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Natural populations of the putative ancient asexual *Darwinula stevensoni* (Crustacea, Ostracoda) differ in their microbiomes

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Abstract

Although ostracods are important components in aquatic ecosystems, little is known about their microbiomes. Here, we analyzed the microbiomes of the putative ancient asexual ostracod species, *Darwinula stevensoni*, in three natural populations from different freshwater habitats in the UK, Belgium and Spain. We applied high throughput amplicon sequencing approaches to analyze the V3-V4 part of the bacterial 16S rRNA region. We tested for host-specific microbiomes by comparing bacterial assemblages of ostracods with those of sediment and water samples from the same locations. Around 2,200 Amplicon Sequence Variants (ASVs) were identified from ostracod samples with universal primers and 1,700 ASVs with endosymbiotic specific primers, illustrating a high microbiome diversity in *D. stevensoni*. Most bacterial taxa were unique to the microbiome of *D. stevensoni* as compared to other freshwater invertebrates and to non-marine ostracods. Alpha diversity of ostracod microbiomes did not differ significantly between the three populations, but PERMANOVA detected significant differences in bacterial compositions. Microbiomes varied highly among ostracod specimens from the same population. Possible factors shaping ostracod microbiomes could be latitude, food, age, and environmental variables. Preliminary functionality analyses showed that *Darwinula*-specific microbiomes contribute to lipid, carbohydrate, nucleotide, and amino acid metabolic processes and the synthesis of co-factors and vitamins.

Keywords: Non-marine ostracod, endosymbiont, freshwater, lake, spring

Introduction

All metazoans harbor microbiomes, greatly influencing their hosts' biology, which is why the entities of a host plus its associated microbes are also called holobionts (Bordenstein & Theis, 2015). Compared to vertebrates and terrestrial invertebrates, the microbiomes of aquatic invertebrates are still understudied, especially those from freshwater habitats (Eckert et al., 2021). Studies on microbiomes of non-marine crustaceans are even more rare, except for a dozen papers focusing on *Daphnia* species (Callens et al., 2016 Macke et al., 2017, 2020; Cooper & Cressler, 2020; Akbar et al., 2022a, b; Pfennig-Butterworth et al., 2022; Rajarajan et al., 2022) and few investigations on other freshwater organisms, including zooplankton groups such as other cladocerans, copepods and rotifers (Ger et al., 2016; Eckert et al., 2021, 2022; Wang et al., 2021), *Hydra* (Taubenheim et al., 2022) and fish (Baldo et al., 2019; Krotman et al., 2020; Riera & Baldo, 2020). For non-marine ostracods, thus far there are only

three studies investigating ostracod microbiomes: one with a focus on endosymbionts (Schön et al., 2019), another on two cultured ostracod species (Olszewski et al., 2020) and the third on an unidentified *Candona* species from a few sites of Lake Baikal (Khalzov et al., 2021). This is surprising, given that ostracods form an essential part of aquatic ecosystems on all continents (except Antarctica) and that about 2,300 living, formally described non-marine ostracod species are currently known (Meisch et al., 2019). The real diversity of non-marine ostracods is probably even higher, as many regions and taxonomic groups are still under-explored (Almeida et al., 2023) and because of the presence of many cryptic ostracod species (e. g. Bode et al., 2010; Martens et al., 2013; Schön et al., 2012, 2014, 2018). Ostracods occur in all types of aquatic and semi-aquatic habitats, sometimes in high abundances. They have the most extensive fossil record of all recent crustaceans and are widely used as proxies for reconstructing past climates and environments (Smith et al., 2015). Parthenogenetic reproduction is common in this group and has evolved multiple times independently in different ostracod lineages (Chaplin et al., 1994; Martens, 1998), while the majority of marine ostracods reproduces fully sexually. The ostracod family Darwinulidae is one of the four remaining examples of putative ancient asexual animals (Schön et al., 2009) next to bdelloid rotifers (Mark Welch & Meselson, 2000), *Timema* stick insects (Schwander et al., 2011), and oribatid mites (Heethof et al., 2007). Fossil data indicate that darwinulids in general might have been asexual for 200 million years (myr) or more (Martens et al., 2003), and the type species of this family, *Darwinula stevensoni* (Brady & Robertson, 1870), for at least 20 myr (Straub, 1952).

Here, we characterize the microbiome of this asexual non-marine ostracod species *Darwinula stevensoni*. We choose this species for three reasons: (1) Ecological studies have shown that *D. stevensoni* has a wide ecological tolerance and can survive an exceptional range of salinities and temperatures (Van Doninck et al., 2003a), most likely because it has a so-called general purpose genotype (Vrijenhoek & Parker, 2009) that has evolved during its long evolutionary history (Van Doninck, 2003a; Schön et al., 2009). It is possible that the microbiome contributes to this wide ecological tolerance. (2) Novel strains of *Cardinium* bacteria have been described from different non-marine ostracods (Mioduchowska et al., 2018; Çelen et al., 2019; Schön et al., 2019), including darwinulids (Schön et al., 2019). In other arthropods, it has been shown that the presence of endosymbionts can significantly influence host biology and microbiome diversity (Li et al., 2022). (3) *Darwinula stevensoni* is geographically widespread, occurring on all continents except Antarctica (Rossetti & Martens, 1996) with different genetic species (Schön et al., 2012). To test if microbiomes of

non-marine ostracods are shaped by environmental factors, as they do in planktonic freshwater crustacean species (Eckert et al., 2021, 2022), and/or also by host genotypes as in *Daphnia* species (Macke et al., 2020; Rajarajan et al., 2022), we here investigate natural populations of *D. stevensoni* representing two genetic species (Schön et al., 2012) from three European freshwater habitats with different limnological characteristics.

More specifically, we aim to test the following research hypotheses: (1) Microbiomes of non-marine ostracods are host specific. (2) Non-marine ostracod microbiomes are stable without significant differences in their composition between geographically different populations or host genotypes. (3) The presence of endosymbiotic bacteria like *Cardinium* shapes microbiome diversity and composition. (4) *Darwinula stevensoni* and its microbiome form a true holobiont, whereby the microbiome contributes to important host functions including the general purpose genotype, which could provide wide ecological tolerance of this ostracod species.

Material and Methods

Sampling

We characterized the microbiome of *D. stevensoni* and its environment (open water and sediment) from different freshwater habitats in Spain, the UK and Belgium, respectively.

The Spanish site, the Ullal Fosc spring, is located in the La Safor wetland, 25 km south of the Albufera Natural Park. This wetland corresponds to the discharge area of aquifers originating in the surrounding karstic Massif Mondúver (Sahuquillo, 2012) and the spring hydrochemistry can be classified as dilute, carbonate-sulphate dominated water (Marco-Barba et al., 2012). The actual pond, into which the spring discharges, has a diameter of 15 m and its depth is between 4 m (point of discharge) and 2 m (most of the pond area). Conductivity is low (590-624 µS/cm) and the pH is alkaline (7.77) (Mezquita et al., 1999). Ullal Fosc has a *Phragmites australis* (Cav.) Trin. ex Steud. belt at the shore, *Lemna* (duckweed) floating vegetation and organic silt and sand sediment; the trophic level of this site has been increasing during the last 30 years, while it was originally oligotrophic (Sahuquillo, 2012). Besides *D. stevensoni*, five other non-marine ostracod species (Mezquita et al., 1999; Mesquita-Joanes, unpublished), as well as eight cladoceran species, five copepods and four rotifer species occur there (Sahuquillo, 2012).

The UK site, Semerwater, is one of the two natural lakes in the Yorkshire Dales. It is an upland, lime-rich lake of glacial origin. It receives drainage from Bardale Gill, Raydale Beck and Cragdale Water (Chiverrell et al., 2008), while the lake has an outlet into the river Bain.

