). This breakdown of insect carcasses liberates bioavailable nitrogen and other nutrients for uptake by host plant tissues, while also supporting inquiline communities (Butler & Ellison, 2007; Karagatzides et al., 2009). In this way, both top-down (macroinvertebrate) and bottom-up (microbial) controls shape availability of key resources (e.g. nitrogen) to promote ecosystem stability and function in the *S. purpurea* ecosystem.

One key macroinvertebrate in the *S. purpurea* aquatic microecosystem is the pitcher plant mosquito, *W. smithii*. Unlike most mosquito species that indiscriminately lay their eggs into small bodies of standing water, adult *W. smithii* females obligately lay their eggs into *Sarracenia* pitchers, where larvae progress through four aquatic juvenile stages (instars) and an aquatic pupal stage before emerging as terrestrial adults (Bradshaw & Creelman, 1984; Heard, 1994). Pitcher-associated microbes play essential roles in *W. smithii* larval development by serving as cues for growth and moulting (Arellano & Coon, 2022), as has been documented in other mosquito species (Coon et al., 2014, 2016a, 2016b, 2020; Valzania et al., 2018). Conversely, *W. smithii* larvae can also affect the composition and trophic structure of inquiline microbial communities (Peterson et al., 2008), with implications for community function (Nemergut et al., 2013). Changes to microbial metabolism and degradative activity may directly impact nutrient supply to host plants, as they are largely dependent on microbes for enzymatic lysis of prey substrates (Young et al., 2018). The effect of *W. smithii* on culturable bacterial diversity and abundance has been previously examined, suggesting that larval grazing on microeukaryotes such as rotifers and protozoa may reduce predation pressure on bacteria, increasing bacterial density and diversity (Cochran-Stafira & von Ende, 1998; Hoekman, 2011; Kneitel & Miller, 2002; Peterson et al., 2008). Even so, other studies have shown that direct bacterivory by larvae may also play a role in structuring bacterial communities (Hoekman, 2007; Hoekman et al., 2009). While models or rationale have been proposed for the role of mosquito larvae in pitcher plant inquiline communities (Buckley et al., 2003; Mouquet et al., 2008), the impact of *W. smithii* on microbial community function in the *S. purpurea* system has not been directly measured in field populations.

In this study, we manipulated *W. smithii* larval density and characterized effects on bacterial community composition and function across a natural population of pitchers over time, from pitcher opening to maturation. Bacterial community composition was characterized by high throughput 16S rRNA gene amplicon sequencing, while microbial community function was characterized by profiling carbon substrate use, bulk metabolic rate, hydrolytic enzyme activity and macronutrient pools. We hypothesized that pitcher-associated bacterial communities would change over time, and that the presence and abundance of mosquito larvae would affect community assembly and function in the pitcher microecosystem.

2 | MATERIALS AND METHODS

2.1 | Study site and experimental design

Experimental manipulation of *S. purpurea* communities was conducted over a 74-day period from June to August 2020 at the Cedarburg Bog in Saukville, WI USA (43°23.2′ N, 88°0.63′ W) (Bott et al., 2008; Grothjan & Young, 2019) with approval from the University of Wisconsin-Milwaukee Field Station advisory committee (Figure 1a). In brief, 60 unopened pitchers of similar size were tagged and allowed to open naturally. Plants were selected throughout accessible areas to capture population variability. Pitchers were mostly located on different plants, but several pitchers shared a common plant and subsequent analyses accounted for potential non-independence by including pitcher and plant ID as random effects (see "Other statistical analyses"). Newly opened pitchers were then flushed and filled with sterile water prior to being provisioned 10 freeze-dried adult *Drosophila melanogaster* flies cultured from commercial stocks (Carolina Biologicals) to standardize initial prey availability (= Day 0). Seven days later, 20 pitchers were assigned to each of three treatments: "Enriched", "Depleted" or "Unmanipulated" (= Day 7). Pitchers were then visited every 10–14 days for a total of 74 days to (i) record the number of mosquito larvae, (ii) maintain a count of 10 larvae in Enriched pitchers, and (iii) remove any natural mosquito larvae background in Depleted pitchers (= Days 7–74). Larval density was surveyed for all pitchers in the field by mixing and transferring pitcher fluid to a clear 50 mL conical tube (Corning Inc., Corning, NY, USA) for counting larvae and measuring volume prior to any sampling (see below). Larvae added were always newly hatched first instars from our laboratory *W. smithii* colony (established from mosquitoes collected from natural populations in Wisconsin) maintained under sterile conditions prior to field introductions. Mixed fluid from each pitcher was sampled on Day 7 (1 week after pitcher opening) and Day 74 (in mature pitcher communities) for total DNA to characterize bacterial communities. Based on previous data (J. J. Grothjan & E. B. Young, unpublished data), this represented early and late succession, although successional trajectories were not specifically examined. Enzymatic activities were measured from samples collected on Days 7, 18, 28, 39, and 74. All other nutrient and functional assays were conducted on pitcher fluid sampled on Day 74. All pitcher samples were transported on ice to the laboratory for immediate processing, while any removed volume was replenished with sterile water at each sampling timepoint to maintain pitchers at ~3/4 maximum volume. Data confirmed that field manipulations were successful in maintaining "high", "medium" and "low" larval abundances in pitchers assigned to the Enriched, Unmanipulated and Depleted treatment groups, respectively, with Enriched pitchers containing significantly higher larval abundances than Depleted pitchers over the entire 74-day experiment (Dunn's test; BH-adj $p < .001$ for all timepoints) (Figure 1b).

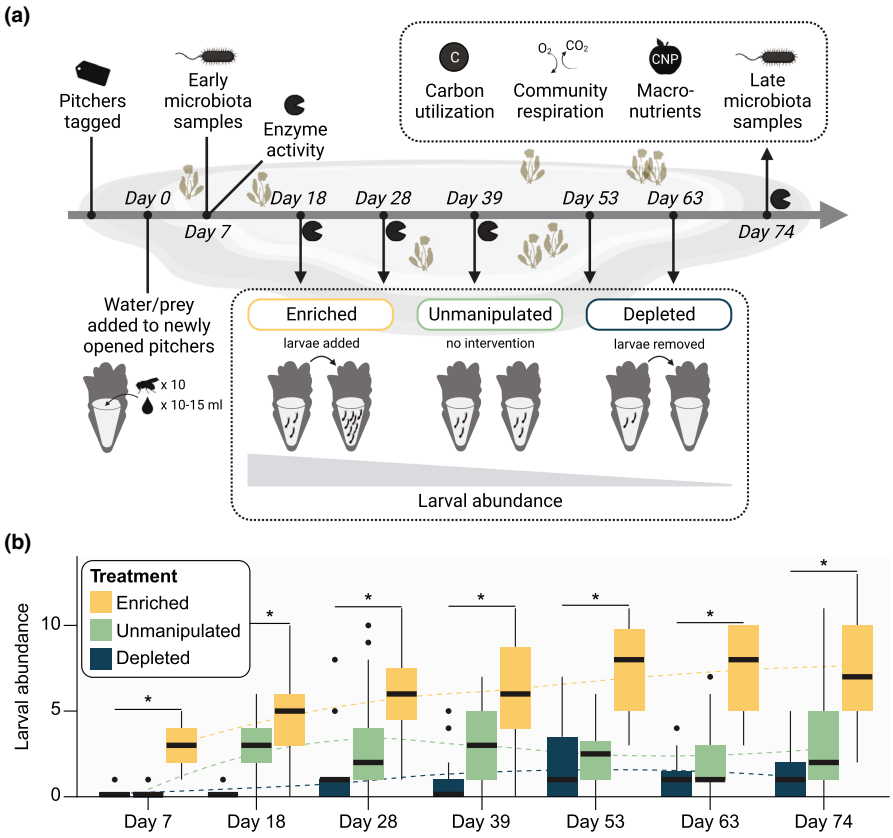

FIGURE 1 Study design for field-based manipulations at Cedarburg Bog, Wisconsin, USA (a), and the effects on larval abundance in pitchers assigned to different treatment groups over time (b). Sixty unopened *Sarracenia purpurea* pitchers were tagged in the field and visited shortly after opening to standardize prey capture by the addition of ten *Drosophila melanogaster* in 10–15 mL sterile water (= Day 0). Seven days later (= Day 7), 20 pitchers were assigned to each of three treatments: "Enriched" (yellow), "Unmanipulated" (green), and "Depleted" (blue). Water was also collected for bacterial 16S rRNA gene amplicon sequencing and enzymatic assays of protease and chitinase activity (= early timepoint, Day 7). At 10–14-day intervals thereafter, pitchers were visited to maintain treatment manipulations (see Days 18, 28, 39, 53 and 63) and to collect samples for additional assays for enzyme activity (see Days 18, 28 and 39). At the late timepoint (= Day 74), all remaining pitcher fluid was collected for bacterial 16S rRNA gene amplicon sequencing and profiling of carbon substrate use, bulk microbial respiration, and macronutrient concentrations. Larvae added to Enriched pitchers were derived from newly hatched eggs laid by adult females in our laboratory *Wyeomyia smithii* colony (established from mosquitoes collected from natural populations in Wisconsin) and were maintained under sterile conditions prior to field introductions. Boxplots show high, low and median values, with lower and upper edges of each box denoting first and third quartiles, respectively. Dashed lines are "loess" fit lines showing the trend of larval abundance in each treatment group over time. Asterisks (*) indicate timepoints where larval abundance was significantly higher in Enriched pitchers relative to Depleted pitchers (Dunn's test; BH-adj $p < .001$).

