Title (max 96 ch): Microbiome dynamics in microscopic marine invertebrates

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Abstract: (100-125 words)

Much of our knowledge on how microbiomes impact the biology and evolution of their hosts comes from two deeply-studied metazoan phyla, chordates and arthropods. To understand interactions on a much broader scale of diversity, we characterized bacterial communities associated with 1,000 microscopic marine invertebrates from 21 phyla. Despite their size, these animals do harbour microbiomes whose composition differs from the surrounding environment. Distantly-related but coexisting invertebrates tend to share many of the same bacteria, suggesting guilds of preferentially host-associated but non-specific microbes are the main drivers of the ecological relationship. Host identity is a minor factor shaping these microbiomes, which show no evidence of long-term co-differentiation, in stark contrast with what has been observed in many large animals.

One-Sentence Summary (max 125 cha):

Bacterial communities harboured by more than 1,000 of the smallest animals on Earth do not correlate with host lineage

Separation from the environment but no phylosymbiosis in the microbiomes of more than 1,000 of the smallest animals on Earth

Main Text:

(Ideally 400-600 words Intro once references are collapsed. Currently ~450)

All macroscopic organisms interact closely with microbes (Gilbert et al 2012; McFall-Ngai et al 2013; Compant et al 2021). While pathogens receive most of the attention, the majority of host-associated prokaryotes are either harmless, beneficial, or some complex combination of the three (Bass et al 2019; Husnik and Keeling 2019). Discrete communities of microbes interacting with each other and with their shared host form distinctive microbiomes (Berg et al 2020), which have become the subject of intense scrutiny over recent decades.

While data on metazoan-associated microbiomes is extensive, it is also very taxonomically skewed, and this limits our ability to answer even some basic questions about broader evolutionary trends. The two largest classes of arthropods, namely insects (Engel and Moran 2013; Kwong and Moran 2016) and crustaceans (REF?; Holt et al 2020), receive a lot of attention, as do corals (Pollock et al 2018; O'Brien et al 2020) and sponges (Hentschel et al 2012; Engelberts et al 2020). But the lion's share of information comes from the gut microbial communities of mammals (Ley et al 2008; Groussin et al 2017) and to a lesser extent of other vertebrates (Colston and Jackson 2016; Levin et al 2021; Mallot and Amato 2021). Mammalian surveys have generally shown a strong correlation between host phylogeny and microbiome similarity (Ley et al 2008; Nishida and Ochman 2017), a pattern dubbed "phylosymbiosis" (Brucker and Borderstein 2013; Brooks et al 2016), the prevalence of which in other animal groups (even non-mammalian vertebrates) is debated (Colman et al 2012; Mazel et al 2018; Colston and Jackson 2016; Fleischer et al 2020; Escalas et al 2021). Other factors often evoked to explain the host distribution of microbial communities include diet (Muegge et al 2011; Colman et al 2012; Youngblut et al 2019; Escalas et al 2021; Levin et al 2021), physiology (Amato et al 2019), social structure (Bennett et al 2016; Moeller et al 2016), surrounding environment (Grond et al 2019; Eckert et al 2021), and geography (Yatsunenko et al 2012; Fleischer et al 2020), all of which can play a role in shaping host-associated microbiomes.

How well our conclusions based on model systems can be generalized to other metazoans is not currently clear, because most of the 33 known extant phyla receive little attention (Bik 2019). Some recent papers have investigated microbiomes in lesser known taxa (e.g., King 2018; Schuelke et al 2018; Vijaian et al 2019; Guidetti et al 2020; Turgay et al 2020), but typically focus on a single phylum, and commonly only on a few species within that phylum, leaving large pockets of diversity unexplored. Here we characterized over 1,000 animals belonging to the most diverse and least-studied category: microscopic marine invertebrates.