The largest part of its catchment area is grazed and less than 5% covered by woodland (Barlow, 1998). The lake has undergone drastic changes in water level, especially since 1939-1940, when the outflow was artificially deepened (Chiverrell et al., 2008). The lake has now a mean depth of 4 m and a maximum depth of 12 m. In summer, it can be as shallow as 0.9 m. Its surface comprises 26.65 ha (Barlow, 1998). Substrate consists of cobble/pebble at the eastern shoreline with few aquatic macrophytes including some stands of *Nuphar lutea* (L.) Sm. in the south-east corner of the lake, where we conducted our sampling. The southern shoreline is mainly characterized by sand and is in summer used for recreation. Semerwater is alkaline (pH of 7.7-7.8) and somewhat eutrophic (Fryer, 1993), with a conductivity of 230-310 µS/cm, alkalinity of 90 mg/l $CaCO_3$, 36.3-52.3 mg/l of Ca and 2.0 mg/l total nitrogen (Fryer, 1993). Semerwater harbors a rich crustacean fauna of up to 69 species (Fryer, 1993), including 17 other ostracod species besides *D. stevensoni*, many cladocerans and the Atlantic stream crayfish *Austropotambius pallipes* (Lereboullet, 1858).

The Belgian study site is an artificial lake situated in the urban Mariadal park of Zaventem, close to Brussels. It lacks vegetation, although there is input of plant material from trees at the shore. The lake is shallow (c. 0.4 m) with mostly silty or sandy sediment and has a surface area of 0.26 ha. Its water is characterized by a pH of c. 8.7, an EC of c. 682 µS/cm, total dissolved organic phosphate of 3.3 µg/l and it is considered as being eutrophic (unpubl. measurements). Three cladoceran species, *Daphnia pulex* Leydig, 1870, *D. longispina* (O.F. Müller, 1776) and *Scapholebris microcephala* G.O. Sars, 1870 (Brans et al., 2017) and six other ostracod species besides *D. stevensoni* are known to occur there (Cours et al., 2021).

At each locality, we took at least three biological replicates by consecutively collecting habitat water (250 ml), scooping up sediment (approximately 50 ml) and sampling ostracods, the latter with a hand net and about 10 sweeps for each replicate. Water samples were filtered in the field with sterile syringes through 0.22 µm sterile filters. Filters and sediment samples were stored in sterile containers at -20°C until further analyses. Ostracod samples were kept in habitat water in bulk while being transported to the laboratory where they were sorted under a microscope in clean water, and frozen at -80°C prior to DNA extractions. For both water and sediment samples for which we would expect a high variability, we took four replicates per locality. We also analyzed three ostracod replicates per population. For the Spanish Ullal Fosc site, an additional nine specimens of *D. stevensoni* were investigated to test the effects of multiple replicates on ostracod microbiome composition.

DNA extractions, amplification and high throughput sequencing of 16S

DNA extractions and PCR amplifications were conducted in the molecular lab of the Royal Belgian Institute of Natural Sciences, and library preparations at the KU Leuven. We extracted DNA from approximately 1.8 g of the sediment samples with the commercial DNA Powersoil (Mo Bio) kit and from the filters with the Gentra Puregene kit (Qiagen); for both sample types, we followed the manufacture's protocols. Because of the small size of *D. stevensoni*, we could not dissect guts or any other body parts prior to DNA extractions. The microbiomes of *D. stevensoni* were thus studied here from complete females. The Blood and Tissue kit (Qiagen) was used for DNA extractions from individual ostracods with a slightly modified protocol (see Schön et al. (2019) for more details). All extractions were conducted in single tubes and lab space and gloves cleaned with 20% bleach in between samples to avoid contamination.

In all subsequent procedures, negative controls were included. We optimized two different sets of primers (Table S1) with tails for Illumina sequencing (Klindworth et al., 2013) to amplify parts of the bacterial ribosomal 16S region with PCR techniques. We used universal 16S primers, which amplify the V3-V4 regions of 16S for a wide taxonomic range of bacteria (Claesson et al., 2010) and were also successfully applied by others to characterize microbiomes (Dethlefsen et al., 2008; Freese & Schink, 2011; De Tender et al., 2015). An additional set of PCR primers (Schön et al., 2019) specifically targeted the V3-V4 16S regions of endosymbionts in non-marine ostracods. Below, we refer to the datasets generated by these two primer sets as V3 and V3 endosymbiotic.

All primers contained tags of 22 nucleotides (see Table S1 for details) that enabled us to apply the Illumina 16S metagenomic sequencing library preparation protocol for high throughput Illumina sequencing of amplicons. The first PCR protocol used 25 µl of the HotStarTaq Master Mix (1.5 mM $MgCl_2$ and 200 µM of each dNTP), 10 pmol of each primer and 1-2 µl DNA (comprising 20-50 ng) in a Biometra Thermal Cycler (Westburg), starting with 15 min at 95°C and 26 (sediment) to 30 (water) or 42 (ostracods) cycles of: 50 sec at 95°C, 50 sec at various annealing temperatures and 1 min at 72°C, followed by a final elongation step of 10 min at 72°C. Annealing temperatures varied from 54°C (for endosymbiont V3-V4) to 56°C-60°C (universal V3-V4). To verify PCR success, electrophoresis of the amplicons was performed on 1.5% agarose gels with Miridori Green staining and gels were photographed under UV.

Successful amplicons were purified with the Agencout AMPure beads kit following standard procedures. A second indexing PCR was conducted for each amplicon with the dual-indexing

system as in Lange et al. (2014) consisting of barcodes of 8 nucleotides each. Index PCR mixes contained 10 µl of Mytaq™ (Bioline), 0.5 µl of each forward and reverse indexing-primer (20 µM) and 9 µl of each amplicon from the first round of PCRs. PCR cycles consisted of initial denaturation for 1 min at 95°C, 15 cycles of denaturation for 15 sec at 95°C, 15 sec of annealing at 51°C and 10 sec of extension at 72°C, followed by a final extension of 5 min at 72°C. The success of the index PCRs was checked with gel electrophoresis, amplicons were purified with Agencourt AMPure XP beads and their concentration quantified with the commercial Quant-iT™ Picogreen® kit (Thermo Fisher). Samples with at least 20 ng of indexed amplicon DNA were pooled in equal concentrations and sequenced on an Illumina MiSeq v3 PE300 at the Genomics Core of the Catholic University Leuven, Belgium. High throughput sequencing was successful for all ostracod samples with the endosymbiont V3 primers and for 99 out of the 103 samples of *D. stevensoni* and its environment with the universal V3-V4 16S primers (see Table 1a for more details).

Analyses of 16S sequence data

Sequences were demultiplexed and analyzed with dada2 (Callahan et al., 2016) on the Nephele (Weber et al., 2018) platform. Barcodes and primers were trimmed, and dada2 analyses were conducted with the following parameters: a maximum expected error (maxee) of 4, no maximal mismatch, a minimal sampling depth of 1500, no trimming of overhangs, trimming 40 nucleotides left and right without any other truncations and a truncation quality score of 3, similar to Schön et al. (2019). Dereplication and denoising of sequences was followed by merging of paired reads and removing of chimeras. Bacteria were classified from the obtained amplicon sequence variants (ASVs) by 99% comparisons to the SILVA database v.138 and their rarefied abundances illustrated in heat maps. Heatmaps were further processed with Morpheus (https://software.broadinstitute.org/morpheus/). Samples were hierarchically clustered into dendrograms by Euclidian distances, bacterial taxa filtered by maximal mean abundance and variance, and the top 100 taxa visualized in heat maps. Further analyses of relative bacterial abundances calculated by the number of ASVs and Kruskal-Wallis tests for significant differences in alpha diversity (estimated as evenness and Shannon indices) were conducted as downstream analyses on the Nephele website with the obtained biome and fasta files from dada2, and a minimum sequencing depth of 1000. The latter was also to rarefy the initial OTU tables with rrarefy in vegan (Oksanen et al., 2022). Barplots and results of the statistical tests were visualized on the QIIME2 website

(https://view.qiime2.org). Bar plots of the top 20 bacterial species were redrawn with phyloseq v4.2.1 (McMurdie & Holmes, 2013) and ggplot2 v3.3.5 (Wickham, 2016) R packages after the removal of ASVs assigned to chloroplasts and mitochondria in R (R Core Team, 2021).