2.2 | DNA extraction, library construction, sequencing and analysis

At each DNA sampling timepoint, 1–5 mL of pitcher fluid was centrifuged at 20,000 rcf (× *g*) for 20 min and supernatant removed prior to storage of pellets at −20°C. Total DNA was extracted from preserved pellets using standard phenol-chloroform methods (Sambrook et al., 1989; Stevenson & Weimer, 2007) prior to one-step PCR amplification of the V4 region of the bacterial 16S rRNA gene using barcoded primers and paired-end sequencing (2 × 250-bp) on an Illumina MiSeq as previously described (Arellano & Coon, 2022). Initial sequence quality control was conducted in QIIME2 (Bolyen et al., 2019), including read-joining using VSEARCH (Rognes et al., 2016). Reads were denoised using q-score based filtering (Bokulich et al., 2013) prior to running Deblur to trim paired-end joined reads (Amir et al., 2017). Taxonomy was assigned using a Naïve-Bayes classifier trained against the Greengenes database (Kozich et al., 2013; McDonald et al., 2012; Pedregosa et al., 2012). Multiple sequence alignment was followed by construction of an unrooted phylogenetic tree using MAFFT and FastTree2 (Katoh & Standley, 2013; Price et al., 2010). All QIIME2 artefacts were converted to phyloseq readable files for data analysis in R (version 4.1.1) (McMurdie & Holmes, 2013). A final phyloseq object was generated following a decontamination procedure using the R package "decontam" (Davis et al., 2018). Contaminant reads, samples with <100 reads, and any reads classified as "chloroplast" or "mitochondria" were then removed prior to conducting downstream analyses.

Species richness and Shannon diversity were estimated using the R packages "breakaway" and "DivNet", respectively (Willis &

Bunge, 2015; Willis & Martin, 2020), while hypothesis testing for both metrics was conducted using the *betta()* function in the "breakaway" package (Willis et al., 2017). Beta diversity ordinations were performed on an Aitchison distance (dissimilarity) matrix using principal component analysis (PCoA) after centered log-ratio (clr) transformation of the feature abundance table, as implemented in the R package "microbiome" (Lahti & Shetty, n.d.; McMurdie & Holmes, 2013). Global dissimilarity was assessed using a permutational multivariate analysis of variance (PERMANOVA) implemented via the *adonis()* function in the R package "vegan" (Oksanen et al., 2020), with pairwise tests corrected using the Bonferroni method (Martinez Arbizu, 2017). Global and Bonferroni-adjusted pairwise differences in variance were assessed using the *betadisper()* function in "vegan" (Oksanen et al., 2020). Differentially abundant taxa across different categorical covariate groups were identified via Wilcoxon rank sum tests (two groups) or Kruskal-Wallis tests (three or more groups) using the R package "ALDEx2" (Fernandes et al., 2014), while relationships between different taxa and continuous covariates were identified via ALDEx2-estimated Pearson correlations (Fernandes et al., 2014). All ALDEx2-generated *p*-values were corrected using a Benjamini-Hochberg false discovery rate (FDR) adjustment.

2.3 | Hydrolytic enzyme activity and macronutrient concentrations

At each sampling timepoint for enzymatic activities, 500 μL of pitcher fluid, filtered through 15 μm mesh, was used to assay chitinase and protease activity within 5 h of collection using fluorometric microplate assays to accommodate small volumes as previously described (Young et al., 2018). In brief, chitinase activity was assayed with 4-methylumbelliferyl N-acetyl-β-D-glucosaminide as a substrate (EX 360 nm, EM 420 nm), and protease activity was measured as leucine-aminopeptidase activity using L-leucine-7-amido-4-methylcoumarin hydrochloride as a substrate (EX 460 nm, EM 380 nm) (all Sigma-Aldrich).

Macronutrient concentrations were assayed using a Hach DR900 kit and reagents (Hach Company, Loveland, Colorado), according to the manufacturer's protocols including blanks and standards. Total organic carbon (TOC) and total phosphorus (TP) concentrations were determined using samples diluted 1:5 with organic-free water (Hach Company, Loveland, Colorado) to 10 mL and 25 mL total volumes, respectively, while total nitrogen (TN) was determined using undiluted samples (2 mL pitcher fluid).

2.4 | Carbon substrate profiling and bulk microbial respiration measurements

A subset of pitcher fluid samples (10 from each treatment) was assayed at the end of the experiment for the capacity of associated bacterial communities to hydrolyze 31 carbon substrates using the Biolog Ecoplate system (Choi & Dobbs, 1999) (Table S1). In brief, each sample was diluted 1:10 with sterile water (pH adjusted to 5.6) and 100 μL of diluted sample was added to each Ecoplate well as previously described (Bittleston et al., 2020). Plates were incubated at 25°C for 3 days and the hydrolysis efficiency for each C substrate was assessed by measuring absorbance at 590 nm (Synergy™ HT; Biotek Instruments, Winooski, Vermont).

The net respiration rate for each pitcher-associated microbial community was also determined at the end of the experiment using a MicroResp™ device (Bittleston et al., 2020; Campbell et al., 2003). In brief, 250 μL of undiluted pitcher fluid was incubated with 100 μL of sterile cricket media slurry prepared as a suspension of food-grade cricket powder (JR Unique Foods) in water at a concentration of 3 g/L (pH adjusted to 5.6) (Bittleston et al., 2020). The deep-well plate was topped with a 96-well plate containing cresol red agar to indicate pH reduction. After 3 days in the dark at 25°C, absorbance of the top plate was measured at 570 nm using a microplate reader. Absorbance values were interpolated to rates of carbon dioxide (CO_2) production using a standard curve generated from incubation of known CO_2 concentrations with indicator medium.

2.5 | Partitioning around medoid clustering analysis

In order to examine community composition independently from our a priori timepoints and treatments, samples were clustered based on community composition using a partitioning around medoid (PAM) algorithm implemented using the R package "biotypeR" (Tap & Mende, 2016). This method identifies tentative clusters ("biotypes") using a dissimilarity matrix and assigns centrally located points with minimal within-cluster dissimilarity as "medoids". Cluster computation is then employed to iteratively minimize the dissimilarity between medoid and non-medoid points. To generate the dissimilarity matrix for PAM clustering, sequence data were collapsed to the genus level prior to clr transformation and estimation of Aitchison distances as described above. Validation of the optimal number of clusters and statistical significance of clustering maximized the computed Calinski-Harabasz (CH) indexes (Calinski & Harabasz, 1974) for 1–20 possible clusters and calculated Silhouette coefficients (Rousseeuw, 1987).

Differentially abundant genera among the two and three biotype clusters generated from samples collected at the early (Day 7) and late (Day 74) timepoints were identified using two independent approaches. First, the "ade4" package in R was used to perform between-class analysis (BCA) of PAM-identified clusters and compute BCA-indices for every taxon in each cluster. High BCA indices for taxa within clusters indicated enrichment relative to other clusters, while low and/or negative values indicated relative paucity. The second approach utilized a Kruskal-Wallis test carried out on the clr-transformed phyloseq object using ALDEx2 in R and identified differentially abundant taxa at the global level (Fernandes et al., 2014). A consensus approach was then used to conservatively identify taxa driving PAM clustering and thus distinguishing biotypes in early and late samples.