Most animal phyla include marine representatives smaller than 1-2 mm (Giere 2009; Brusca et al 2017), which numerically dominate and play crucial roles in the ecology of nearly all marine ecosystems (Giere 2009). While their minute size is almost the only feature they have in common, it is by itself important in determining many physiological and ecological traits, such as low dispersal capabilities, high body wall permeability, and sensitivity to environmental change (Snelgrove 1999; Danovaro et al 2008; Giere 2009). Microscopic marine invertebrates overlap in size with unicellular protists, and are occasionally preyed upon by them. Whether such tiny organisms have the capacity to host complex, differentiated microbiomes is currently unclear, since the topic has never been extensively examined. If they did, these animals would

represent most of the metazoan host diversity, and their microbial communities would provide critical points of comparison for more familiar systems (Hammer et al 2019).

(Ideally 1,000-1,500 words for Results once references are collapsed. Currently **~1600**)

Results

Collection of 1,000 animals from 21 phyla

We isolated, imaged, and preserved marine invertebrates measuring approximately 100-2,000 μm from five temperate (British Columbia, Canada) and tropical (Curaçao, in the Caribbean) locations (Fig. 1A). We collected 46 samples in three main habitats (sediment, water column, and intertidal macroalgae), characterizing for each the background environmental microbial community as well as the microbiomes of 15-30 animals (tables S1, S2). Our collection consisted of 1,040 individual specimens and more than 11,000 high-quality pictures, representing 21 phyla (Fig. 1B, fig. S1). Missing phyla are primarily symbiotic (eg, Cycliophora), require very specific sampling techniques (eg, Loricifera), or have generally larger representatives (eg, Brachiopoda). Detailed taxonomic identifications were performed on groups that were highly abundant in our survey, of particular interest, or especially underrepresented in the literature (table S2). We assigned specimens to the least inclusive taxon that could be determined based on morphology, avoiding over-specific assignments that might be dubious, considering that the surveyed regions are underexplored and that many collected specimens might belong to undescribed species or genera. The degree of taxonomic detail obtained was also lineage-dependent, so that for example most annelids and nematodes were assigned to families (70% and 91% respectively), while most kinorhynchs were assigned to species (89%). Since the number of isolated organisms per species roughly reflects its abundance in each sample, taxa vary widely in frequency (table S2), altogether giving the data set a great deal of taxonomic breadth, as well as considerable sampling depth for certain lineages.

Microscopic marine invertebrates harbor distinct bacterial microbiomes

Microbiomes associated with hosts clearly differed from environmental microbial communities. In analyses of the entire dataset, the two groups clustered separately in Principal Coordinates Analysis (PCoA) (Fig. 2A), their difference being the most obvious factor influencing the ordination. Animal-associated microbiomes (Fig. 2B) were only weakly and/or insignificantly affected by environmental features such as habitat (ANOSIM. R: 0.000140, p-value=0.476) or location (ANOSIM. R: 0.172, p-value < 0.001), which, not surprisingly, more strongly impacted environmental controls (ANOSIM. Habitat, R:0.662, p-value <0.001; Location, R:0.366, p-value < 0.001) (Fig. 2C).

Animal-associated microbiomes were also consistently and substantially less diverse and species-rich than their environmental counterparts (Fig. 2D-G; fig. S2). Bacterial diversity was clearly impacted by host phylum, location, and habitat (ANOVA. Host phylum, p-value < 2e-16. Location, p-value = 5.86e-7. Habitat, p-value = 0.0302). Shannon index values within each phylum (Fig. 2D), as well as within smaller taxonomic ranks, like families in Annelida and Nematoda (Fig. 2E), varied extensively, but never approached environmental microbial

communities from the same location (Fig. 2F) or habitat (Fig. 2G). The same conclusions apply to other measures, such as the number of Amplicon Sequence Variants (ASVs) observed in each microbial community (ANOVA. Host phylum, p-value < 2e-16. Location, p-value = 1.4e-10. Habitat, p-value = 0.0165) (fig. S2). Neither diversity nor richness of invertebrate-associated microbiomes correlate with those of their corresponding environmental controls (fig. S3), altogether highlighting differential and independent dynamics acting on the two types of microbial communities.

