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Major article: Multi-scale ecological filters shape the crayfish microbiome

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Running headline: The crayfish microbiome

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Abstract

1) Communities of symbiotic microbes obtained from the environment are an integral component of animal fitness and ecology. Thus a general and practical understanding of the processes that drive microbiome assembly and structure are paramount to understanding animal ecology, health, and evolution.

2) We conceptualized a series of ecological filters that operate at the environment, host, and host tissue levels during microbiome assembly and discuss key ecological processes that structure animal microbiomes at each level.

3) We conducted a survey of crayfish across four sites within the contiguous range of the of stream-inhabiting crayfish *Cambarus sciotensis* in western Virginia, USA, to characterize multiscale variation in the crayfish microbiome. We also conducted an in situ experiment to assess local drivers of microbial diversity on the closely related *Cambarus chasmodactylus.* We used a combination of DNA fingerprinting and next-generation sequencing to characterize microbiome diversity and composition from crayfish carapaces and gills to identify key filters affecting microbiome structure.

4) Field survey showed that local environment and host tissues interact to create patterns of microbial diversity and composition, but the strongest effects on microbial community structure were observed at the level of host tissue. Our field experiment confirmed strong effects of host tissue, and also showed that a metazoan ectosymbiont which feeds on biofilms (Annelida; Branchiobdellida) had significant effects on microbial composition of the host carapace. Crayfish carapaces were colonized by diverse and taxonomically even microbial communities that were similar to, and correlated with, microbial communities of the ambient environment. Conversely,

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40 crayfish gills were colonized by less diverse communities and dominated by two families of
41 bacteria with potentially significant functional roles: Comamonadaceae and Chitinophagaceae.

42 5) Our results suggest that microbial assembly of the carapace is driven by external biotic and
43 abiotic processes, whereas assembly on the gills appears to be coupled to host biology that favors
44 interactions with few specific taxa. Our work shows how multi-scale studies of symbiont
45 community assembly provide valuable insights into how the animal microbiome is structured
46 under conditions of natural complexity and help identify other symbiont taxa, i.e., the
47 branchiobdellidans, that may further influence microbiome assembly and structure.

48 **Key-words:** bacteria, branchiobdellida, cleaning symbiosis, defensive symbiosis, disease
49 ecology, metacommunities, parasites

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Introduction

Recent advances in our understanding of the intimate and diverse interactions between animals and their associated microbiota have blurred the lines that define individual organisms and fundamentally changed the way we think about organismal biology, ecology, and evolution (Gilbert, Sapp & Tauber 2012). Simultaneously, observational studies utilizing new sequencing technologies have highlighted patterns of symbiont microbial diversity and composition across a range of contexts from specific host body parts to geographic regions (e.g. Costello *et al.* 2009; Dominguez-Bello *et al.* 2010; Kuczynski *et al.* 2010). Increasingly, frameworks that embrace the multi-scale processes of community structure are being used to explain symbiont diversity (e.g. Dethlefsen, McFall-Ngai & Relman 2007; Pedersen & Fenton 2007; Graham 2008; Mihaljevic 2012). Though promising, these frameworks are still in need of empirical evaluation, and particularly experimental evaluation.

Mounting evidence is showing that functionally significant relationships with microbial symbionts are ubiquitous among metazoans (Scheuring & Yu 2012; Clay 2014). While the attention payed to animal microbiome research has been biased towards terrestrial vertebrates, especially humans, microbial symbionts are important in other host-associated systems. For instance, skin microbiota are thought to serve as protective agents against amphibian pathogens (Harris *et al.* 2009; Loudon *et al.* 2014). By unlocking novel food resources, or competitively excluding potentially harmful pathogens beneficial microbial symbionts are essential to a diverse and growing list of aquatic invertebrates as well, including sponges, corals, and arthropods (Scheuring & Yu 2012; Peerakietkhajorn *et al.* 2015). Thus important and yet undiscovered relationships between crayfish and microbial symbionts seem likely. Crayfish have a world-wide distribution in freshwaters, serve as keystone species and ecosystem engineers (e.g. Creed 1994;

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Statzner *et al.* 2000; Usio & Townsend 2002; Statzner, Peltret & Tomanova 2003; Creed & Reed 2004; Usio & Townsend 2004) , comprise many threatened and endangered species (Taylor *et al.* 2007; Helms *et al.* 2013; Owen *et al.* 2015), as well as several globally invasive nuisance species, and are one of the most frequently used model organisms in organismal biology (Holdich & Crandall 2002). Part of the interest in crayfish research stems from their amenability to field and laboratory studies, including experimental studies of symbiotic associations (Skelton *et al.* 2013). Despite the wealth of interest and intense study of crayfish, we are unaware of previous investigations focused on the crayfish microbiome.

In this study, we conceptualized the bacterial microbiome of stream inhabiting crayfish as the result of a series of nested filters operating at scales from the geographic region, to the host tissues (microsite community, Figure 1). At the coarsest scale, physical and biological characteristics of a local environment filter out a subset of all bacterial taxa that could potentially colonize the habitat from the regional species pool (Figure 1 – a). This subset of the regional pool represents the environmental pool of potential microbial symbionts for hosts living in a given local habitat. Similarly, it is likely that only a subset of the environmental pool is suited for life in symbiosis with a particular host and thus microbiome community membership is filtered at the interface between host and environment (Figure 1 – b). Furthermore, variation in microbial habitat quality and interactions among symbionts that occur in or on the tissues that form different parts of the host body, here called “microsites”, may further filter colonizing microbial symbionts (Figure 1 – c). Under this framework of nested filters, we implemented field surveys and an *in-situ* experiment to characterize the bacterial microbiome of crayfish and explore multi-level drivers of diversity. We examined the strength of environmental filtering on microbial symbiont communities by comparing environmental and symbiotic bacterial communities from

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four watersheds. We then relate environmental patterns to the patterns observed in the microbiome of crayfish from each site. To asses environmental filtering at the level of host tissue, we compared microbial community patterns observed on tissues which are highly interactive with crayfish physiology and one that is less interactive; the gills and carapace, respectively. Finally, we conducted a field experiment with manipulated co-infection treatments of *Cambarincola ingen* (Annelida: Branchiobdellida; Hoffman, 1963), an obligate ectosymbiont that feeds on crayfish biofilms, to explore the effects of symbiont-symbiont interactions on the diversity and composition of the crayfish microbiome. Our results demonstrate the necessity of a complete multi-level framework to understand patterns of microbial symbiont diversity and to identify key interactions that may influence microbiome assembly and structure.