In Excel, OTUs assigned to Eukaryote, chloroplast and mitochondrial phyla were removed from the ASV abundance tables along with columns detailing OTU taxonomy and normalized using the Total Sum Scaling method. In R v4.1, non-metric multidimensional scaling (NMDS) was performed with the Bray-Curtis dissimilarity metric on the normalized ASV abundance data in vegan (v2.6.2) (Oksanen et al., 2022) and visualized with ggplot2 (v3.3.5) (Wickham, 2016). Subsequently, Permutational Multivariate Analysis of Variance analyses were performed with the *adonis2*() function of vegan to test for significant differences among the microbial communities of samples grouped by either location and/or sample type and based on the Bray-Curtis dissimilarity index.

More specifically, a two-way PERMANOVA was conducted (1) with the V3 dataset to compare the microbiome of ostracod samples to the microbial communities from their surrounding environments in a model that included both the type and geographic origin of samples, as well as the interaction between both, as independent variables. (2) A subset of the V3 dataset including only ostracod samples was analysed in a second PERMANOVA that only included the geographic origin of samples as an independent variable to compare ostracod microbiomes across locations. (3) Another two-way PERMANOVA was applied to the V3 endosymbiotic dataset to compare endosymbiont communities between ostracods from the three geographic locations in a pairwise manner as *Cardinium* endosymbionts were only known from the populations in Zaventem and Ullal Fosc (Schön et al., 2019). The *adonis2()* function was ran separately for the three comparisons and resulting p-values were corrected using the Bonferroni method to account for repeated testing.

Because effect significances obtained from PERMANOVA analyses are also influenced by potential differences in the variation of sample groups in multivariate space, homoscedasticity was also tested during each of these analyses with the *betadisper()*and *permutest()* functions of vegan. This R program is an implementation of Marti Anderson's PERMDISP2 procedure (Anderson, 2006) in which the original, non-euclidian distances between samples and group centroids are first reduced to principal coordinates. Distances of group members to their group centroids are then subjected to Analysis of Variance (ANOVA) to test if group dispersions are significantly non-homogenous. As interaction effects between the geographic origin and type of samples in the first PERMANOVA was deemed

significant, a column with the concatenated values of both variables was created and used in *betadisper*() as a grouping factor. betadisper objects were visualized using the ggplot2 package.

Indicator values were calculated for the ASVs using the *indval*() function of the labdsv v2.0-1 package (Roberts, 2019). This was done for the full V3 and V3 endosymbiont datasets, as well as for a subset of the V3 dataset containing only ostracod samples. The *indval*() function used the previously normalized and subsets of ASV abundance data and a table which listed the origin of each sample. A subset of the table acquired from this function was then selected to contain only ASVs for which the IndVal value belonged to the 20 most statistically significant values of its dataset. This ensured that the p-values of the IndVal values only had to be corrected for 20 repeated tests, a step that was done using the fdr method in the *p.adjust*() function (Benjamini & Hochberg, 1995) of R.

Microbiome functionalities of the V3 and the V3 endosymbiotic datasets were characterized with PICRUSt2 2.4.1 (Douglas et al., 2020) on the Nephele server (Weber et al., 2018) using the biome and sequence files created by dada2, a maximal nsti (nearest-sequenced taxon index) of 2, a minimum of 1000 reads, and at least 2 samples. Analyses by PICRUSt2 consisted of sequence placement, hidden-state prediction of genomes, and prediction of metagenomes and pathway-levels (Douglas et al., 2020). PICRUSt2 predicts Enzyme Commission (EC) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) functions of each ASV with nsti values being weighted by their abundance and predicts pathway abundances based on the abundances of (predicted) EC number in heatmaps. We manipulated the PICRUST heatmaps similar to the taxonomic ones as described above, clustered samples hierarchically in dendograms by Euclidian distances and grouped and visualized the top 100 pathways and their community-wide abundances according to their means and variances. The top 100 predicted pathways were broadly categorized according to the KEGG pathway Database (https://www.genome.jp/kegg/pathway.html#energy) and the frequency of these categories calculated for each dataset.

Raw sequences and their metadata have been submitted to GenBank (BioProject accession number PRJNA957963).

Although we accept the recommendations made by Naselli-Flores et al. (2022) to include names of the describer(s) to species names, we have here refrained of doing so for the microbial taxa, as this is not generally done in other contemporary papers on microbiomes.

Results

Our high throughput sequencing approach was successful and yielded between 107,714 and 932,172 sequencing reads (Table 1a). Water and sediment had higher numbers of sequencing reads and ASVs than ostracod samples. For ostracod samples, the highest number of sequencing reads and ASVs was generated with the V3 primers as compared to the V3 endosymbiotic primers (Table 1a). If we compare the 12 ostracod samples from Ullal Fosc to the V3 dataset with 3 ostracod samples each from the three populations, the total number of sequencing reads was somewhat higher, while only half as many ASVs were identified (Table 1a). For all sample types and the two molecular datasets, the asymptotic plateau was reached in rarefaction curves indicating that the sequencing depth of the current study was adequate (Figure S1a, b).

We also identified reads which were assigned to chloroplasts or mitochondria in all samples, a characteristic of the dada2 pipeline. Depending on the sample type and the molecular data, chloroplasts contributed between 1.35% and 2.47%, and mitochondria between 0.03% and 0.22% to the total number of ASVs (Table 1b). Water samples had the lowest abundance of both chloroplasts and mitochondria, and ostracod and sediment samples the highest.

Bacterial taxonomic diversity of ostracods and their freshwater environments

Based on the phylum identity of the top 20 microbial species for the V3 dataset, Proteobacteria and Bacteriodota were most frequent (Figure 1a) in the three sample types and localities. Bacterial phyla Actinobacteriota and Deinococcota were only among the top taxa in ostracods, and Chloroflexi, Cyanobacteria and Firmicutes in sediment samples. If we compare the genus identity of the top 20 bacterial species of all sample types for the V3 data (Figure S3), there are pronounced differences between the three sample types although unidentified genera were also present in most samples (Figure S3).

The genus identities of the top 20 microbial species are described in detail for the ostracod samples (see below, Figure 1b) as these were the focus of the current study. Overall, we found a high variability in both the relative frequency of different bacterial taxa as well as the identity of the top 20 taxa at the phylum level among the three sample types and among the three different localities for the same sample types. Details of all identified ASVs are provided in Tables S2 and S3a and S3b in the supplementary material. Box plots showed a lower alpha diversity of bacterial communities in ostracods as compared to sediment and water samples (Figure S4 for evenness). Kruskal-Wallis tests detected overall significant

differences of alpha diversity between the three sample types (evenness: H = 22.40; p<0.0001; Shannon: H = 15.75, p<0.0001).

Bacterial communities of ostracods and their freshwater environments

In the NMDS plot based on the V3 data, bacterial communities of all sample types and localities clustered separately without overlapping with each other (Figure 2a). One exception is a single sediment sample from Ullal Fosc, which grouped together with the sediment samples from Semerwater. Also the PCoA dispersion plot of the V3 dataset showed a separate grouping of the ostracod samples as compared to environmental (sediment and water) samples for all three study sites (Figure 2b). The two-way PERMANOVA analysis of the V3 dataset (Table 2) confirmed that the composition of bacterial communities differed significantly between the environmental and ostracod samples (p = 0.001), between the three localities (p = 0.001) and the interaction of these two factors (p = 0.001). Likewise, sampling localities and the interactions between the sample type and locality showed significant differences for these two comparisons (p = 0.002 and 0.001, respectively, for localities, and p = 0.001 for interactions of sample type and locality; Table 2).