Guilds of non-specific host-associated bacteria dominate the microbiomes of microscopic invertebrates

To identify the causes of the strong separation between animal-associated and environmental microbial communities, we focussed on the most sample-rich location, Quadra Island. First we compared the proportion of bacterial ASVs shared between individual invertebrates and their corresponding environmental controls (Fig. 3A-C). This turned out to be relatively low, implying that limited taxonomic overlap contributes to the separation of host-associated and environmental communities. The habitat was a significant determinant of the extent of this overlap (ANOVA. P-value = 0.0473), but there were no significant differences when comparing different host phyla within the same habitat (ANOVA. Sediment, p-value = 0.147. Macroalgae, p-value = 0.0669. Plankton, p-value = 0.209) (Fig. 3A-C). This suggests that although the observed trend widely varies in magnitude, it is not predominantly under the control of the animals.

In order to assess if the relatively few bacteria shared by invertebrates and the wider environments were, as a rule, the ecologically important ones, we first determined the ‘core’ bacterial communities in environmental controls by constructing microbial ecological networks (Fig. 3D). We identified and ranked keystone environmental ASVs according to their eigen-centrality in the network – i.e. the number of other nodes (ASVs) to which each node is linked and additional links made by those to subsequent nodes. We then assessed the prevalence and relative abundance of the key environmental ASVs with the highest eigen-centrality, arguably the most ecologically important, in the microbiomes of animals from the same habitat and location. Both prevalence and abundance were found to be generally low in animal-associated communities, and several keystone ASVs were altogether absent (Fig. 3E). This was true of all habitats sampled from Quadra (fig. S4).

The microbiomes of microscopic invertebrates therefore share little overlap with the microbial communities in the environment, and likely differ in the composition of the most ecologically important bacteria. In contrast, and more surprisingly, individual invertebrates shared a higher proportion of bacterial ASVs with even distantly-related animals isolated from the same sample (Fig. 3F-H). The proportions of ASVs shared among co-occurring specimens and with the environment are correlated (fig. S5A), but the increments are not driven by the same ASVs (fig. S5B), which suggests that the trend is not significantly affected by ubiquitous generalists. The phenomenon was also not only consistent across the spectrum of habitats, but held true after agglomerating ASVs into bacterial genera (fig. S6) (although all percentage values, predictably, increased). Overall, host-associated bacteria do not make up a substantial proportion of the environmental microbial community, but tend to be shared among unrelated animals, altogether suggesting the existence of guilds of preferentially host-associated, yet non-specific, bacteria.

Lack of phylosymbiosis from phylum to genus level

The term phylosymbiosis describes a correlation between microbiome similarities and host phylogeny, something we observed no evidence for in our data at the highest taxonomic ranks. PCoA ordinations show no clustering of microbiomes from invertebrates of the same phylum (ANOSIM. R: 0.0115, p-value = 0.173) (Fig. 2B). Examining the relationship from the opposite perspective supports the same conclusion: microbial community compositions are extremely poor predictors of broad host taxonomy according to random forest models (fig. S7). These models can reliably distinguish animal-associated from environmental microbial communities, and fare reasonably well in discriminating animal-associated microbiomes from different locations and habitats (although these are predicted far better by corresponding environmental communities). In contrast, however, out-of-bag error rates are extremely high when attempting to classify microbiomes according to host phyla, classes, or orders, overall providing no support for phylosymbiosis at these levels.

To investigate whether host-taxon correlations became more apparent with less inclusive taxa, we focussed on lineages with the most specimens and best annotations, namely Annelida and Nematoda (Fig. 4). Within these phyla, microbiomes did not cluster according to host family (ANOSIM. Annelida, R = -0.1044, p-value=0.964. Nematoda, R = 0.0655, p-value = 0.025). Moreover, pairwise dissimilarity values were particularly high, with three quarters of comparisons scoring above 0.95 and 0.91 in the two phyla, respectively, and did not decrease substantially when comparing hosts from more closely-related (or the same) families (Fig. 4).