Materials and methods

Field survey: We examined the crayfish microbiome from 4 sampling sites within the New River drainage near Blacksburg, Virginia, USA; Sinking Creek (37°18'9.34'' N, 80°29'6.9'' W), Big Stoney Creek (37°24'53.33'' N, 80°34'53.58'' W), Tom's Creek (37°14'23.82'' N, 80°27'30.55'' W), and Spruce Run Creek (37°15'54.43'' N, 80°35'52.67'' W). Six adult crayfish (*Cambarus sciotensis;* Rhoades, 1944) of similar size (35 mm mean carapace length [CL], ± 5 mm SD) from each site were chosen from which to collect gill and carapace biofilm samples. Terminal restriction fragment length polymorphism (TRFLP) analysis of the 16S rRNA gene was used to estimate bacterial diversity and compositional variation (Thies 2007) of these samples, in addition to three replicate samples of benthic substrate biofilms from each site. TRFLP is a largely automated process suited for high sample through-put and is useful for tracking changes in microbial community structure at coarse taxonomic scales over time and space (Schutte et al. 2008). We limited our sampling to the crayfish species *C. sciotensis* of

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similar size to minimize effects of host age on microbiome composition; 35 mm mean carapace length (CL), ± 5 mm SD. All sampling sites were within the contiguous range of this species (Hobbs, Holt & Walton 1967). Sampling of benthic substrate consisted of swabbing a 1 cm^2 upward facing surface of randomly selected cobbles from the streambed.

Cleaner symbiont experiment: We conducted a field experiment to specifically characterize the microbial communities of the crayfish gills and carapace, and to assess the influence of metazoan ectosymbionts on the crayfish microbiome. Twenty crayfish enclosures ("cages") were installed in the South Fork of the New River near the campus of Appalachian State University in Boone, N.C. in June 2012. Cages were constructed of welded aluminum frames with solid aluminum sides and bottoms. Two layers of hardware clothe mesh (12 mm) were on the front and backs of the cages permitted unimpeded flow of stream water while isolating individual crayfish for recapture and preventing transmission of ectosymbionts to caged crayfish from external crayfish (Brown *et al.* 2012). Steel bars (rebar, 2 per side) were driven into the streambed on both sides of each cage to hold it in place during against high discharge events. Approximately 40 L of mixed substrate collected immediately downstream was added through hinged, mesh-covered lids on top. Cages were arrayed according to a randomized block design, with 5 blocks perpendicular to stream flow to allow estimation of upstream/downstream effects of cage placement. Each block contained four cages, wherein a single crayfish received one of four treatments: no worms (all branchiobdellidans removed), 6 worms, 12 worms, or immediate harvesting for assessment of initial microbial communities at the experiment's outset.

Crayfish (*Cambarus chasmodactylus*; James, 1966) were collected from nearby tributaries of the New River, and chosen based on carapace length (30-35mm), and presence of all their appendages. All crayfish were cleaned of branchiobdellidan worms in the laboratory via

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manual removal followed by 5 min submersion in a 0.5M $MgCl_2$ solution (Brown et al. 2002). Crayfish were then immediately relocated to experimental cages to allow reconditioning of gill and carapace surfaces with native microorganisms. After 13 days, we began the experiment by transplanting large worms (*C. ingens*) collected from local tributaries onto crayfish at treatment levels. Midway through the 43 d experiment, crayfish were physically examined and worm treatments adjusted by reapplying missing worms as necessary to achieve initial treatment levels. Debris was removed from the exterior of cages every other day throughout this period to prevent accumulation of sediment or altered flow in/around cages.

Microbial sampling protocols: Sampling of microorganisms was performed in-field using flame-sterilized equipment. Carapace communities were sampled by wiping one randomly selected lateral half of the carapace with a sterile swab for 10s and immediately preserving the swab head in a microcentrifuge tube preloaded with a sucrose lysis buffer (pH = 9) (Mitchell and Takacs-Vesbach 2008). Similar techniques were used to sample a 1 cm^2 area of cobbles comprising the benthic substrate of our field survey. For gill samples, the carapace was removed and the rear-most gill tuft from one lateral side (randomly chosen) was clipped and stored in a tube of buffer. All samples were kept on ice and moved to -80°C storage within hours. All remaining crayfish carcasses were preserved in 70% ethanol for later observation.

DNA extraction from gill and carapace samples was performed using a conservative technique optimized for potentially low-biomass and low diversity environmental samples (Geyer et al. 2013). Briefly, DNA was extracted using a cetyltrimethylammonium bromide (CTAB) procedure that involves a mixture of 1% CTAB, 10% sodium dodecyl sulfate, phenol/chloroform/isoamyl alcohol (pH=7.5), lysozyme (0.2µg/µL), and proteinase K (20µg/µL) with either a swab head or gill tissue. Extracted DNA was resuspended in Tris buffer (pH=8.0)

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and quantified via spectrophotometry (NanoDrop 2000; Thermo Scientific, Wilmington, DE, USA). The average recovery of DNA from carapace swabs was ~50ng DNA/µL and ~500-1000ng DNA/µL per gill filament (a majority of which was assumed to be crayfish DNA).

TRFLP Analysis: PCR amplification of extracted DNA took place in triplicate (25µL reaction volume) using a standard 2uL of diluted template, 5 units/µL of Taq Hot Start Polymerase (Promega Corporation, Madison, WI, USA), and the universal bacterial primers 8F (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R (5'-ACCGCGGCTGCTGGCAC-3'), the forward primer labeled with a 5' 6-FAM fluorophore (Integrated DNA Technologies, Coralville, IA, USA). Amplification was optimized for concentrations of $MgCl_2$ (2.5mM per reaction), BSA (1µL/reaction), annealing temperature (53°C), and final extension time (5min). Amplification replicates were pooled and cleaned using a QuickClean II PCR Extraction Kit (GenScript, Piscataway, NJ, USA). Amplifications were digested with HaeIII (New England BioLabs, Ipswich, MA, USA) in triplicate (20µL reaction volume) for 3 hours at 37°C following manufacturer's suggested protocols. Digestion replicates were then pooled and cleaned using GenScript extraction kits. Fragment separation/quantification took place in quadruplicate with an ABI 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) and fragments binned using the GeneMarker software AFLP protocol. Resulting sample profiles were standardized using the procedures outlined in Dunbar (2001) to produce both a consensus profile among replicates and final normalization of all sample profiles by total sample fluorescence.