Patterns of bacterial diversity in ostracod hosts

If we inspect the genus identity of the top 20 bacterial species in the ostracod samples for the V3 dataset, *Limnobacter* is among the top in all samples with varying abundances (Figure 1b). Ostracods from Semerwater and Ullal Fosc furthermore had *Hymenobacter* among the top 20 while *Blastococcus* and *Deinococcus* were only found in ostracods from Ullal Fosc and *Thiotrix* only in *D. stevensoni* from Zaventem. The bacterial genus *Candidatus* Paenicardinium was among the top 20 bacterial species in ostracods from Ullal Fosc and Zaventem but not present in all individuals.

Among the phyla of the top 20 bacterial species of the endosymbiotic V3 dataset, we noticed Proteobacteria in all samples (Figure 3a, b) in high and *Firmicutes* in varying abundances. Furthermore, Actinobacteria were found in all but one ostracod sample. Also the phylum Bacteroidota was among the top 20 bacterial species in ostracods from Ulall Fosc and one ostracod specimen from Zaventem. Spirochaeta were only present among the top 20 bacterial species in Zaventem. We observed some additional genera in the top 20 bacterial species of the endosymbiotic V3 dataset (Figure 3b), which were not detected with the V3 data.

The V3 endosymbiotic dataset confirmed the presence of *Candidatus* Paenicardinium in ostracods from Ullal Fosc and Zaventem and *Limnobacter* in Ullal Fosc, similarly to the V3

dataset (see above). It is obvious that ostracods from the three investigated populations differed greatly in the relative frequencies of bacterial genera and top 20 taxa based on both molecular datasets. We also noticed variation between individuals from the same populations. This is also the case for ostracods from Ullal Fosc where we had analyzed 12 individuals (Figure S5).

Besides *Cardinium* (here identified as *Candidatus* Paenicardinium), we also retrieved ASVs in some ostracod samples from all three localities which were assigned to *Rickettsia*, another potential endosymbiont, in low sequence read numbers in both molecular datasets (see supplementary material, Tables S3a, b).

We found no significant differences in the alpha diversity of bacterial communities among *D. stevensoni* specimens from the three localities based on the V3 dataset with Kruskal-Wallis tests (evenness: $H = 5.42$; $p = 0.066$; Shannon: $H = 5.60$; $p = 0.061$) or the V3 endosymbiotic dataset (evenness: $H = 5.42$, $p = 0.066$; Shannon: $H = 3.82$, $p = 0.148$).

Both the NMDS (Figure 2a) and the dispersion plots (Figure 2b, c) showed a clear separation of ostracod microbiomes for each of the three study sites based on the V3 data set. The two-way PERMANOVA analysis confirmed that the composition of bacterial communities differed significantly between the ostracod samples from the three localities for the V3 dataset (Table 2; $p = 0.004$ for information on the betadisper results, see Table S4). Also for the V3 endosymbiotic dataset, most samples clustered separately in the NMDS (Figure S6a) and the dispersion plot (Figure S6b) according to locality although some overlap of the samples from the same locality is visible in the NMDS plot. However, the pairwise PERMANOVA analyses showed no significant differences in the endosymbiont composition of ostracods from the three locations (Table 2), neither for comparisons of locations with (ZA and UF) nor without (SW) known *Cardinium* prevalence (Schön et al., 2019), i.e., Ullal Fosc versus Zaventem (corrected $p = 0.2$), Semerwater versus Zaventem (corrected $p=0.666$) and Semerwater versus Ullal de Fosc (corrected $p = 0.2$). When conducting the PERMANOVA analysis with the V3 endosymbiotic dataset, the program indicated that sample size might be insufficient as implied by the near zero stress value (see Figure S6a).

In the dendrogram of the heatmap for the ostracod V3 dataset (Figure S7a), two samples from Ullal Fosc clustered together while the third was grouped with the three ostracod samples from Semerwater; the three samples from Zaventem formed another cluster. There were noticeable differences among individual ostracod samples for most of the top 100 bacterial taxa ranked according to maximum variance. In the dendrogram of the heatmap for the V3

endosymbiotic dataset (Figure S7b), two samples each of Zaventem and Semerwater clustered together, while the three samples from Ullal Fosc fell into two separate groups. We identified several bacterial taxa with the IndVal analyses which were indicative for the three different sampling sites. Based on all sample types and p values =0.05 after fdr corrections, we found for Semerwater Rhizobiales and different ASVs being identified as Burkholderiales (Chitinibacteracae and Comamonadaceae) to be indicative, for Ullal Fosc Rhizobiales and Spirochaetales, and for Zaventem, other Burkholderiales (Rhodocyclaceae plus an unidentified family) and Leptospirae (Leptospirales) (see Table 3a). Based on the V3 ostracod data, also different ASVs belonging to Burkholderiales were indicative for Semerwater, Ullal Fosc and Zaventem with significant p values (Table 3b). *Darwinula stevensoni* from Semerwater was furthermore also characterized by Cytophagales and ostracods from Zaventem also by Chitinophagales, Peptostreptococcales-Tissierellales and Bacteriodales. With the endosymbiotic V3 data for which we obtained the lowest numbers of sequencing reads and ASVs (see Table 1a), only indicative bacterial taxa for *D. stevensoni* from the Ullal Fosc population were observed with significant p values (see Table 3c), including again, as for the other two data sets, Burkholderiales and Peptostreptococcales - Tissierellales, but also Flavobacteriales, Azospirillales, Oscillospirales, Fuscobacteriales and Staphylococcales as being indicative for this population.

Functionality of ostracod microbiomes

Details of all predicted KO functions for ostracods based on the V3 dataset are provided in Table S5. We also show the heatmaps of the top 100 predicted pathways of the V3 ostracod dataset ranked according to their means (Figure S8). When their identity of the KEGG pathway database is used to group individual pathways, we notice five types of pathways to be especially abundant in ostracod microbiomes (Table 4): amino acid and nucleotide metabolisms (both 25%), carbohydrate and lipid metabolisms (17 and 15%, respectively) and co-factor and vitamin metabolism (10%). Energy metabolism, secondary metabolisms, carbon fixation and glycan metabolism are represented each with one or two pathways only. When comparing abundances of pathways among ostracod samples, two samples from Semerwater (SW3 and SW1) and one from Ullal Fosc (UF1) show the highest means (Figure S8). Overall, abundances of the top 100 pathways are rather heterogeneous among the analyzed ostracod samples, illustrating a high variability of microbiome functionality.

Discussion

The current study characterized, for the first time, the microbiomes of a non-marine ostracod species in natural populations from different freshwater habitats with high throughput 16S sequencing. This approach generated hundreds of thousands of 16S sequencing although the frequencies of identified chloroplasts in the current study (Table 1b; Figure S2) are somewhat higher than in other microbiome studies which only analyzed water or zooplankton (see for example Eckert et al., 2021).

Bacterial diversity of ostracods and their freshwater environments

We found that bacterial alpha diversity and microbiome composition of ostracod hosts significantly differed from those of their freshwater environments (water and sediment) in all study sites. Similar differences were described for microscopic marine invertebrates (Boscaro et al., 2022) and for marine (De Corte et al., 2018) and freshwater zooplankton (Hegg et al., 2021; Wang et al., 2021). In contrast, 50 to 90% of bacterial taxa were shared between planktonic rotifer or cladoceran species and their surroundings in, for example, the study by Eckert et al. (2021), emphasizing the role of the host environment as source for its microbiome.