Even among families of the same phylum we could not find the typical signatures of phylosymbiosis, which were instead only detected at a very low taxonomic level, below genus. For example, microbiomes from the flatworm *Astrotorhynchus* (Fig. 5A) as well as the kinorhynch *Echinoderes* (fig. S8) seemingly clustered according to host species. It is however worth noting that species in these genera were sampled from different locations and/or habitats, making it difficult to distinguish whether the observed effect is due to host taxonomy or correlated environmental factors.

Potential symbionts and host taxa-specific associations

Phylosymbiosis concerns trends that apply to the whole microbiome composition, and its absence does not preclude the possibility that specific bacteria preferentially associate with certain host taxa, as is the case in many symbioses. We found however that most phyla did not contain any prevalent bacterial ASVs (among those with 0.05% relative abundance or more) that were not also prevalent elsewhere (fig. S9). Those that did were all represented by relatively few specimens (Hemichordata, Ctenophora, Chaetognatha, Chordata, Tardigrada), most of which belonged to a single genus (e.g., *Meioglossus* in Hemichordata) or species (e.g., *Florarctus antillensis* in Tardigrada) in our survey.

To examine symbioses at less inclusive ranks, we plotted the relative prevalence of ASVs in well sampled genera or species, considering as potential host-specific symbionts those that were present in at least half the specimens in each host subset. This filter produced seven candidate host genera/species of interest, shown in Fig. 5B, which were then reduced to three after disregarding ASVs that were also present in other invertebrates (and therefore not taxa-specific).. Three ASVs were identified as candidate symbionts in *Astrotorhynchus regulatus*

(Platyhelminthes) (Fig. 5C), two in *Florarctus antillensis* (Tardigrada) (Fig. 5D), and one in an unknown *Meioglossus* species (Hemichordata) (Fig. 5E), all of which are similar to environmental and/or marine animal-related reference sequences (table S3). None were present in all specimens of the putative host. ASVs prevalent in, but not exclusive to, specific taxa, that could have been interpreted as symbionts in a survey with fewer investigated hosts, tend to be linked to environmental conditions, providing an explanation for their distribution independent from host identity. For example, an ASV identified as *Psychrosphaera* (order *Alteromonadales*) in *A. regulatus* is also found at a relative abundance of over 1% in other invertebrates, all collected from Calvert Island.

Looking beyond strict taxon-specificity, our database also revealed the presence of a number of known bacterial symbionts inhabiting a wide range of host organisms. Members of the specialized intracellular order *Rickettsiales*, for example, were present in 15 different host phyla and include a number of unidentified lineages as well as genera such as *Neorickettsia*, MD3-55 and "*Candidatus* Megaira". We also found a series of ASVs from the common symbiont of animals *Endozoicomonas* in several host phyla.

(Ideally 300-600 words Discussion once references are collapsed. Currently ~750)

Discussion

Our data shows that even the smallest invertebrates, some barely larger than eukaryotic microbes, do harbour associated microbial communities. Their microbiomes are clearly influenced by habitat and geographic location, but they are distinct from environmental communities by both composition and key ecological players. The similarities with microbiomes from larger metazoans, however, mostly end here.

Habitat and proximity play a much larger role than the identity of the host in shaping the microbiomes of microscopic invertebrates, despite extreme evolutionary divergence between some of the hosts. Co-occurring animals share a considerable proportion of bacterial species with each other, which only partially overlap with those in the environment. Overall, many bacterial taxa are preferentially host-associated, but do not show a strong affinity for any specific host or host lineage. Those that do, have a patchy distribution and might not be quantitatively dominant, to the point where their signal is drowned by other components of the microbial community. Since the investigated animals differ enormously in anatomy, diet, physiology, and presumably selective pressures, we can speculate that in this situation selection applies primarily to the bacteria, making them the drivers of the ecological relationship (REF; Husnik and Keeling 2019).