Next-generation Sequencing: Illumina MiSEQ amplicon sequencing was used to examine the diversity of bacterial communities. Approximately 150ng of 48 DNA extracts were provided to the Virginia Bioinformatics Institute at Virginia Tech for 150bp paired end sequencing of the V4-V6 region of the16S rRNA bacterial gene. The result was ~500k reads per sample with an

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189 average length of 253bp after stitching of paired reads. PANDAseq was used to merge forward
190 and reverse reads, correct errors in the region of overlap, and reject any reads that failed to
191 overlap sufficiently (Masella et al. 2012). Taxonomy was assigned to all unique sequences using
192 the uclust method and GreenGenes reference database (13_8 release) (DeSantis et al. 2006) in
193 QIIME (1.7.0) (Caporaso et al. 2010). USEARCH was used to cluster all dereplicated reads into
194 OTUs at the 97% similarity level and filter chimeras (Edgar 2010). The UPARSE pipeline was
195 followed for all data denoising. (Edgar 2013). A total of 10,489 OTUs were successfully
196 annotated and formed the basis of multivariate analysis.

197 *Statistical methods:* To assess bacterial diversity observed on carapaces and gills, we calculated
198 Simpson diversity for each sample from normalized TRFLP fragment peaks using the *diversity*()
199 function of the vegan package for R v2.0 – 10 (Oksanen *et al.* 2013). Simpson diversity indices
200 were converted to effective numbers of taxa following Jost (2006). This conversion
201 approximates the total number of equally abundant taxa in a community and makes comparisons
202 among communities more interpretable and intuitive than raw diversity indices (Jost 2006). We
203 used linear regression to correlate the observed diversity of crayfish samples with bacterial
204 diversity of the local substrate and to compare diversity observed on gills to that of carapaces.
205 We first calculated the average diversity of all substrate samples from each sampling locality,
206 and then used average substrate diversity as a continuous predictor and crayfish tissue (gill
207 versus carapace) as a categorical factor, with an interaction term. We also used linear regression
208 to assess the effects of sample type (gills versus carapaces) and branchiobdellidan treatment level
209 with an interaction term in our symbiont experiment. In this case, diversity was calculated as
210 described above using the number of reads obtained from each OUT obtained from
211 bioinformatics of NGS sequence data.

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We examined the main and interactive effects of host tissue (gills versus carapaces) and sampling site on the TRFLP fragment composition (a proxy for bacterial community composition) using permutations multivariate analysis of variance (PERMANOVA; Anderson 2001) implemented using the *adonis*() function in the R package Vegan v2.0 - 10 (Oksanen *et al.* 2013), with a Bray-Curtis dissimilarity matrix and 10,000 permutations. Compositional effects were visualized by non-metric multidimensional scaling (NMDS) using the metaMDS() function in vegan v2.0 – 10. Similar methods were used to assess the effects of branchiobdellidan treatment levels on the bacterial composition of gills and carapaces in the symbiont experiment. We also used Mantel tests to assess the strength of multi-variate correlations between average within-site TRFLP profiles from substrate samples, to those of gills and carapaces across all 4 sites. This was done by taking the average peak height for each fragment length from each sample type (gills, carapaces, and cobbles) within each site to create 3 site by fragment length matrices; 1 matrix each for cobbles, gills and carapaces. We then used 2 Mantel correlations to correlate gills and cobble, and carapaces and cobbles across sites (mantel() function; vegan 2.0 – 10; Oksanen *et al.* 2013).

Results

Field survey: TRFP results recovered an average of 37.9 (min = 21, max = 47) and 20.3 (min = 8, max = 36) OTU taxa from carapace and gill tissues, respectively, suggesting greater overall phylum-level richness of carapace biofilms (Fig 2). Cobbles sampled from the stream bed had an average of 30.2 taxa (min = 13, max = 50). There was considerable variation in microbial diversity within host tissues, among sampling locations (Fig 2), and marginally significant, interactive effects of environmental (non-host) microbial diversity and host microsite on

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symbiotic microbial diversity (Table 1). Among-site variation in microbial diversity of the carapace was correlated with the microbial diversity of local substrate, however among-site variation in the diversity of the gills was not related to substrate diversity (Fig 2).

Host microsite accounted for most of the compositional variation among samples (PERMANOVA, $F_{1,33} = 23.86$, $p < 0.001$, $R^2 = 0.33$). Additionally, there was significant among-site variation in microbial composition (PERMANOVA, $F_{3,33} = 2.68$, $p = 0.003$, $R^2 = 0.11$), and interactive effects of sampling site and microsite on composition (PERMANOVA, $F_{3,33} = 2.54$, $p = 0.003$, $R^2 = 0.10$). NMDS ordination recovered 2 convergent solutions after 12 tries and a final stress of 0.12 (Fig 3). The bacterial composition of the carapace biofilm was largely indistinguishable from that of local substrate biofilms, whereas the composition of the gills was distinct from the substrate (Fig 3). Across sampling sites, there was a significant correlation between the bacterial composition of the benthic substrate and the carapace microbiome (Mantel $r = 0.812$, $p = 0.034$) and a marginally significant correlation between substrate composition and the composition of the gills biofilm (Mantel $r = 0.527$, $p = 0.089$).

Cleaner symbiont experiment: Similar to the TRFLP results from the field survey, Illumina sequencing of the experimental crayfish biofilms confirmed that the bacterial communities of carapaces were much more diverse (mean = 35.9) than those of the gills (mean = 4.76). There were no significant effects of branchiobdellidan treatments on the microbial diversity of the carapace or the gills (Table 2). Again, similar to the results of our field survey, the microbial composition of the carapace was distinct from that of the gills. Bacterial communities of the carapaces contained a large number of evenly abundant taxa, whereas the gills contained many fewer taxa and were dominated by two bacterial families; Comamonadacea and Chitinophagacea (Fig 4). Comamonadacea had the highest relative abundance of all taxa present in gill samples,

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comprising an average of 41.0% (± 3.9% SE) of reads in gill samples. Second was Chitinophagacea which comprised an average of 15.5% (± 2.4% SE) of reads from gill samples. Conversely, these taxa comprised and average of 4.0% (±0.007% SE) and 0.4% (± 0.002% SE) of reads from carapace samples respectively. Branchiobdellidan treatments had no detectable effects on the microbial composition of the crayfish gills (PERMANOVA $F_{2,12} = 0.732$, $p = 0.782$). In contrast, there was a significant effect of worm treatment on the bacterial composition of the carapaces in which crayfish exposed to high symbiont densities were distinct from controls and medium density treatments (Fig 5; PERMANOVA $F_{2,16} = 1.646$, $p = 0.007$, $R^2 = 0.17$).