Our results thus indicate that specific bacterial communities are present in *D. stevensoni* which are different to the bacteria in their surroundings. It was already known that some ostracods (including *D. stevensoni*) harbor specific endosymbiotic bacteria (Schön et al., 2019). The present study shows that non-marine ostracods from natural freshwater habitats also host diverse microbiomes extending beyond endosymbiotic bacteria.

The estimated alpha diversity of bacterial communities in ostracods was significantly lower than that of bacterial communities in their environments (Figure S4). This was also found in studies on the microbiomes of skins from freshwater fish (Krotman et al., 2020) and in freshwater zooplankton (Wang et al., 2021). Macke et al. (2020) interpreted such a lower alpha diversity of hosts as compared to their environment as evidence for selective rejection of bacteria from the surroundings, which would further support the hypothesis of ostracod-specific microbiomes. Our results also match the study by Olszewski et al. (2020), who described distinct microbiomes in two other (cultured) ostracod species and are thus in line with the predictions of the holobiont concept (Bosch & Miller, 2017) suggesting strict relationships between hosts and their microbiomes.

We found the phyla Proteobacteria and Bacteroidota among the top 20 bacterial species in all three freshwater sample types, i.e. sediment, water and ostracods, and Actinobacteria in the

three ostracod populations (see Figure 1a). Both Proteo- and Actinobacteria have also been reported as being common in marine waters and their zooplankton (De Corte et al., 2018) and in freshwater zooplankton (Wang et al., 2021; Hegg et al., 2021; Akbar et al., 2022a). Proteobacteria were also observed in cichlid fishes from South America and East Africa (Riera & Baldo, 2020).

Some of the most abundant bacterial phyla and genera in the non-marine ostracod *D. stevensoni* were also found in high frequencies in the three thus far available microbiome studies on Ostracoda. When using the same endosymbiotic V3 primers, also Schön et al. (2019) found Proteobacteria and Bacteroidetes to be common in four non-marine ostracod species including *D. stevensoni*. We can unfortunately not discuss possible similarities at lower taxonomic levels, because many bacterial taxa in the study by Schön et al. (2019) remained unidentified as SILVA 132 was used as reference database, whereas in the current study, SILVA 138 was available. Proteobacteria and Bacteroidetes also occurred in high frequencies in *Candona* spec. from Lake Baikal, from which furthermore Actinobacteria, Gracilibacteria, and Cyanobacteria were reported (Khalzov et al., 2021). Proteobacteria were also prominently present in two ostracod species *Sclerocypris tuberculata* (Sars, 1924) and *Potamocypris mastigophora* (Methuen, 1910) (see Olszewski et al., 2020), while certain Beta- and Gammaproteobacteria contributed most to differences in microbiome composition between these two ostracod species. Interestingly, of the three abundant bacterial families reported for *S. tuberculata* and *P. mastigophora* by Olszewski et al. (2020), only Comamonadaceae was also found in *D. stevensoni*. This family was indicative for the Semerwater and Ullal Fosc ostracod populations, respectively (Table 3a, b, c). Compared to the study of Khalzov et al. (2021) on *Candona* spec., we also detected *Thiotrix* and Flavobacteriales in *D. stevensoni* (Figure 1b), but no Holosporales. These differences between ostracod microbiomes can probably be attributed to at least three major factors: (1) taxonomic differences between the investigated ostracods, (2) geographic origin of the samples, and (3) investigating ostracods from natural populations or from lab cultures. *Darwinula stevensoni* belongs to the Darwinulidae which split c. 360 myrs ago from the Cyprididae and Candonidae (Maddocks, 1982; Martens et al., 1998) to which the species investigated by Olszewski et al. (2020) and Khalzov et al. (2021) belong. Furthermore, the three studies differ in their habitat types and sampling locations: Khalzov et al. (2021) investigated ostracods from Lake Baikal near a methane seep at 420 m depth, while the three localities of our study comprised one natural and one artificial lake and a spring. Olszewski et al. (2020) raised ostracods from dry resistant eggs of sediment samples from Botswana,

Africa. Our study and that of Khalzov et al. (2021) screened ostracods collected in the field while Olszewski et al. (2020) cultured the hatched ostracods in the laboratory under controlled conditions for months before characterizing their microbiomes. Hegg et al. (2021) observed drastic changes in microbiome compositions of *Daphnia magna* (Straus, 1820) when previously cultured animals were released into a mesocosm in a natural lake, making it difficult to directly compare the results of our study to those of Olszewski et al. (2020). When comparing our results to the microbiome compositions of freshwater zooplankton, it is obvious that almost none of the most abundant bacterial genera in zooplankton were also detected in the ostracod hosts. For example, Eckert et al. (2022) found that the genus *Limnohabitans* of the Comomonadaceae was the major component of the microbiome core in *Daphnia* spp. We also found Comomonadaceae in *D. stevensoni*, but we did not detect the genus *Limnohabitans*, nor Chitinophagaceae, Enterobacteriaceae, *Pseudomonas* or Optitutaceae, which Eckert et al. (2022) cited as core OTUs of all investigated rotifers. In fact, only Burkholderiaceae occurred in both rotifer species (Eckert et al. 2022) and in *D. stevensoni* (the present study). This is not surprising, given the different life style and feeding behavior of *D. stevensoni* as compared to zooplankton. This ostracod species cannot swim and is purely benthic.

As already mentioned above, the presence of *Candidatus* Paenicardinium as one of the most abundant bacterial taxa in *D. stevensoni* (in the boxplots with the genus identity of the top 20 bacterial species of both molecular data sets - Figure 1b & 3b) matches the results of Schön et al. (2019) on *D. stevensoni* and studies on other non-marine ostracods (Schön et al., 2019; Khahlzov et al., 2021). Our present study also confirms the patchy distribution of *Cardinium* in infected populations of *D. stevensoni*, like the results by Schön et al. (2019). However, *Candidatus* Paenicardinium was not indicative in the IndVal analyses for any of the three populations of *D. stevensoni* (see Table 3 b, c). The study by Khahlzov et al. (2021) reported the presence of the potential endosymbiont *Rickettsia* in the investigated *Candona* ostracods in high frequencies. Our study confirms that this bacterium is also present in *D. stevensoni* in all three investigated populations, but this in low frequencies. The PCR approach of Schön et al. (2019) did not pick up *Rickettsia*, which is potentially related to the low prevalence of *Rickettsia* in individual ostracods (see Table S3 a, b).

Patterns and causality of bacterial diversity in ostracod hosts

Although we obtained evidence for specific ostracod microbiomes as compared to their environment, we also observed significant differences in the bacterial diversities among the

three studied ostracod populations. This is similar to results obtained for freshwater *Hydra* (Taubenheim et al., 2022) or bacterial communities of fish skins (Krotman et al., 2020). Several studies on freshwater zooplankton also reported flexible or dynamic microbiomes (Macke et al., 2017, 2020; Eckert et al., 2021, 2022; Taubenheim et al., 2022). That the microbiome of *D. stevensoni* is indeed highly dynamic, is further illustrated by the large variability of the microbiome composition between individuals from the same population, even for Ullal Fosc where we investigated 12 ostracod specimens (Figure S5). These high interindividual differences cannot be compared to other invertebrate or ostracod studies, because individuals from the same population are usually pooled in other studies (Olszewski et al., 2020; Eckert et al., 2021, 2022; Khahlzov et al., 2021) and therefore, no data on variability between individuals are available. It might be worthwhile to further investigate interindividual differences in the future, also for other small aquatic invertebrates, in order to obtain a better idea about the real dynamics of host microbiomes in natural populations.
The significant differences of ostracod microbiomes according to locality provide the strongest evidence that the composition and diversity of ostracod microbiomes is to a large extent variable. Hegg et al. (2021) observed equally dynamic microbiomes in *Daphnia magna* from a natural lake. Despite the large variability of host microbiomes in our present study, we still identified some bacterial taxa which appear to be indicative for ostracods from the three study locations (see results of the IndVal; Table 3b, c) and could suggest the presence of a limited core microbiome for each population of *D. stevensoni*, as was also found for certain freshwater zooplankton species (Eckert et al., 2021, 2022).