2.6 | Other statistical analyses

Analyses of carbon substrate hydrolysis and macronutrient concentrations by a categorical group, treatment, or biotype, were conducted using one-way ANOVA followed by post-hoc Tukey–Kramer honest significant difference (HSD) tests with Bonferroni correction of *p*-values. Bulk respiration rate data failing to conform to assumptions of parametric tests were analysed using omnibus Kruskal-Wallis tests followed by post hoc Dunn's tests with Benjamini-Hochberg (FDR) *p*-value adjustment. Differences in the proportions of samples assigned to specific biotypes and the distribution of treatment groups among biotypes were examined using Chi-square or Fisher's exact tests. Differences in larval abundance between treatment groups or biotypes were examined using Mann–Whitney *U* tests or omnibus Kruskal-Wallis tests followed by post hoc Dunn's tests with FDR *p*-value adjustment.

Relationships between different functional assay outputs and larval abundance in individual pitchers were examined using either simple linear regression or linear mixed effects models estimated via the R package "lme4", with pitcher and plant included as nested random effects. Larval abundance was defined in one of two ways, depending on the response variable: (i) as the total number of larvae in a given pitcher at the time of sampling (response variable = protease or chitinase activity), or (ii) the total number of larvae counted in a given pitcher over the entire sampling period (all other response variables; hereafter referred to as "cumulative larval abundance"). Significance of fixed effects in models was calculated using Type III ANOVA with Satterthwaite's method for approximation of degrees of freedom (implemented via the "lmerTest" package) (Kuznetsova et al., 2017), while simulation-based restricted likelihood ratio tests (implemented via the "RLRsim" package) (Scheipl et al., 2008) were used to determine if the variance of random effects significantly differed from zero. The only exception to the above pertained to the relationship between measures of microbial respiration and cumulative larval abundance, which was non-linear and therefore better approximated by a second order polynomial function.

3 | RESULTS

3.1 | Patterns of bacterial diversity and treatment effects in field manipulations over time

We identified a total of 1103 bacterial ASVs from 16 phyla across all samples (Table S2), with four phyla comprising 87% of total ASVs: Proteobacteria (54%), Bacteroidetes (19%), Actinobacteria (8%) and Firmicutes (5%) (Figure 2a). Alpha diversity, measured by estimates of both species' richness and evenness (Shannon's *H* index), significantly increased between early (Day 7) and late (Day 74) samples, independent of any treatment effects (betta test, $p < .001$) (Figure 2b; Table S2). Among early samples, Enriched pitchers (with high larval abundances were maintained throughout the experiment; Figure 1b) exhibited significantly lower species richness values–but not Shannon's *H* index values–relative to Depleted pitchers (in which larvae were absent or present at very low abundances) (betta test, $p < .001$) (Figure 2b; Table S2). A similar, but more dramatic pattern was observed among late samples, with Enriched pitchers exhibiting significantly lower species richness values (betta test, $p = .006$) and both Enriched and Unmanipulated pitchers (in which larval abundances naturally varied from 0 to 11 throughout the experiment) exhibiting significantly lower Shannon's *H* index values than Depleted pitchers (betta test, $p < .01$) (Figure 2b; Table S2).

Bacterial communities also significantly differed in composition between early and late samples, with 102 ASVs identified as differentially abundant between Day 7 and Day 74 (Table S3). Early samples contained notably greater proportions of taxa within the Proteobacteria (BH-adj $p < .001$) and Bacteroidetes (BH-adj $p < .001$), while late samples contained greater proportions of taxa within the Planctomycetes (BH-adj $p < .001$), Acidobacteria (BH-adj $p < .001$) and Verrucomicrobia (BH-adj $p = .02$) (Figure S1). These observations were further supported by ordination analysis using a beta diversity index (Aitchison distance), which revealed significant clustering of samples by timepoint (i.e. early vs. late; Figure S2A). In contrast, no taxa were identified as differentially abundant between Enriched, Unmanipulated, and Depleted pitcher samples at either timepoint, although ordination analysis using the same beta diversity index revealed a marginally significant treatment group effect in late samples driven by differences between Enriched and Depleted pitchers (Figure S2B,C; Table S4).

3.2 | Association of specific bacterial taxa with mosquito larval abundance in individual pitchers

To address whether specific taxa are associated with the presence and abundance of mosquito larvae in pitchers independent of the pitchers' assigned treatment group, we also conducted differential abundance testing using models that assessed whether individual taxa in pitchers sampled during early and late timepoints covaried with the total number of larvae in each pitcher at the time of sampling. We identified two ASVs that were negatively associated with larval abundance, corresponding to the genera *Gordonia* (Order: Actinomycetales; BH-adj $p = .001$) and *Sphingomonas* (Order: Sphingomonadales; BH-adj $p = .02$). Nine bacterial ASVs were positively correlated with larval abundance, four of which could be identified to genus: *Cryocola* (Order: Actinomycetales; BH-adj $p = .008$), *Rhodanobacter* (Order: Xanthomonadales; BH-adj $p = .002$), and *Novosphingobium* (Order: Sphingomonadales; BH-adj $p = .001$). An additional five positively correlated ASVs were identified to families Acetobacteriaceae (Order: Rhodospirillales; BH-adj $p = .04$), Methylocystaceae (Order: Rhizobiales; BH-adj $p = .04$), Isosphaeraceae (Order: Gemmatales; BH-adj $p = .03$), Sinobacteraceae (Order: Xanthamonadales; BH-adj $p = .02$) and Comamonadaceae (Order: Burkholderiales; BH-adj $p = .001$). One other positively correlated ASV had a taxonomic assignment resolved to the order level: Burkholderiales (BH-adj $p = .04$).