Discussion

Although some microbial symbionts are transmitted vertically from parent to offspring, or horizontally from host to host, most are obtained from environmental sources (Bright & Bulgheresi 2010; Walke *et al.* 2014). In this study, we examined ecological filters that operate at multiple levels as the crayfish microbiome is assembled from environmental sources. We found significant sources of variation at each level, from geographic sampling location, to interactions among metazoan and microbial symbionts within specific microsites. By far, the strongest effects were observed at the tissue-specific interface between host body and environment.

Host habitat level: In our field survey we found correlative evidence for environmental controls over the crayfish microbiome. We observed significant variation in microbial composition among sampling sites, and correlations between the microbial composition of environmental samples and composition of the gill and carapace symbiont communities. Similarly, microbial diversity of environmental samples was positively correlated with carapace communities, but environmental and gill communities were not correlated. Thus, the taxonomic composition of bacteria on the gills and carapace are influenced to some degree by the local environment.

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However, only carapace bacterial diversity was correlated with local environment, and bacterial diversity of the gills showed no relationship with environmental diversity. This result suggests that a process acting at a finer level limits membership of the gills, and gill community diversity, but not carapace diversity, was saturated at all sampling sites. We suspect that the observed variation among sites in both environmental and crayfish samples may be attributed to physical characteristics of the sampling sites that influence environmental microbial species pools. For instance, global patterns in soil and stream bacteria composition and diversity are shaped by pH gradients at both world-wide and local spatial scales (Fierer *et al.* 2007; Lauber *et al.* 2009; Rousk *et al.* 2010). Although we did not assess physiochemical characteristics of our sampling sites in this study, pH seems to be a likely underlying environmental driver of among site variation based on the results of work from another temperate watershed in the eastern United States (Fierer *et al.* 2007).

Host-habitat interface: As microbial symbionts are acquired from the surrounding environment, membership of the microbiome may again be filtered at the interface of host and environment. Not all microorganisms present in an environment are suited for symbioses with an animal host and variation among host species and individuals select for different subsets of the environmental pool (McKenzie *et al.* 2012; Kueneman *et al.* 2014). Ecological and host-mediated differences among microsites on or in the host body may select for a more specialized subset of the global pool. For example, microbial communities of human skin vary widely among parts of the human body (Costello *et al.* 2009; Grice & Segre 2011). Therefore the communities of bacteria found in association with a host tissue may represent a heavily restricted subset of the global pool of potential colonists. Our results suggest that filtering is strong at the gill microsite, but weak or nonexistent on the carapace. Carapace diversity was comparable, or

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304 even greater than bacterial samples taken from local substrates. Conversely, microbial
305 communities of the gills were shown by TRFLP analysis of survey data to be less diverse than
306 local substrate and carapaces at all sampling sites, and again found to be less diverse than
307 carapace biofilms by direct sequencing of experimental animals. Thus the strong tissue-specific
308 filtering that occurs at the gills imposes a strong filter on microbial symbiont communities, but
309 similar filtering is weak or non-existent on the less physiologically active carapce.

310 *Microsite processes:* We predicted that the microbial communities of more inert tissues would be
311 largely composed of opportunistic, environmental symbionts, whereas the microorganisms
312 colonizing more active tissues would include specialist symbionts. Our results support this
313 prediction. Crayfish gills are a vital interface between the crayfishes' internal physiology and
314 external environment. The gills exchange respiratory gases with the environment, are sites of
315 nitrogenous waste excretion, and regulate ion exchange. Consequently, the gills are composed of
316 thin, un-sclerotized epithelia. Conversely, the carapace is composed of heavily sclerotized and
317 calcified cuticle, and is approximately 300 times less permeable than the gills (Pequeux 1995).
318 Our field survey suggest that the microbial communities of the crayfish carapace are similar to
319 the surrounding substrate in terms of both diversity and composition. Moreover, variation among
320 sampling sites revealed correlations between bacterial communities of local substrate and the
321 crayfish carapace, indicating that the microbiota of the crayfish are probably composed of mostly
322 opportunistic taxa from the surrounding environment. Congruent to survey of DNA fingerprint
323 data, sequence data from our field experiment revealed relatively even, highly diverse
324 communities composed of ubiquitous environmental taxa. In contrast to the carapace, crayfish
325 gills were found to have less diverse microbial communities that were dominated by few taxa.
326 While carapaces were characterized by many evenly distributed taxa, the gills were dominated

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by two bacterial families: Comamonadacea and Chitinophagacea. Reduced bacterial diversity, and constrained microbial membership of the crayfish gills may be at least in part attributable to antimicrobial agents with crayfish hemolymph. Previous work has shown that the hemolymph of some crayfish species can inhibit the growth of some bacterial taxa in culture and may reduce bacterial accumulations on crayfish gills (Farrell, Creed & Brown 2014).

Evidence from other freshwater animals suggests that some taxa found in this study may serve a significant function in crayfish biology. Recent experimental work has demonstrated a positive effect of bacterial symbionts on the population growth rates of a small freshwater crustacean, *Daphnia* (Peerakietkhajorn *et al.* 2015). Similar to our study, these beneficial microbial communities were largely composed of Betaproteobacteria belonging to the Comamonadacea; a finding congruent to previous investigations of the bacterial symbionts of *Daphnia* (Qi *et al.* 2009; Freese & Schink 2011). Moreover, Comamonadacea and Chitinophagacea are often dominant members of the skin microbiomes of aquatic amphibians (Harris *et al.* 2009; McKenzie *et al.* 2012; Kueneman *et al.* 2014; Walke *et al.* 2015), yet the processes that led to their ubiquity and dominance in aquatic animal microbiomes, and their potential functional significance are not resolved.