Studied habitats and their abiotic variables

As mentioned above, we observed significant differences in ostracod microbiomes among the three study sites from different latitudes based on the V3 data. Microbiome studies on, for example, freshwater *Hydra* (Taubenheim et al., 2022), and *Daphnia magna* (Frankel-Bricker et al., 2020) also found locality-specific differences in host microbiomes. In addition, stochastic effects influencing the composition and dynamics of microbiomes have been proposed by Boscaro et al. (2022) and Obrestad et al. (2022), and these most likely also relate to the observed variability in the present study.
The three sites of the current study furthermore varied in multiple abiotic variables such as conductivity, pH, temperature, sediment type, the presence of water plants and other crustaceans, depth, size of the water body and habitat type (see above). Several abiotic variables such as pH, dissolved organic carbon, conductivity, temperature, and oxygen have

been shown to influence the diversity of microbiomes in other freshwater taxa including cladocerans, rotifers and fish (Akbar et al., 2022a; Krotman et al., 2020; Riera & Baldo, 2020; Eckert et al., 2021; Wang et al., 2021). Likewise, the trophic state and habitat type of the water body, which both clearly varied between our three study sites, can impact microbiome composition as shown for freshwater *Hydra* (Taubenheim et al., 2022) and zooplankton (Eckert et al., 2022). Water temperature can apparently have a stronger effect on the microbiome composition than geographic location or depth (Sehnal et al., 2021) or host biology (Li et al., 2023), and we expect that temperatures differed between the three sites of the current study. Although we took all samples in the same month, also other seasonal differences might have occurred as the study sites were located at different latitudes; such seasonal effects on host microbiomes were for example reported by Hegg et al. (2021) for *Daphnia magna*.

Genotype

In the best studied freshwater zooplankton genus *Daphnia*, interactions between environment, genotype and food have been put forward as the main factors shaping its microbiome (Akbar et al., 2022b). We did not find any obvious influence of host genotype on bacterial beta diversities as the PERMANOVA showed significant differences among all three ostracod populations although two (Semerwater and Zaventem) belonged to the same genetic (cryptic) species (Schön et al., 2012). The lack of any effect of host genotypes is also different from the results of microbiome studies on freshwater snails (Takacs-Vesbach et al., 2016) and on *Daphnia* (Akbar et al., 2022b; Rajarajan et al., 2022).

Host biology including endosymbionts and age

We could not identify any clear effect of endosymbionts on the microbiome diversity and composition of *D. stevensoni* as the three populations differed significantly from each other in the PERMANOVA analysis (Table 2), despite results of the current study and of Schön et al. (2019) reporting *Cardinium* to be absent from the Semerwater population.

Boscaro et al. (2022) suggested additional factors which could potentially cause host microbiome plasticity, such as life-cycle differences, lack of an adaptive immune system and impacts of the hosts' health. Indeed, to the best of our knowledge, also ostracods lack an adaptive immune system and have different life cycle stages. Given the exceptionally long generation time of *D. stevensoni* of at least one year in Belgium (Van Doninck et al., 2003b), it is possible that the ostracod populations in October 2017 consisted of a mix of adults from

2016 and new adults of the sampling year; thus, also the age of the hosts could somewhat have affected microbiome composition. The effect of age on microbiomes has so far only been studied in long-lived fish (Zhang et al., 2018; Zhao et al., 2020) as zooplankton species usually have short generation times. The influence of life cycle stages and age on microbiome composition could be further studied in *D. stevensoni* by directly comparing individuals of different developmental stages.

Food

Like other benthic ostracods, *D. stevensoni* feeds on bacterial films and microscopic particles (Smith et al., 2015). By analyzing ostracods from natural populations, we cannot rule out the possibility that some of the detected bacteria in ostracod microbiomes are in fact transient and originate from indigested particles. This could, for example, be apparent in the varying abundances of chloroplasts which we mainly found in sediment and ostracod samples (see Table 1b). Thus, some of the variation among ostracod specimens and populations could possibly be attributed to transient bacterial taxa originating from ingested particles. Even such transient microorganisms have been shown to influence residential microbiomes (Amor et al., 2020).

Several other studies have also reported indirect effects of food on host microbiomes. In cichlid fishes (Baldo et al., 2019; Riera & Baldo, 2020), clear differences in microbiomes were observed according to the hosts' feeding guilds. Food availability can furthermore change the strengths of the mutualistic relationship between hosts and their microbiomes in *Daphnia magna* (Callens et al., 2016, and also diet and feeding behavior can cause large differences in host-associated bacterial communities as in *Daphnia dentifera* Forbes, 1893 and *Daphnia magna* (Pfennig-Butterworth et al., 2022). Seasonal changes in the microbiome composition of *Daphnia magna* in a natural lake (Hegg et al., 2021) have been attributed to changes in food through associations with phytoplankton.

Functionality of ostracod microbiomes

Even if its composition is flexible, we found evidence that the microbiome of *D. stevensoni* can provide important functionalities for its host. Most abundant was the metabolism of amino acids, nucleotides, fatty acids and vitamins and co-factors, possibly providing essential nutrients for the hosts (see for example the review by Akbar et al., 2022a on *Daphnia*). Especially the synthesis of essential vitamins (Putnam & Goodman, 2020; Sokolovskaya et al., 2020) is obviously beneficial. The synthesis of certain amino acids could be equally

advantageous as their availability has been shown to enhance development and increase the total number of produced eggs in *Daphnia* (Fink et al., 2011). In a metagenomic analyses of *Daphnia magna*, Cooper and Cressler (2020) also found the microbiome to be involved in metabolic pathways including amino acid exporters which were specifically mentioned as important for host fitness, together with the synthesis of key vitamins.

We also found that microbiomes of *D. stevensoni* are involved in carbohydrate metabolism, indicating a possible role of ostracod microbiomes in carbon cycling which should be further explored, especially in view of climate change. The functionality of microbiomes for fatty acid, carbon and nitrogen metabolisms in zooplankton has been shown by Wang et al. (2021). At least two of these, fatty acid and carbon metabolism, are confirmed by our results. Wang et al. (2021) further mentioned Methylobactericeae as methane users, Sphingomondales as possible degraders of aromatic compounds and *Pseudomonas* as nitrogen reducers. Some of these taxa were also present in the microbiomes of *D. stevensoni* and especially Methylobactericeae were very abundant.

The possible functions of ostracod microbiomes of the current study matched, at least to some extent, the results of Olszewski et al. (2020) on other ostracod species. They also found evidence for metabolic functions including lipids, nucleotides, vitamins and co-factors and glycan. In their study, however, these functions differed between the two investigated ostracod species, *Sclerocypris tuberculata* and *Potamocypris mastigophora*. This could possibly indicate that these functions are not omnipresent in ostracod microbiomes. It further remains unclear if the study by Olszewski et al. (2020) found any contribution of microbiomes to amino acid synthesis as we observed in the current study. Koskella et al. (2017) indicated the importance of microbiomes in regulating metabolic host flexibility; the large variation in functionality in our samples (Figures S8 and the differences to the study by Olszewski et al. (2020) suggest that this might also be the case for ostracods.

Compared to the metagenomic study on marine zooplankton by De Corte et al. (2018), we identified some similar microbiome functions which are related to energy metabolism and carbon fixation. However, the metabolic functions of the *D. stevensoni* microbiome described above were listed under genes for nutrition and cell protection by De Corte et al. (2018). In contrast, we did not find any evidence that the microbiomes of *D. stevensoni* are involved in membrane-associated functions like pH- and homeostasis-regulation as De Corte et al. (2018) observed. We would have expected such membrane-associated functions if microbiomes would contribute to the ecological tolerance to for example wide salinity ranges and thus also to the general-purpose genotype of *D. stevensoni* (Van Doninck et al., 2003a).