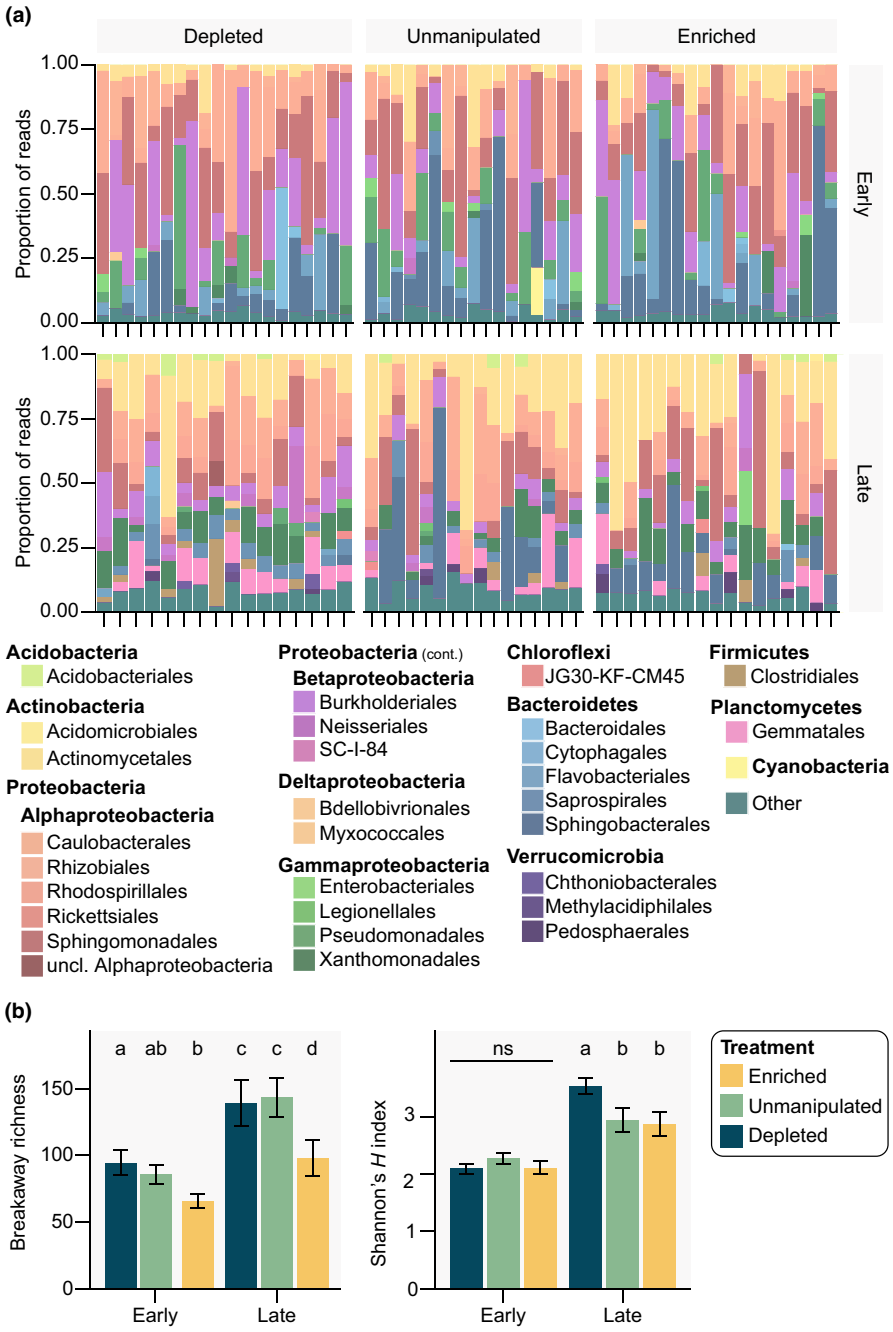

FIGURE 2 (a) Relative abundance of bacterial orders in fluid collected from "Enriched", "Unmanipulated", and "Depleted" pitchers at the early (*upper*, Day 7) and late (*lower*, Day 74) timepoint. Coloured bars present the proportion of sequencing reads assigned to a given order. Low abundance orders (<2%) are represented by the "Other" category. (b) Differences in alpha diversity between communities in Enriched, Unmanipulated and Depleted pitchers as measured by breakaway richness (left) and Shannon's H index (right). Mean values ± standard errors are shown. Different letters indicate significant differences between treatment groups (betta test, $p < .01$).

3.3 | Associations between larval abundance and measures of microbial community function

Studies in other aquatic systems support the capacity of environmental alterations to induce changes in microbial community performance that may or may not be associated with changes in community composition (Bier et al., 2015; Comte et al., 2013). In this way, *W. smithii* larvae have the capacity to shape ecosystem function in the *S. purpurea* microbial community via impacts on individual taxa and/or community-level processes. We therefore sought to elucidate larval impacts on key measures of pitcher microbial community function, including measures of community hydrolytic enzyme activity, carbon substrate use capacity, and respiration, as well as macronutrient concentrations in pitcher fluid. The activity of two hydrolytic enzymes involved in prey digestion (protease and chitinase) was characterized in fluid sampled from individual pitchers at five timepoints throughout the experiment (Figure 1a), while the capacity of pitcher-associated communities to hydrolyze each of 31 carbon substrates, community bulk respiration rates, and TN, TOC and TP concentrations in pitcher fluid were only characterized for the late timepoint at the end of the experiment (Figure 1a).

Linear mixed effects models identified time as well as both discrete and continuous measures of larval abundance (i.e. treatment group and larval abundance counts at the time of sampling) as significant fixed effects in predicting protease activity (Table 1; Table S2), with Enriched pitchers and pitchers with higher larval abundances

TABLE 1 Model parameters for mixed effects models of enzymatic activity.

Response	Fixed effect	Fixed effect coefficient estimate	Test statistic and *p*-value	Random effect	Test statistic and *p*-value
log(Protease)	Treatment	Unmanipulated-Depleted: −0.018 Unmanipulated-Enriched: −0.196	t: −0.259; $p = .796$ t: −2.688; $p = .008^{**}$	Pitcher ID	RLRT < 0.001; $p = .481$
	Sampling date	−0.006	$F_{1,243}$: 30.405; $p < .001^{***}$	Plant ID	RLRT = 0.922; $p = .148$
log(Chitinase)	Treatment	Unmanipulated-Depleted: 0.059 Unmanipulated-Enriched: −0.079	t: 0.758; $p = .450$ t: −0.992; $p = .323$	Pitcher ID	RLRT < 0.001; $p = .481$
	Sampling date	−0.009	$F_{1,239}$: 53.904; $p < .001^{***}$	Plant ID	RLRT = 3.677; $p = .024^{*}$
log(Protease)	Larval abundance	−0.068	$F_{1,247}$: 18.719; $p < .001^{***}$	Pitcher ID	RLRT <0.001; $p = 1$
	Sampling date	−0.008	$F_{1,232}$: 27.912; $p < .001^{***}$	Plant ID	RLRT <0.001; $p = 1$
	Interaction	0.002	$F_{1,245}$: 7.938; $p = .005^{**}$		
log(Chitinase)	Larval abundance	−0.049	$F_{1,254}$: 9.326; $p = .002^{**}$	Pitcher ID	RLRT <0.001; $p = 1$
	Sampling date	−0.011	$F_{1,233}$: 50.692; $p < .001^{***}$	Plant ID	RLRT <0.001; $p = 1$
	Interaction	0.001	$F_{1,243}$: 8.851; $p = .003^{**}$		

$^{***}0 < p < .001$; $^{**}.001 < p < .01$; $^{*}.01 < p < .05$.

exhibiting significantly lower measures of protease activity than Depleted pitchers or pitchers with lower larval abundances (Table 1; Table S2). In contrast, only time was identified as a significant fixed effect in predicting chitinase activity when larval abundance was coded as a discrete variable (Table 1; Table S2), while both time and larval abundance were identified as significant fixed effects in predicting chitinase activity when larval abundance was coded as a continuous variable (Table 1; Table S2).

We also observed significant interactions between time and larval abundance in predicting both protease and chitinase activity, with temporal effects on enzymatic activity being differentiated by larval abundance early in our sampling (Table 1; Figure S3; Table S2). This was further consistent with the observation that measures of both protease and chitinase activity generally peaked early in our sampling and declined over time (Figure S3; Table S2).

Communities in pitchers assigned to different treatment groups also significantly differed in their capacity to hydrolyze certain carbon substrates, namely glycogen (ANOVA on log-transformed dependent variable, $F_{2,24} = 3.67$, $p = .04$), with measures of glycogen hydrolysis efficiency being significantly lower in Enriched pitchers than in Depleted pitchers (Tukey–Kramer HSD test, Bonferroni-adj $p = .03$) (Figure 3a; Table S2). Fluid collected from pitchers assigned to different treatment groups further contained different concentrations of the macronutrient nitrogen (ANOVA on square root-transformed dependent variable, $F_{2,24} = 3.82$, $p = .04$), with TN concentrations being significantly higher in Enriched pitchers than in Depleted pitchers (Tukey–Kramer HSD test, Bonferroni-adj $p = .03$) (Figure 3a; Table S2). Consistent with both of these treatment group effects, linear regression models revealed a significant negative association between cumulative larval abundance and the efficiency of the associated community's capacity to hydrolyze glycogen (linear regression on log-transformed dependent variable, $R^2 = .39$, $p < .001$) (Figure 3b; Table S2). These models also revealed significant positive associations between cumulative larval abundance and both TN concentration (linear regression on square root-transformed dependent variable, $R^2 = .36$, $p < .001$) and L-phenylalanine hydrolysis efficiency (linear regression, $R^2 = .13$, $p = .04$) (Figure 3b; Table S2), although L-phenylalanine hydrolysis did not significantly differ across pitcher treatment groups. Unmanipulated pitchers showed intermediate values for each of these functional measures, falling between values obtained from Enriched and Depleted pitchers (Figure 3a; Table S2). In contrast, measures of community respiration as a proxy for microbial metabolic activity exhibited a more quadratic relationship with larval abundance, with bulk respiration rates being significantly higher in Enriched pitchers than in Unmanipulated pitchers (Dunn's test, BH-adj $p = .002$) but statistically similar as rates in Depleted pitchers (Figure 3a,b; Table S2). There were no significant differences in TOC, TP or hydrolysis of the other profiled carbon substrates between treatment groups or as a function of larval abundance (Table S2). Furthermore, no particular taxonomic group or ASV was significantly correlated with measures of enzymatic activity, bulk respiration, or carbon substrate hydrolysis ($p > .05$).