The numerical dominance of Comamonadacea and Chitinophagacea found here and in other aquatic microbiome suggest they may serve as defensive symbionts. Increasingly, microbiome studies are focused on non-random microbiome community assembly, with a particular focus on beneficial microbial taxa (e.g. Bäckhed *et al.* 2005; Dethlefsen *et al.* 2006; Dethlefsen, McFall-Ngai & Relman 2007; Huttenhower *et al.* 2012; Scheuring & Yu 2012; Shafquat *et al.* 2014). Most beneficial microbes provide either nutritional advantages or defense against invading pathogens (Bäckhed *et al.* 2005; Huttenhower *et al.* 2012). Defensive microbes

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most often protect their hosts by establishing as competitive dominants through interference composition by producing antibacterial compounds (Scheuring & Yu 2012). Recent work suggests that hosts favor the competitive dominance of defensive microbes through resource provisioning (Scheuring & Yu 2012). In this study, we found very little evidence that the microbial communities of the crayfish carapace assemble non-randomly or contain functionally significant taxa. In stark contrast to carapaces, the microbial communities of the gills comprised a very limited set of microbial taxa. From recent theoretical developments (Scheuring & Yu 2012), we speculate that microbial resources released through the thin and permeable cuticle of the gills fuels the competitive dominance of Comamonadacea and Chitinophagacea, and that one or both taxa may produce antimicrobial compounds to maintain dominance and protect the host from invading pathogenic taxa. The gill cuticle itself could be a resource for Chitonphagacea, as several strains within this family are known to degrade chitin (Rosenberg 2014). Similar relationships between animals and protective microbes are common in nature and have been described in many marine invertebrates such as sponges and corals (Scheuring & Yu 2012; Clay 2014), and are therefore likely to be common freshwater invertebrates. Though the nature of the symbioses between Comamonadacea, Chitinophagacea, and their hosts are currently speculative, these relationships deserve further attention.

The study of symbioses continues to expand beyond concepts based on pairwise species interactions and is embracing the realistic complexity of symbiosis. However, perspectives that transcend microbe-microbe or microbe-host interactions are not typically considered. Recent synthetic work has highlighted the importance of direct and indirect interactions among symbionts during symbiont community assembly (Graham 2008; Skelton, Creed & Brown 2015; Skelton *et al.* 2016; Thomas *et al.* 2016). Crayfish growth and survivorship can be increased by

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hosting branchiobdellidan worms (reviewed in Skelton *et al.* 2013). Several lines of evidence suggest that the positive effect of worms on their host is mediated by interactions with microbial communities that develop on the gills. Branchiobdellidans such as *Cambarincola ingens* often enter the gill chamber and consume organic matter that accumulates therein, in addition to grazing on the exoskeleton biofilm (Brown, Creed & Dobson 2002; Brown *et al.* 2012). Accumulations of organic matter, particularly bacterial flocs, present a major challenge to crayfish in their natural benthic environment (Bauer 1998; Rosewarne *et al.* 2014). Thus researchers have hypothesized that some branchiobdellidans increase the growth and survival of their hosts by consuming harmful organic matter, including bacteria flocs, from the gill epithelia (Brown, Creed & Dobson 2002; Brown *et al.* 2012). Strong support for this hypothesis has come from multiple studies demonstrating context-dependent outcomes in the interactions between crayfish and their worms (Lee, Kim & Choe 2009; Brown *et al.* 2012; Skelton, Creed & Brown 2014; Thomas *et al.* 2016)

In this study, branchiobdellidans had a significant effect on the composition of carapace microbial communities, but no detectable effect on microbial composition of the gills. Bacterial richness was also unaffected on both gills and carapaces. The methods used in this study could only detect changes in bacterial composition and not bacterial biomass, so it remains possible that branchiobdellidans reduce bacterial biomass on the gills without specific effects on the composition of bacterial communities. Because the gills were found in this study to be dominated by a few taxa and perhaps influenced directly or indirectly by the host, any effects of worms on gill bacterial communities is more likely to be quantitative than qualitative. In contrast to the gills, microbial communities of the carapace appear to be more variable, and composed of abundant opportunistic colonizers whose relative abundances are influenced by local factors such

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as environment and branchiobdellidan presence. The effect of branchiobdellidans could be either from direct grazing, indirect effects of grazing via resource alteration, or perhaps an effect of adhesive compounds released by the worms' duogland adhesive organs used to attach to their hosts. Whatever the cause, the disparity between worm effects on gills and carapaces suggests that gill microbial communities are less susceptible to alteration from local biological factors, and that the symbiosis between crayfish and the bacterial communities of their gills is more tightly coupled to host biology than environmental context.

Conclusions

Our goal for this study was to examine the effects of ecological processes on patterns of microbial symbiont communities at multiple scales, from across watersheds to specific host tissues. We provide the first characterization of the crayfish microbiome and identify taxa with potential functional significance to host biology. We found significant variation in microbial symbiont communities at each scale, i.e. among watersheds, among individual crayfish, and among host tissues. We also provide experimental evidence that coinfection of a metazoan symbiont may influence microbiome composition of some tissues. However, the vast majority of variation in microbial composition and diversity was explained by host microsite, i.e., gills versus carapaces. This result was consistent across DNA fingerprint analyses in our field survey (TRFLP), and the results of sequencing from experimental samples. This result is also congruent with microbiome work from other animals including humans (e.g. Grice & Segre 2011) and suggests that the predominant ecological processes that dictate membership of the crayfish microbiome operate at the finest spatial scales, where microorganisms meet tissue. Furthermore, the microbial communities of the gills were found to be less responsive to local environmental context and dominated by two bacterial families identified as symbionts of crayfish in this study

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and symbionts of other aquatic animals elsewhere, suggesting that these organisms may represent important specialized aquatic animal symbionts. Future work may uncover functional significances of these microbial taxa that are closely associated with the gills of crayfish and the skins of frogs and salamanders.