Phylosymbiosis, defined as a correlation between microbiome similarity and host phylogeny, indubitably exists, as it has been convincingly demonstrated in many systems through both descriptive and experimental approaches (Brucker and Borderstein 2013; Brooks et al 2016; Nishida and Ochman 2017; Pollock et al 2018; O'Brien et al 2020). But our understanding of its underlying mechanisms and frequency is based on a small fraction of diversity, almost exclusively macroscopic metazoans with multiple body compartments (REF?), the digestive system being the one most often investigated. Our survey does not distinguish microbes associated with different parts of the body of these microscopic invertebrates, which moreover have much higher surface:volume ratios and reduced capability to regulate their

internal state, possibly leading to less discrete compartmentalization. At the same time, our data encompass a greater phylogenetic range of animal hosts than has ever been examined, and we find the features of their microbial communities correspond to those predicted for the absence of phylosymbiosis (Brooks et al 2016): microbiomes not significantly more similar within than among taxa, microbiome composition predominantly influenced by the host surroundings, no correspondence between host phylogeny and microbiome similarities (except possibly at the narrowest taxonomic scale). The enormous variability we observe instead may be due to many factors, including stochastic processes driving microbial assemblages in an aquatic environment (Burns et al 2016), microbiome plasticity during invertebrate life cycles (Xiong et al 2019; Holt et al 2020), or the impact of host health and dysbiosis (Bass et al 2019), the combination of which seems to obfuscate most of the potential host influence.

It has also been argued that constraints related to ecological factors mirror (and hence confuse) the phylogenetic signal even in systems where phylosymbiosis is observed, which would cast doubt on phylogeny as an independent factor and coevolution as a mechanism explaining the observed patterns (Mazel et al 2018). In light of the overall lack of phylosymbiosis across this wide taxonomic range of animal host, and considering the growing number of studies with similar conclusions in specific lineages (Grond et al 2019; Fleischer et al 2020; Eckert et al 2021; Escalas et al 2021), we suggest that phylosymbiosis, however common within some well-studied groups, should not be the default assumption for all metazoans. It certainly does not seem to be the case in the hyper-diverse microscopic marine invertebrates, spanning most phyla, where the microbial communities are primarily made up of ecological guilds of bacteria that are adapted to living in association with animals, but have low host-specificity. This raises interesting questions about the roles of the various parties in the relationship, and specifically which is its primary beneficiary.

The scope of our analysis was intentionally very broad in order to complement other studies that mostly focus on a particular system, bolster general lack of information from most invertebrate phyla, and to provide a baseline and framework for future studies. We predict that delving more deeply into any one of the examined lineages might reach similar general conclusions at all but the narrowest taxonomic units, but will also shed additional light on specific trends by taking into account different niches occupied by related hosts. It is also important to stress the difference between overall host-associated microbiomes vs. specific microbial symbionts. Examples of well-defined symbiotic associations between bacteria and small hosts, including marine invertebrates (REF) as well as protists (Boscaro et al 2017; Husnik et al 2021; Graf et al 2021), abound, and might display different evolutionary paths. Such relationships could be very common, and yet still only account for a fraction of the total interactions between animal hosts and bacteria, most of which we conclude are non-specific host-associated microbes.

References should be cited in parentheses with an italic number (*1*). Multiple reference citations are separated by commas (*2*, *3*) or if a series, en dashes (*4*–*6*). References are cited in order by where they first are called out, through the text, text boxes, figure and table captions, reference notes and acknowledgments, and then the supplementary materials.

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Supplementary Materials

Materials and Methods

Supplementary Text S1

Figs. S1 to S9

Tables S1 to S3

References (## ##)

Fig. 1. Over 1,000 specimens representing most animal phyla. (**A**) Surveyed areas in British Columbia and Curaçao. Numbers in small rectangles represent samples collected from each marine habitat (sediment, water column, or macroalgae); large pie charts represent the number of individual specimens from each habitat (including a few, in white, collected elsewhere); small pie charts represent the number of corresponding environmental controls. (**B**) Animal diversity. The cladogram includes phyla with at least some marine free-living representatives. Phyla in bold were covered by our survey, and photos (not in scale) show examples of collected specimens (see text S1 for description). Pie charts depict the distribution of invertebrates from each phylum across different sampled areas and habitats, and their size correlates with the number of analyzed specimens.