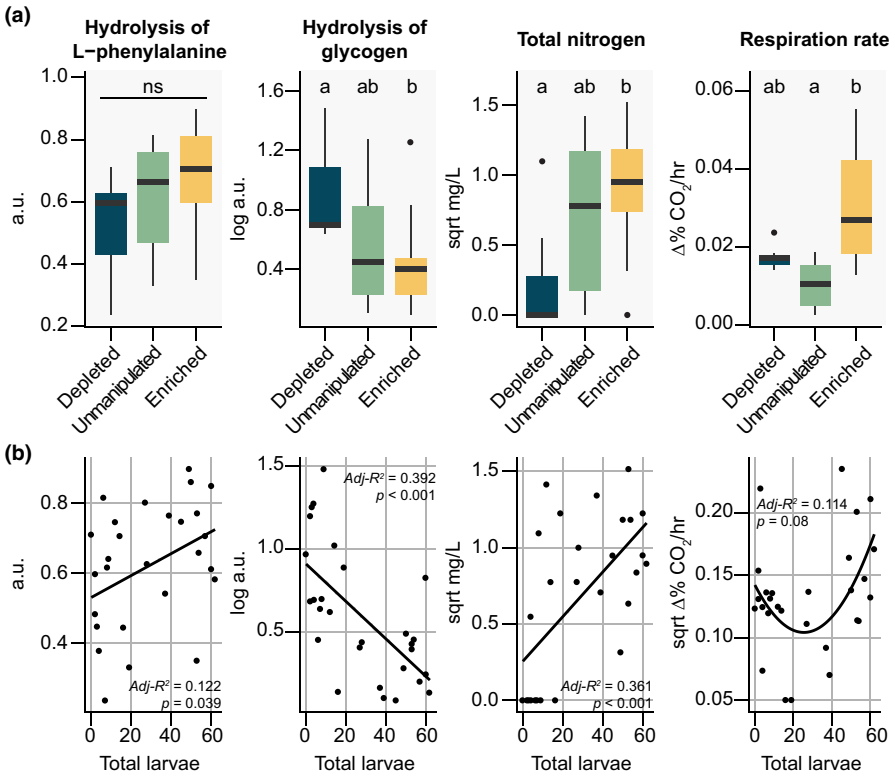

FIGURE 3 (a) Differences in select measures of microbial community function within "Enriched", "Unmanipulated" and "Depleted" pitchers. Boxplots show high, low and median values, with lower and upper edges of each box denoting first and third quartiles, respectively. Different letters indicate significant differences between treatment groups (Tukey–Kramer HSD test, $p < .05$). (b) Regression analyses between the same functional measures (*y*-axis) and cumulative larval abundance in individual pitchers (*x*-axis). Significant relationships were identified between cumulative larval abundance and measures of Lphenylalanine hydrolysis efficiency ($p = .039$, $R^2 = .122$), glycogen hydrolysis efficiency ($p < .001$, $R^2 = .392$), and TN concentration ($p < .001$, $R^2 = .361$), but not respiration ($p = .08$, $R^2 = .114$).

3.4 | Unsupervised clustering of samples into biotypes that support mosquito effects on the composition and function of pitcher-associated microbial communities

Owing to known limitations associated with traditional approaches to microbiome data analysis, which can result in overgeneralization (e.g. by comparing observations by alpha diversity measures) and/or overspecification (e.g. by performing differential abundance tests for every taxon, which incurs a heavy penalty for multiple testing) of the data, our last series of analyses employed an unsupervised clustering method to identify naturally occurring clusters of samples. To this end, we used the PAM clustering method–a distance-based method originating from studies of host-associated microbiomes (Arumugam et al., 2011) and that has been proposed as a robust approach to cluster microbial samples from a range of environments (Cheng & Ning, 2019).

PAM clustering of communities across the entire dataset identified two distinct biotypes corresponding to samples from the early and late timepoints, with the exception of a single early (Day 7) sample that clustered with late (Day 74) samples (Figure 4a). Re-clustering of communities separated by timepoint subsequently identified two and three distinct biotypes among early and late samples, respectively (hereafter referred to as "Early Biotype 1", "Early Biotype 2", "Late Biotype 1", "Late Biotype 2" and "Late Biotype 3"; Figure 4b,c). Early biotypes were distinguished by five taxa from the orders Actinomycetales, Micrococcales and Xanthamonadales that were enriched in Early Biotype 1 relative to Early Biotype 2 (Table S5). Estimates of both species' richness and evenness (Shannon's *H* index) were also significantly higher for communities assigned to Early Biotype 1 than for communities assigned to Early Biotype 2 (betta, $p < .05$; Table S2). However, communities assigned to either early biotype were equally likely to have been derived from Enriched, Unmanipulated or Depleted pitchers ($\chi^2 = 0.37$, $p = .831$) with statistically similar measures of cumulative larval abundance and hydrolytic enzyme activity (Mann–Whitney *U* test, $p > .05$). In contrast, the vast majority (~83%) of late communities assigned to Late Biotype 1 were derived from Enriched or Unmanipulated pitchers, while most (~64%) communities assigned to Late Biotype 2 were derived from Depleted pitchers (Figure 4c). Accordingly, measures of cumulative larval abundance were significantly higher in pitchers harbouring communities assigned to Late Biotype 1 than in pitchers harbouring communities assigned to Late Biotype 2 (Dunn's test, $p = .04$). Communities assigned to Late Biotype 1 were also more likely to be derived from pitchers assigned to Early Biotype 2, although this association was only marginally significant (Fisher's exact test, $p = .049$).

Consistent with the observation that late timepoint samples from Enriched pitchers harboured communities of lower alpha diversity than samples from Unmanipulated or Depleted pitchers, estimates of alpha diversity were significantly lower for communities assigned to Late Biotype 1 than for communities assigned to Late Biotype 2 or Late Biotype 3 (betta test, $p < .001$) (Table S2). Forty-four taxa were also identified as significant drivers of late biotypes, including genera like *Novosphingobium* and *Sphingomonas* that were

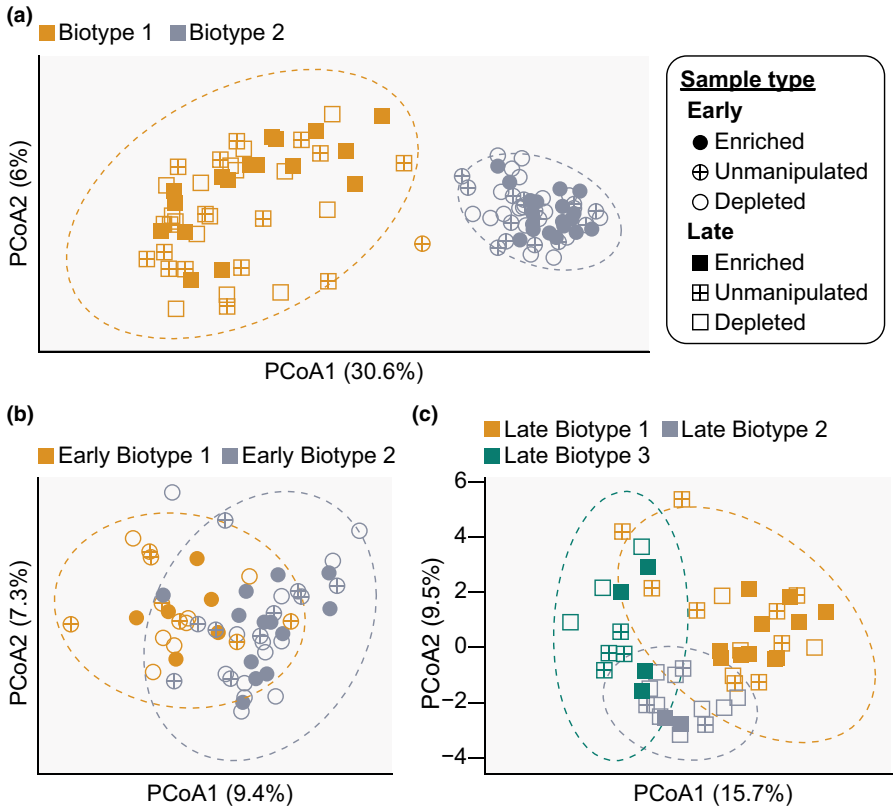

FIGURE 4 BCA visualization of biotypes as identified by PAM clustering of communities across the entire dataset (a) or separated by early (b, Day 7) and late samples (c, Day 74). Symbols in each panel are coloured by biotype, while the legend in the upper right of the figure designates pitcher treatment group ("Enriched", "Unmanipulated" or "Depleted") and timepoint of sampling (early or late) by symbol fill and shape, respectively. Ellipses (dashed lines) represent 95% confidence intervals.

enriched in Late Biotype 1 and Late Biotype 2 and were previously shown to be positively and negatively correlated with larval abundance, respectively (Figure 5; Table S5). However, ASV diversity remained significantly lower for communities assigned to Late Biotype 1 than for communities assigned to Late Biotype 2, regardless of whether ASVs assigned to any biotype-enriched genus or only genera specifically enriched in communities assigned to Late Biotype 1 were considered (Tukey–Kramer HSD test, Bonferroni-adj $p < .05$; Figure S4; Table S2).