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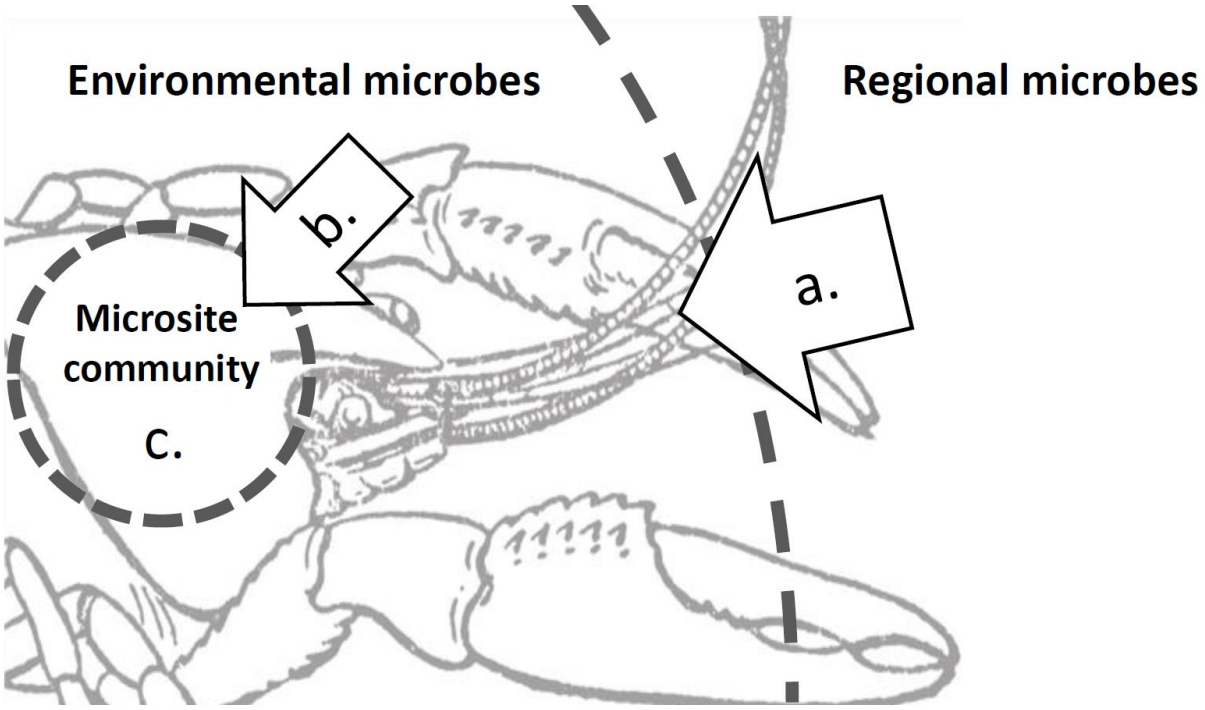
Figure 1: Conceptual diagram showing multi-level filtering of microbial symbiont community during symbiont community assembly. Colonizing potential symbionts are filtered by habitat characteristics such as water chemistry and pH at the host habitat level (a), by host characteristics at the host-habitat interface (b), and by symbiont interactions and tissue-specific characteristics at the host microsite level (c). The community structure observed at any microsite within the host is the product of a series of ecological filters that operate at scales from geographic to cellular.



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638 Figure 2. Field survey results depicting the relationship between microbial diversity originating
639 from crayfish (gills[grey symbols]; carapace[black symbols]) and surrounding environment
640 (substrate) from four sampling sites, ± 1 SE. Diversity is shown as the effective number of
641 equally abundant unique fragment lengths, derived from Simpson's Index of relativized TRFLP
642 data. Grey reference shows 1:1 relationship. Diversity of carapace was typically higher than, and
643 positively correlated with substrate diversity. Gill diversity was typically lower than, and
644 uncorrelated with substrate diversity.