Fig. 2. Impact of host taxonomy and environmental variables on microbiome dynamics. (A-C) Principal Coordinates Analyses (PCoA) according to Bray-Curtis dissimilarity. (**A**) Animal-associated and environmental microbial communities. (**B**) Animal-associated microbiomes colour-coded according to host phyla as in (D). (**C**) Environmental communities according to location (colour) and habitat (shape). (D-G) Shannon diversity of the microbiomes, grouped according to: (**D**) host phylum; (**E**) host order within the phylum Annelida and Nematoda respectively; (**F**) location; and (**G**) habitat. Corresponding environmental controls are indicated by a grey box in (F) and (G). Black circles indicate the average for that row, whereas solid lines represent overall averages. Specimens not associated with controls were removed from habitat comparison (G). The dashed box separates phyla with less than five sampled specimens.

Fig. 3. Weak association between environmental bacteria and host-associated microbiomes. (A-C) Proportion of bacterial ASVs shared between individual invertebrates from Quadra and their environment, separated by habitat: (**A**) sediment; (**B**) macroalgae; and (**C**) water column. (**D**) SPIEC-EASI co-occurrence network of environmental ASVs found in Quadra sediment samples. Each node represents a single ASV. Lines connecting two nodes (edges) indicate an

association between the two ASVs. Node size is scaled to eigen-centrality, which considers the number of connecting nodes as well as their subsequent connections. (**E**) Prevalence and abundance (both as %) of key environmental ASVs in animals from the same habitat and location. Individual ASVs (on the x-axis) are ordered according to their eigen-centrality in the network. Grey arrowheads indicate ASVs that are absent in host-associated microbiomes. Point colour indicates host phyla (see A-C). (F-H) Proportion of bacterial ASVs shared between individual specimens and all other animals from all phyla in the same sample, separated by habitat: (**F**) sediment; (**G**) macroalgae; and (**H**) water column. Solid, black lines in circular plots indicate overall average. Black circles plot phylum average.

Fig. 4. Host phylogeny does not correlate microbiome composition at family-level ranks. Annelida (top) and Nematoda (bottom) are used as examples due to dense sampling and more precise identification. Heatmaps (centre) show intra-phylum pairwise comparisons of Bray-Curtis dissimilarity scores of microbiomes for families with a minimum of five specimens. Tiles are coloured according to dissimilarity score (half of each heatmap is shown as the matrix is symmetrical). Families in each phylum are arranged according to their phylogenetic relationship, and light grey boxes on the diagonals indicate self-self comparison. Top and bottom Principal Coordinates Analysis (PCoA) show nematode families (top) and annelid families (bottom). Both show no clustering by family. Host family, habitat, and location are displayed above and below corresponding half-matrices.

Fig. 5. Weak signals of host taxa-specific bacterial sequence variants at genus and species level. (**A**) Principal Coordinates Analysis using Bray-Curtis dissimilarity of microbiomes from *Astrotorhynch*us species largely clustered according to host identity. Ellipses group animals of the same species (and suggest identities for the few unassigned specimens). (**B**) Average relative abundance of bacterial sequence variants in host genera/species plotted against its overall prevalence (as % of specimens) within that host taxon. Sequence variants with a prevalence of at least 50% are identified as potentially host-specific (light grey box). (C-E) Taxonomic composition of bacterial microbiomes from host taxa displaying highly prevalent bacteria: (**C**) *Astrotorhynchus regulatus* (Platyhelminthes); (**D**) *Florarctus antillensis* (Tardigrada); and (**E**) an unidentified species belonging to the genus *Meioglossus* (Hemichordata). Solid colours indicate bacterial sequence variants with a prevalence of at least 50% in their respective host genus/species. Striped bar segments identify bacterial variants that are also common in other hosts and/or environmental controls and, therefore, are not taxa-specific.