Finally, fluid collected from pitchers harbouring communities assigned to Late Biotype 1 contained higher TN concentrations than pitchers harbouring communities assigned to Late Biotype 2 (Tukey–Kramer HSD test, Bonferroni-adj $p = .02$; Table S2), consistent with TN concentrations being significantly higher in fluid collected from pitchers with higher larval abundance than in fluid collected from Depleted pitchers (Figure 3a; Table S2). Communities assigned to Late Biotype 2 also exhibited significantly higher capacities to use Tween40 as a carbon substrate than communities assigned to Biotype 1 (Tukey–Kramer HSD test, Bonferroni-adj $p = .003$) (Table S2), consistent with hydrolysis efficiencies for other complex carbohydrate polymers (e.g. glycogen) being (i) significantly lower for communities from Enriched pitchers than for communities from Depleted pitchers, and (ii) negatively correlated with cumulative larval abundance in our previous analyses (Figure 3a; Table S2). In contrast, communities assigned to Late Biotype 2 exhibited protease and chitinase activities that did not statistically differ from levels of communities assigned to Biotype 1 (ANOVA, $F_{1,26} = 1.863$ and 3.681; $p = .184$ and .070 for protease and chitinase, respectively; Table S2), despite measures of protease activity being significantly higher in Depleted pitchers than in Enriched pitchers in our treatment group comparisons (Table 1; Table S2).

4 | DISCUSSION

Here, we used the model aquatic microecosystem hosted by the carnivorous purple pitcher plant, *S. purpurea*, to explore the top-down effects of an inquiline predator species (*W. smithii*) on microbial community composition and function. We leveraged a local field population of *S. purpurea* pitcher plants and our laboratory colony of *W. smithii* mosquitoes to manipulate mosquito larval abundance (and therefore the intensity of any top-down effects) in replicate pitchers over a 74-day period spanning the natural progression of community assembly (from pitcher opening to mature or late succession communities). Tandem characterization of bacterial community composition and hydrolytic enzyme activity throughout pitcher community assembly and maturation, along with the profiling of key microbial community functions and macronutrient concentrations in mature pitchers, then allowed us to address novel questions pertaining to the impact of *W. smithii* larvae on essential *S. purpurea* microecosystem processes. The results supported our hypothesis that variability in mosquito larval abundance over succession would affect microbial community composition and function in *S. purpurea* pitchers, leading to functionally distinct mature community states with implications for ecosystem function.

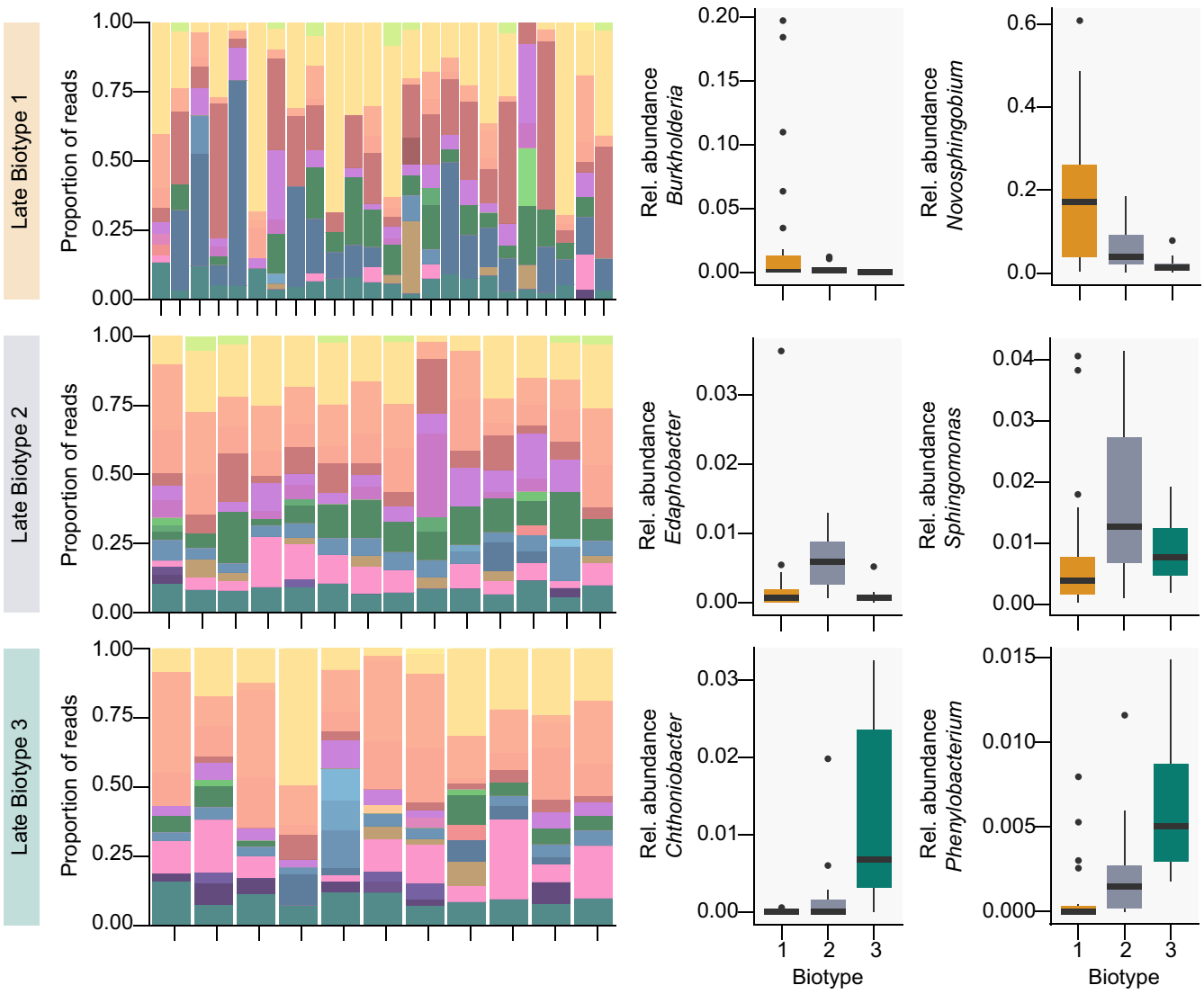

FIGURE 5 Relative abundance of bacterial orders in late (Day 74) communities assigned to "Late Biotype 1", "Late Biotype 2", and "Late Biotype 3" (*left*), along with the relative abundances of select genera that were enriched in each biotype (*right*). Coloured bars in the left panel present the proportion of sequencing reads assigned to a given order, with low abundance orders (<2%) being represented by the "Other" category. Boxplots in the right panel show high, low, and median values, with lower and upper edges of each box denoting first and third quartiles, respectively. Only boxplots for the two genera with the highest BCA coefficients in each biotype are presented. Other taxonomic drivers of biotypes can be found in Table S5.