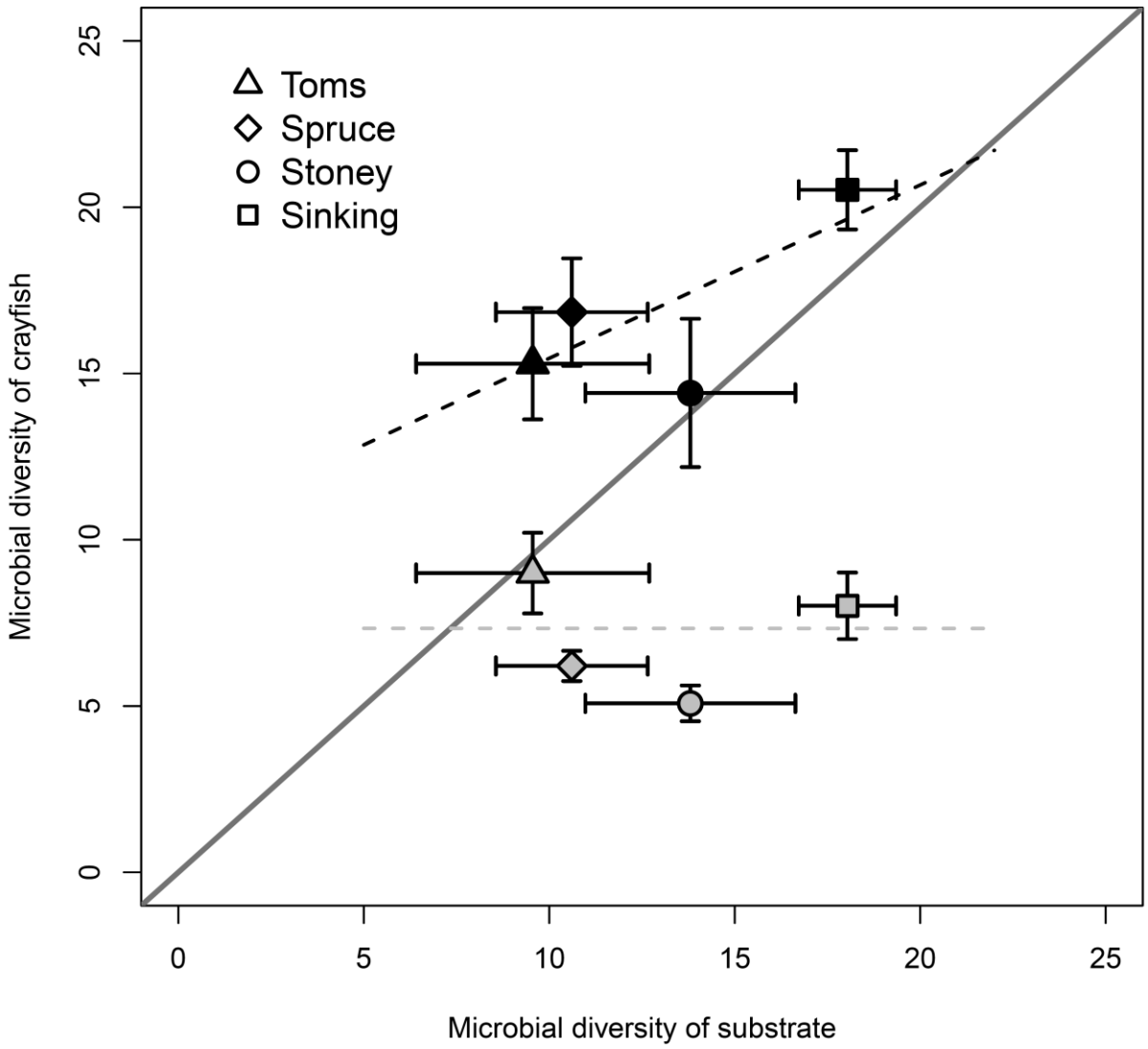

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Figure 3: Non-metric multidimensional scaling (NMDS) of field survey results showing relationships among the bacterial biofilm community of gills (squares), carapaces (circles) and substrate (shaded ellipses) from four locations; red = “Sinking Creek”, blue = “Big Stoney”, green = “Spruce run”, grey = “Toms Creek”. Symbols represent centroid (mean) of each group. Ellipses represent 95% confidence envelope for centroid of substrate samples. Community composition of the carapaces was less variable, but within the range of local substrate. Gill composition was variable at some sites, but always distinct from substrate.