The current study provides first data on microbiome functionality in *D. stevensoni* by indirectly interfering functionality from the identified bacterial taxa and their known functions. However, unidentified taxa cannot be taken into account with such an approach. To obtain definitive insights into the functionality and flexibility of ostracod microbiomes, more direct approaches need to be applied such as shot gun sequencing for metagenomic studies or qPCR techniques to assess the activity of specific bacterial genes.

The phylosymbiosis hypothesis

The phylosymbiosis hypothesis (recently reviewed by Kohl, 2020) proposes that similarities of host-associated microbiomes mirror the phylogenetic relationships of their hosts. Several studies on marine and freshwater plankton have provided evidence against this hypothesis (see for example Eckert et al., 2021, 2022; Boscaro et al., 2022). Here, we can only draw indirect conclusions on the validity of this hypothesis for non-marine ostracods as we only investigated one species. If we compare our results to the other two microbiome studies of non-marine ostracods (Olszewski et al., 2020; Khalzov et al., 2021), we found several unique bacterial taxa in *D. stevensoni.* Also, the two ostracod species investigated by Olszewski et al. (2020) differed in their microbiomes from each other and from *Candona* spec. (Khalzov et al., 2021). These differences could be owing to the phylogenetic distances between the investigated ostracod species and possibly support the phylosymbiosis hypothesis. However, given the large differences in host biology and habitat characteristics of the three microbiome studies on non-marine ostracods, additional field and laboratory experiments are required to test the phylosymbiosis hypothesis more thoroughly. We would recommend investigating the microbiome of sympatric non-marine ostracod species simultaneously, similar to the study design of Eckert et al. (2021).

Conclusions

We successfully characterized the microbiomes of a non-marine ostracod species from different field populations with high throughput sequencing. We found that *D. stevensoni* harbored specific microbiomes which were different to its environments, but also highly variable and dynamic. Ostracod core microbiomes were small although certain bacterial taxa were indicative for ostracod microbiomes from each study site.

Given the rarity of microbiome studies on non-marine ostracods, this study provides important insights into the microbiome of a putative ancient asexual ostracod. However, additional investigations are needed to test how common the patterns observed here are in

other non-marine ostracods from natural populations and which environmental or host-related factors are the major factors shaping ostracod microbiomes. Future ostracod field studies should be designed to replicate sampling sites with similar abiotic variables and habitat types and to compare microbiomes between different ostracod species living in the same habitat. We also still have to further assess, for example with germ free ostracods or laboratory experiments, if the relationships between ostracods and their microbiomes are transient or long-lasting and where they are situated in the range from obligatory to symbiotic relationships.

With this study, we could confirm that microbiomes of non-marine ostracods are host specific (hypothesis 1) but also highly dynamic, thus refuting hypothesis 2. We found no effect of the presence of endosymbiotic *Cardinium* on microbiome diversity and composition (hypothesis 3). *Darwinula stevensoni* and its microbiome seem to form, at least to some extent, a true holobiont (hypothesis 4), whereby the microbiome contributes to important metabolic host functions, but not necessarily to the wide ecological tolerance (general purpose genotype) of this ostracod species.

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Conflict of interest

The authors have no competing interests to declare that are relevant to the content of this article.

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Figure 1-3

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Figure 1a: Relative abundance and phylum identity of the top 20 bacterial species of all sample types and localities based on V3 data.
Different phyla are indicted by different colours. OST = ostracod; SED = sediment; WAT = water; SW = Semerwater; UF = Ullal Fosc; ZA = Zaventem. Numbers indicate replicates from the same study site.

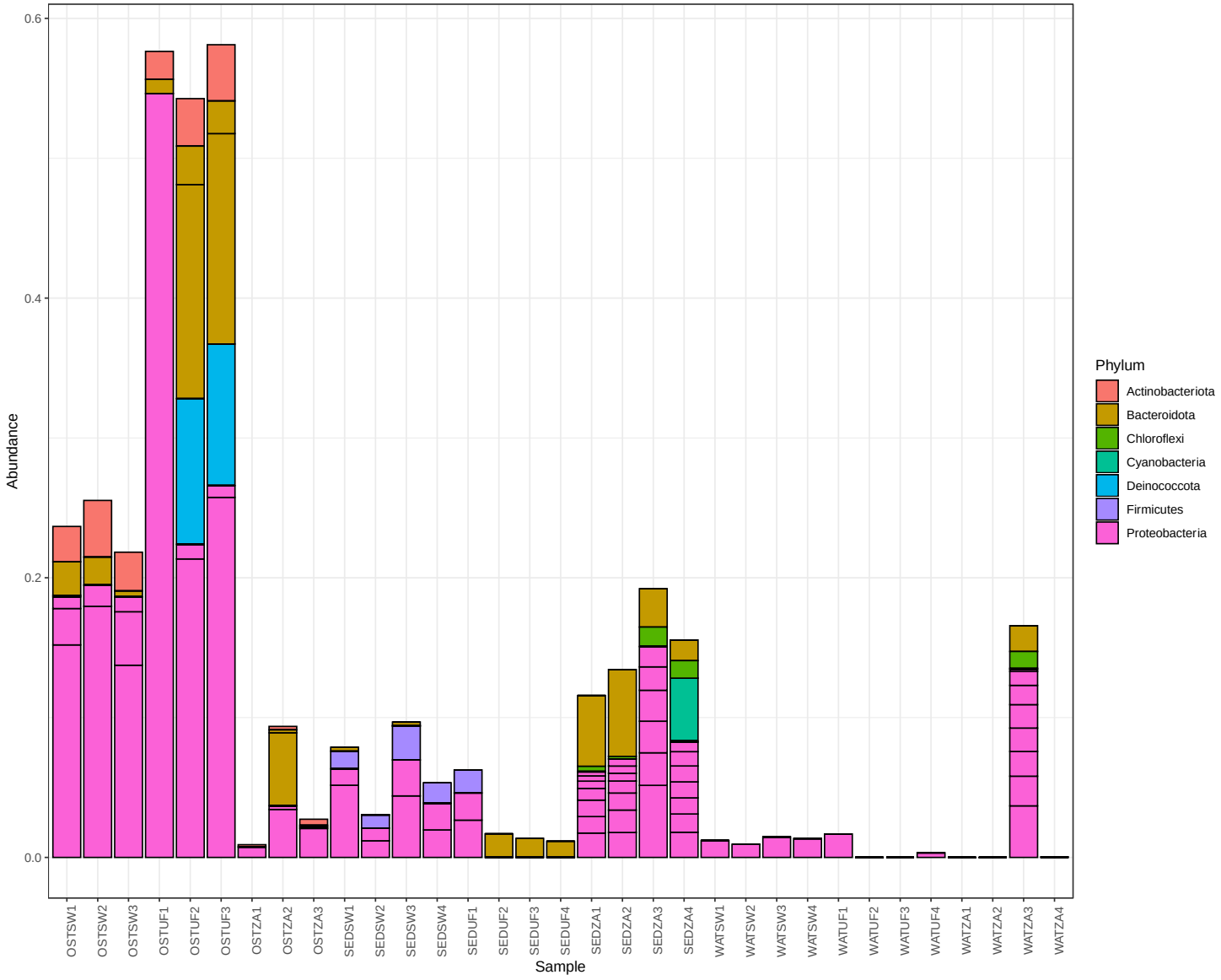

Figure 1b: Relative abundance and genus identity of the top 20 bacterial species of ostracod samples from the three localities based on V3 data.
Different genera are indicted by different colours. SW = Semerwater; UF = Ullal Fosc; ZA = Zaventem. Numbers indicate replicates from the same study site.