4.1 | Microbial community assembly and composition is shaped by mosquito larval abundance in pitchers

Bacterial taxa identified across the field data set were consistent with previous characterization of inquiline bacterial communities in *S. purpurea* (Bittleston et al., 2018, 2020; Freedman et al., 2021; Grothjan & Young, 2019, 2022; Northrop et al., 2017; Paisie et al., 2014), sharing broad patterns such as the presence of diverse members of the Alphaproteobacteria (Rhodospirillales, Rhizobiales, Caulobacterales, Sphingomonadales), Betaproteobacteria (Burkholderiales, Neisseriales) and Gammaproteobacteria (Enterobacteriales, Pseudomonadales, Xanthomonadales), as well as members of the Bacteroidetes (Saprospirales, Sphingobacteriales, Flavobacteriales), Actinobacteria (Soilrubribacterales, Actinomycetales) and Firmicutes (Clostridiales). Bacterial community diversity (both species' richness and evenness) also increased from early (7 days after pitcher opening) to late succession (74 days), with significant shifts in bacterial composition observable up to the phylum level. These results are consistent with observations for inquiline communities in other pitcher plant species (Armitage, 2017), but differ from previous studies in *S. purpurea* reporting either variable shifts in bacterial diversity (Grothjan & Young, 2022) or stable diversity through ecological succession (Miller & TerHorst, 2012). The latter study (Miller & TerHorst, 2012) used culture-based methods to assess bacterial diversity, as opposed to the culture-independent, sequencing-based methods used here, while the former study (Grothjan & Young, 2022) assessed bacterial diversity in pitchers on plants maintained under greenhouse conditions. Greenhouse experiments may incorporate effects of isolation from wetland microbial sources, reiterating the importance of dispersal processes in shaping pitcher community assembly (Boynton et al., 2019; Kneitel & Miller, 2003) and the relative loss of diversity in *S. purpurea* pitchers outside of a field context (Arellano & Coon, 2022). Diversity of late timepoint samples differed by treatment group, with communities in pitchers with higher mosquito larval abundances having fewer bacterial taxa with more uneven community compositions relative to those in pitchers with few or no larvae. That mosquito larval abundance influenced bacterial diversity from early to late timepoints suggests a role for top predators in mediating successional trajectories via top-down controls on bacterial communities, expanding on former reports of local correlations between *W. smithii* larval colonization and bacterial diversity (Peterson et al., 2008). Previous studies have suggested that the presence and abundance of other macroinvertebrates—but not mosquito larvae—is associated with higher alpha diversity in pitchers over a continental scale (Freedman et al., 2021), but our observation of lower species richness in pitchers with higher larval abundance within a single wetland site also suggests that the nature of *W. smithii*-microbiota interactions may vary across the *S. purpurea* range. Indeed, our results are more consistent with the hypothesis that increasing bacterial diversity within pitcher plant inquiline communities with increasing latitude (Buckley et al., 2003) may be driven by release of filter-feeding pressure by *W. smithii*, as pitcher mosquito larval density also decreases with increasing latitude (Bradshaw & Holzapfel, 1989). Interestingly, late timepoint communities in Enriched pitchers had significantly lower richness than communities in Unmanipulated and Depleted pitchers, but similar evenness (measured as Shannon's *H* index) relative to Unmanipulated pitchers. Elevated richness in Depleted pitchers may suggest a release from predation pressure under field conditions.

While higher larval abundances were associated with significantly lower estimates of alpha diversity for both early and late timepoint communities, only mature communities at the late timepoint were distinguished by treatment group a priori–varying in composition and sample heterogeneity. This may suggest that mosquito-mediated shifts in bacterial composition intensified over time when larval abundance treatment differences were maintained with regular manipulations. Eleven bacterial ASVs exhibited relative abundances that scaled significantly with mosquito larval abundance. This could relate to previous empirical findings that gnotobiotic *W. smithii* larvae show different developmental outcomes with different bacterial taxa present (Arellano & Coon, 2022). Although specific relationships between each of these taxa and larval developmental outcomes are unclear, this is consistent with our understanding that *W. smithii*-microbe interactions are more specific than in other mosquito species (Coon et al., 2014, 2016a, 2016b, 2020; Valzania et al., 2018). This also supports our previous finding that pitcher bacterial communities are curated and actively dispersed by *W. smithii* mosquitoes (Arellano & Coon, 2022).

4.2 | Mosquito larvae affect microbial community function

In addition to mosquito-mediated impacts on bacterial community composition, mosquito larval abundance was linked to changes in several community-level processes important for ecosystem function, and these associations were generally stronger than those observed between larval abundance and bacterial diversity. This suggests that, in addition to top-down effects of larval predation, the presence of larvae may influence bacterial gene expression related to community functions. Mature pitcher communities differed by treatment in several functional measures, including the capacities of communities to hydrolyze glycogen, bulk community respiration rates and TN concentrations in pitcher fluid. With the exception of community respiration, these relationships seemed to scale linearly with larval abundance; higher larval abundance was correlated with lower community-level glycogen hydrolysis rates and higher TN concentrations, suggesting mosquito larvae influence nutrient cycling and thus the chemical environment, resulting in responses of microbial metabolic capacities. Decline in glycogen hydrolysis rates with more larvae could relate to larval excretion and egestion providing alternative carbon and nitrogen-rich substrates that influence expression of different bacterial catabolic pathways, as occurs in nitrogen salvage by gut bacteria during host hibernation in mammals (Regan et al., 2022). Indeed, higher larval abundances also correlated

with higher capacities for communities to hydrolyze L-phenylalanine. While declines in the capacities of bacterial communities to utilize different substrates could relate to lower bacterial densities due to predator grazing, the positive relationship between larval abundance and L-phenylalanine hydrolysis efficiency also strongly suggests that mosquito-mediated decline in bacterial cell density was not the sole feature dictating metabolic features of late succession microbial communities.

As noted above, *W. smithii* mosquitoes have a unique association with *S. purpurea* pitchers, with larvae of most other mosquito species developing in a variety of natural and artificial water container habitats (Day, 2016). Adult females of most mosquito species also must usually feed on blood from a vertebrate host, which, together with teneral reserves acquired during larval feeding, provides essential nutrients for egg production and underlies the capacity of some species to transmit disease-causing organisms to humans (Takken & Verhulst, 2013; Timmermann & Briegel, 1999). However, some mosquito species, including *W. smithii*, have evolved to be autogenous, with adult *W. smithii* females in northern populations (including those in Wisconsin) producing eggs without ever blood feeding (Bradshaw, 1980; Bradshaw et al., 2022; Wolff & Riffell, 2018). Previous studies in other autogenous species implicate enhanced nutrient acquisition as larvae, along with genetic factors, in facilitating the evolution of autogeny (Lounibos et al., 1982; Steffan & Evenhuis, 1981; Tsuji et al., 1990). Our previous work further supports roles for mosquito-associated microbes in shaping teneral reserves and mobilization of key nutrient stores (particularly protein and lipid stores) during oogenesis in adult females (Coon et al., 2016a, 2020). In the *S. purpurea* system, pitcher-associated microbial communities are known to have distinct genomic potential for amino acid metabolism (Bittleston et al., 2018), the expression of which is at least partially related to prey capture (Northrop et al., 2017). A positive correlation between mosquito larval abundance and hydrolysis of L-phenylalanine suggests that *W. smithii* larvae may affect amino acid turnover in pitcher communities, particularly of a metabolically expensive aromatic essential metabolite for which bacteria are frequently auxotrophic (D'Souza et al., 2014; Zengler & Zaramela, 2018). In addition to the potential implications for the composition and underlying interactions of pitcher-associated communities, phenylalanine, along with tyrosine, has previously been identified as a key metabolite in the metabolism of non-blood-feeding relative to blood-feeding subspecies of the common house mosquito, *Culex pipiens* (Beintema et al., 1994; Siperstein et al., 2022; Wheeler & Buck, 1996). Whether phenylalanine hydrolysis is also important for the maintenance of a non-blood-feeding life history in *W. smithii* warrants further study, especially in light of results from a previous study that failed to detect differences in phenylalanine metabolism between blood-feeding and non-blood-feeding *W. smithii* populations (Bradshaw et al., 2018). Whether the symbiotic association of *W. smithii* with *S. purpurea* and inquiline microbial communities may have resulted in an "out-sourced" route to a non-blood-feeding life history also warrants further study, given the broader implications for our understanding of the factors driving mosquito evolution away from life histories important for disease transmission.

Higher TN in the presence of *W. smithii* larvae is consistent with other studies reporting higher ammonia release in pitcher fluid related to presence of inquiline larvae (*W. smithii* and/or the pitcher plant midge, *Metriocnemus knabi*) (Bradshaw & Creelman, 1984). However, prior research suggested the presence of a "complete" pitcher-associated food web (i.e. predatory pitcher plant flesh fly, *Fletcherimyia* spp., larvae; *W. smithii* larvae; *M. knabi* larvae; and other pitcher-associated eukaryotes) may not translate to greater nitrogen pools within host plant tissue (Bradshaw & Creelman, 1984; Butler et al., 2008). The influence of *W. smithii* larval density on nitrogen concentrations may also depend on interactions with intermediate trophic levels and vary as a function of prey (bacterivorous protozoa or rotifers versus direct bacterivory) (Mouquet et al., 2008). This study offers evidence that *W. smithii* can influence key macronutrient pools in the *S. purpurea* inquiline ecosystem, although some nitrogen may be used within the community. While explicit consideration of other food web components could help, our results are consistent with the idea that nitrogen release from captured insect prey is facilitated first by mechanical break-down by inquilines with a shredding feeding modality (i.e. *Fletcherimyia* spp. and *M. knabi*), followed by enzymatic hydrolysis by bacteria. Filter feeding *W. smithii* may then act to promote mineralization of nitrogen by preying upon microorganisms and releasing bioavailable nitrogen as a bottom-up effect in addition to the top-down effects of grazing (Bradshaw & Creelman, 1984; Heard, 1994).