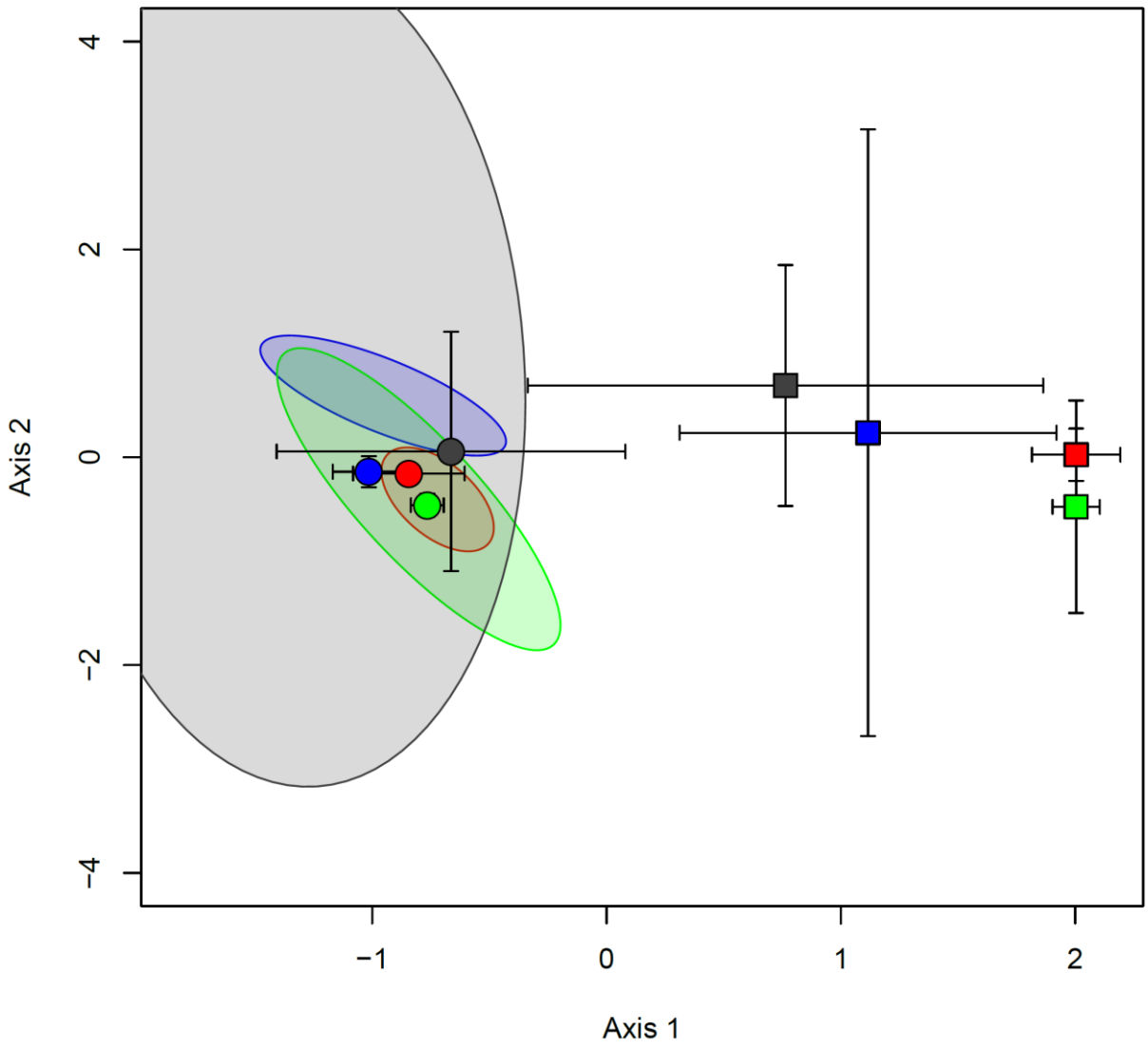

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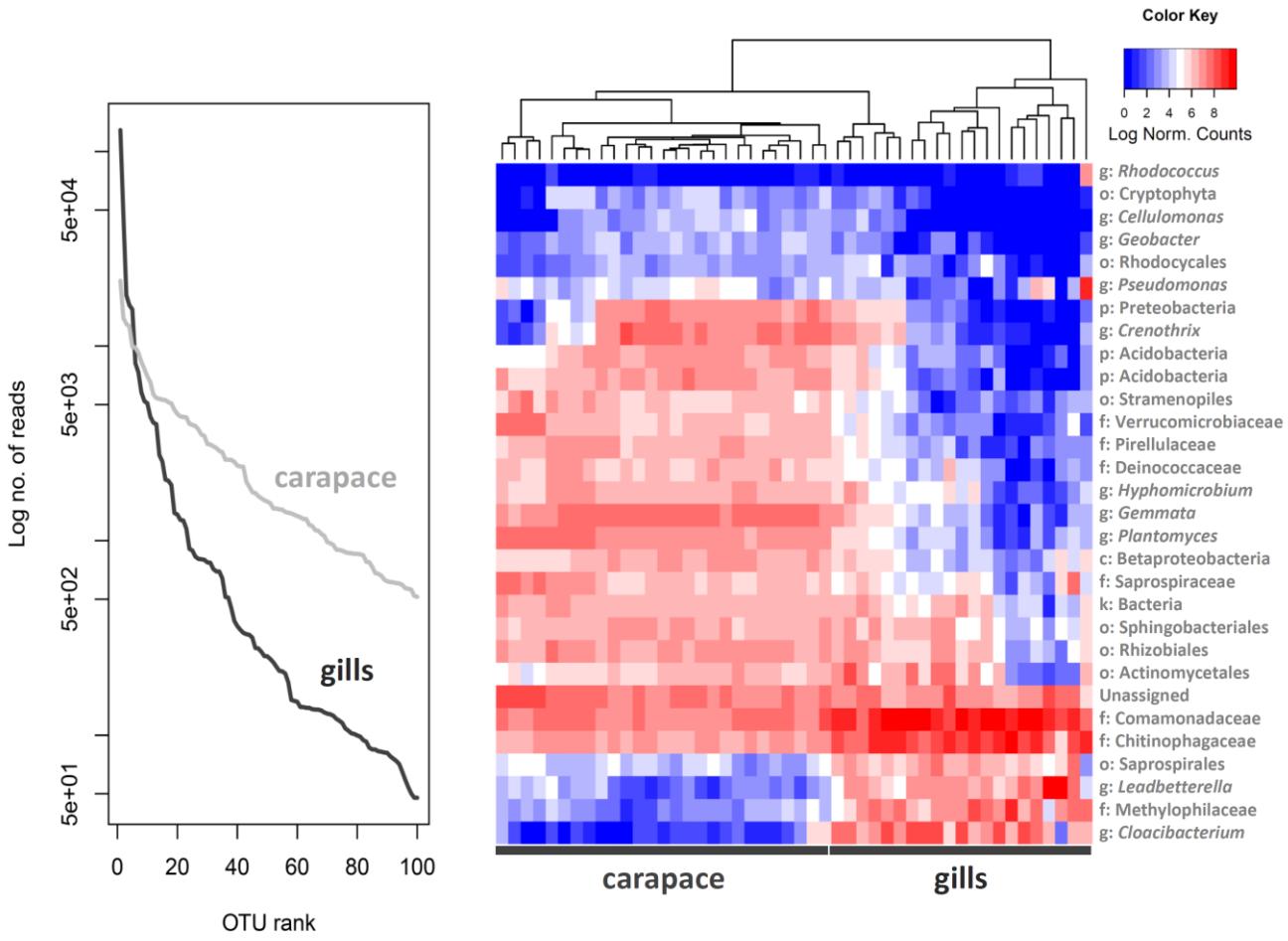
Figure 4. Comparison of microbial communities of the crayfish carapace and gills. (left) Rank abundance curves for the 100 most frequently observed taxa showed that carapace communities were more even than those of the gills, and gills were largely dominated by few taxa. (right) Heat map of showing the log counts of reads for 30 most frequently observed OTUs revealed obvious compositional differences between gills and carapaces. Columns are arranged by similarity using hierarchical clustering. Many taxa were equally abundant on the carapace, however, the gills were dominated by two taxa, Comamonadacea and Chitnophagacea. Letters before taxa indicate finest taxonomic rank resolved from bioinformatics pipeline (e.g., g=genus, f=family, o=order).



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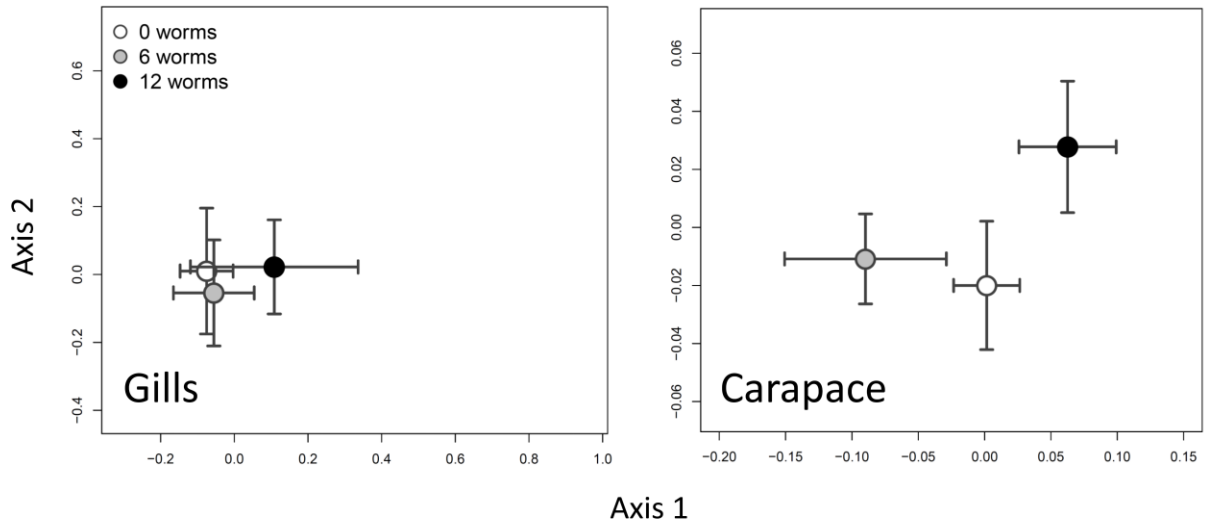
663 Figure 5. Non-metric multidimensional scaling ordination showing significant effects of
664 branchiobdellidan treatment on carapace (right; stress = 0.14) but not gill (left; stress = 0.08)
665 microbial communities.



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668 Table 1: Linear regression for effects of average substrate diversity and crayfish tissue type on

669 bacterial diversity. The overall model was highly significant ($F_{3, 37} = 30.21$, $p < 0.001$).

Coefficients	Estimate	t statistic	p value
Intercept	10.25	3.685	< **0.001***
Sample type (Gills)	-2.91	-0.718	0.477
Mean substrate diversity	0.521	2.507	**0.017***
Sample type × substrate diversity	-0.521	0.300	0.090

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672 Table 2: Linear regression for effects of branchiobdellidan worm treatment and crayfish tissue

673 type on bacterial diversity. The overall model was highly significant ($F_{3, 28} = 80.13$, $p < 0.001$).

Coefficients	Estimate	t statistic	p value
Intercept	36.18	18.045	< **0.001***
Worm treatment	0.187	0.254	0.466
Sample type (Gills)	-30.179	-9.663	< **0.001***
Sample type × worm treatment	--0.258	-0.658	0.516

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