Activity of the hydrolytic enzymes chitinase and protease changed over time, consistent with past experiments characterizing enzymatic activity in response to insect prey additions (Young et al., 2018). Despite strong temporal trends, a mixed effects modelling approach supported a trajectory of pitcher enzymatic activity as partially attributable to *W. smithii* larval abundance, whereby higher larval numbers correlated with lower chitinase and protease activity. Notably, for chitinase activity, plant identity was important, potentially due to pitcher differences in natural prey capture over time after the initial standardized feeding (Grothjan, 2021). However, while we did not explicitly control for variability in prey capture rates over the season, our selected pitchers were within an area of similar prey availability and both plant and pitcher ID were included as random effects to at least partially account for potential prey capture biases. Protease production can be stimulated by relatively limiting N availability (Young et al., 2018); higher TN with more larvae may alleviate N limitation, reducing protease expression. Degradative activity in *S. purpurea* pitchers is largely attributable to microbial enzymes (Adlassnig et al., 2011; Young et al., 2018); thus, lower activity with higher larval abundance may also result from larval predation decreasing microbial biomass, although several functional assays scaled non-linearly or positively with larval abundance at the late timepoint. For example, significantly higher bulk microbial community respiration was observed in pitchers with higher mosquito larval abundance. The parabolic relationship between larval abundance and respiration suggests that microbial respiration is highest under

low and high predation, but is lowest under intermediate predation, or larval abundance, so community respiration rate may relate to an interaction of physiological status related to nutrient resources as well as cell density, related to predation. A similar relationship has been proposed for protozoan predation on bacteria under N-limited conditions (Hunt et al., 1977), analogous to the relatively low nutrient pitcher environment (Grothjan & Young, 2022). Furthermore, enhanced nutrient mineralization and stimulation of microbial respiration have been observed in response to higher predation pressure (Barsdate et al., 1974; Stout, 1973).

Overall, the more pronounced shifts in function with varying mosquito larval abundance despite more marginal shifts in bacterial community composition suggest differential expression of functions in response to larval abundance. However, while we were able to observe marked differences in bacterial composition between Days 7 and 74, more frequent sampling of community composition is necessary to demonstrate successional patterns and contextualize the higher temporal resolution of our enzymatic activity data. Experiments are also warranted to better understand how changes in abiotic conditions interplay with mosquito larval abundance to influence pitcher-associated microbial communities in the field, although such changes will be difficult to resolve at the short time scales relevant for most microbes without disrupting community succession. We further recognize that, while our functional assays reflected the activity of complete pitcher-associated microbial communities, our DNA sequencing focused only on bacteria and non-bacterial components may contribute to metabolic activity (Young et al., 2018). Hydrolytic enzyme activity has been associated with bacteria (Young et al., 2018), but respiration and carbon substrate use could have contributions from fungal or other microeukaryotes, which could also be affected by top-down pressures of *W. smithii* (Boynton et al., 2019; Paisie et al., 2014). As noted above, our results could also reflect release from bacterivore predation by other microeukaryotes such as rotifers and protozoa that, like bacteria, are directly grazed upon by *W. smithii* (Cochran-Stafira & von Ende, 1998; Hoekman, 2011; Kneitel & Miller, 2002; Peterson et al., 2008). Indeed, future research should explore the varying degrees of *W. smithii* omnivory and its role in mediating potential trophic cascades in this system (sensu Paine, 1980). Varied intra- and inter-trophic processes may indirectly influence mosquito-bacterial interactions and thus pitcher-associated microbial community composition and function.

4.3 | Mosquito-mediated impacts on diversity and function are recapitulated by unsupervised clustering of pitcher microbial communities

Our last series of analyses employed unsupervised clustering to assess whether pitcher microbial communities independently cluster into biotypes that associate with measures of mosquito larval abundance and community function in the field. Indeed, our unsupervised clustering results largely recapitulated patterns observed in our previous analyses, particularly for late timepoint communities. This serves as an independent line of evidence supporting top-down structuring of late succession communities by mosquitoes. Several prevalent genera identified in mosquito-enriched or mosquito-depleted biotypes were also represented in larval abundance-associated ASVs, with consistent directional responses to mosquitoes. Taken together with lower richness under mosquito-enriched conditions and fewer detectable ASVs in mosquito-enriched biotypes, this supports potential strain-level curation by mosquito larvae resulting in a winnowing of genetic diversity. While the impacts on specific strain-level function this curation may have is unclear, members of one mosquito-associated taxon, *Novosphingobium*, have been previously observed in symbiosis with insects for the detoxification of secondary metabolites, enabling close association with plant tissue (Cheng et al., 2018). Another mosquito-associated taxon, *Burkholderia*, is also comprised of members that are well known to engage in symbiotic associations with diverse insect species (Kaltenpoth & Flórez, 2020). Indeed, field-derived isolates of the *Burkholderia-Paraburkholderia* complex have also been shown to support robust development of gnotobiotic *W. smithii* larvae in a laboratory setting (Arellano & Coon, 2022), suggesting they may also be functionally significant in this symbiosis. Functionally, the mosquito-depleted Late Biotype 2 also demonstrated low TN relative to the other biotypes, reinforcing the positive correlation between larval abundance and TN. In contrast, biotypes identified via unsupervised clustering failed to recapitulate associations between mosquito larval abundance and hydrolytic enzyme activity, despite previous analyses identifying a significant negative association between mosquito larval abundance and protease activity. These differences underscore how myriad factors in the field are likely to additionally shape late succession communities, as has been explored in previous studies investigating temporal trajectories of pitcher or pitcher-derived bacterial communities in field and greenhouse settings, respectively (Bittleston et al., 2021; Grothjan & Young, 2022). That early biotypes non-randomly associated with late biotypes in our study is also consistent with previous work in pitcher systems characterizing the importance of historical contingency in community progression (Bittleston et al., 2020). Future work is necessary to address how different variables in the field shape pitcher microbial communities independent of, or in concert with, top-down effects of *W. smithii* to affect community-level processes underlying key functions in the *S. purpurea* model microecosystem.

5 | CONCLUSIONS

This study supports a role for *W. smithii* mosquitoes in top-down regulation of the *S. purpurea* system through modulation of bacterial diversity, community composition and ecosystem function. Our novel findings link functional traits of the pitcher-associated microbial community to larval abundance over time, even in the presence of strong temporal structuring and field conditions. Functional

analyses captured shifts in microbial community respiration and substrate hydrolysis capacities important for digestion of captured insect prey. Increases in nitrogen subsidies under high larval abundance are also of great importance in this nitrogen-depleted food web. That bacterial richness was consistently lower in pitchers with higher larval abundances, even when considering only those taxa that were enriched in the presence of larvae, further supports a role for *W. smithii* larvae in curating the *S. purpurea* microbial community to promote functions that benefit both the host plant and resident mosquitoes. Altogether, our results provide the first experimental evidence to support predation and nutrient release as mechanisms through which mosquitoes mediate ecosystem processes in field pitchers, with broad implications for our understanding of how interkingdom interactions shape microbial communities in this and other systems. Our results also provide novel insights into the biology of a group of mosquitoes of fundamental interest because adult females have evolved to produce eggs without blood feeding.

AUTHOR CONTRIBUTIONS

Aldo A. Arellano, Erica B. Young, and Kerri L. Coon conceived and designed the experiments. Aldo A. Arellano, Erica B. Young, and Kerri L. Coon performed the experiments. Aldo A. Arellano carried out the data analysis. Aldo A. Arellano wrote the initial manuscript, and Erica B. Young and Kerri L. Coon contributed to revisions.

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CONFLICT OF INTEREST STATEMENT

All authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw Illumina reads are available in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under BioProject ID PRJNA1033702. Scripts used for analysis and figure generation are available in the Coon laboratory's GitHub repository (https://github.com/kcoonlab/cedarburg-bog-expt-2020). All other data generated by this study are available as Supporting Information herein.

ORCID

Kerri L. Coon https://orcid.org/0000-0002-2701-5195

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SUPPORTING INFORMATION

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