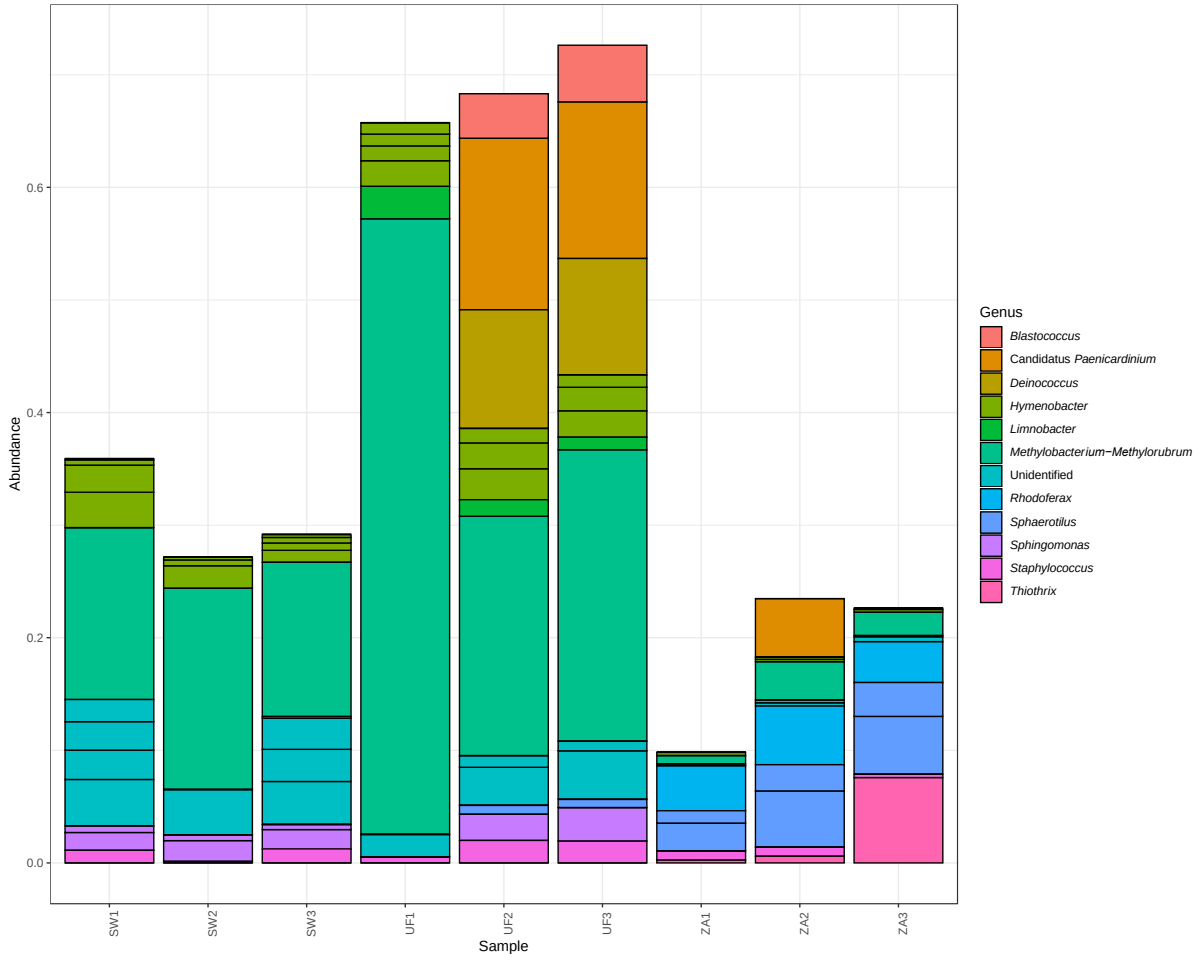

Figure 2a: Non-metric multidimensional scaling plot of the composition of bacterial communities based on V3 data.
Study sites are indicated by different colours, sample types by different symbols. Number of random restarts: 2; Specified number of axes: 2; Value of stress: 0.061.

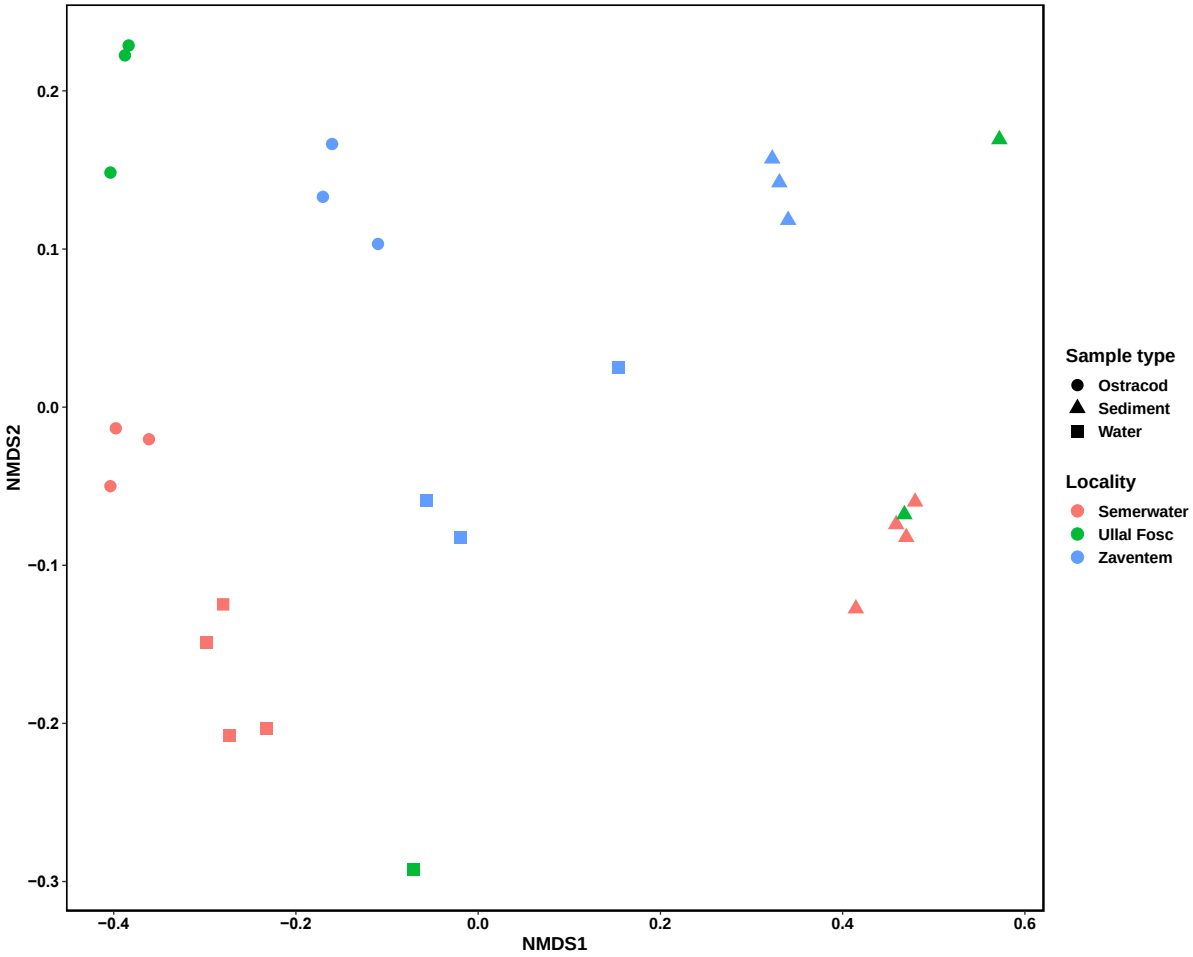

Figure 2b: PCoA dispersion plot of the composition of bacterial communities based on V3 data.
Environmental data comprise both, data from water and sediment samples.
SW = Semerwater; UF = Ullal Fosc; ZA = Zaventem**.**

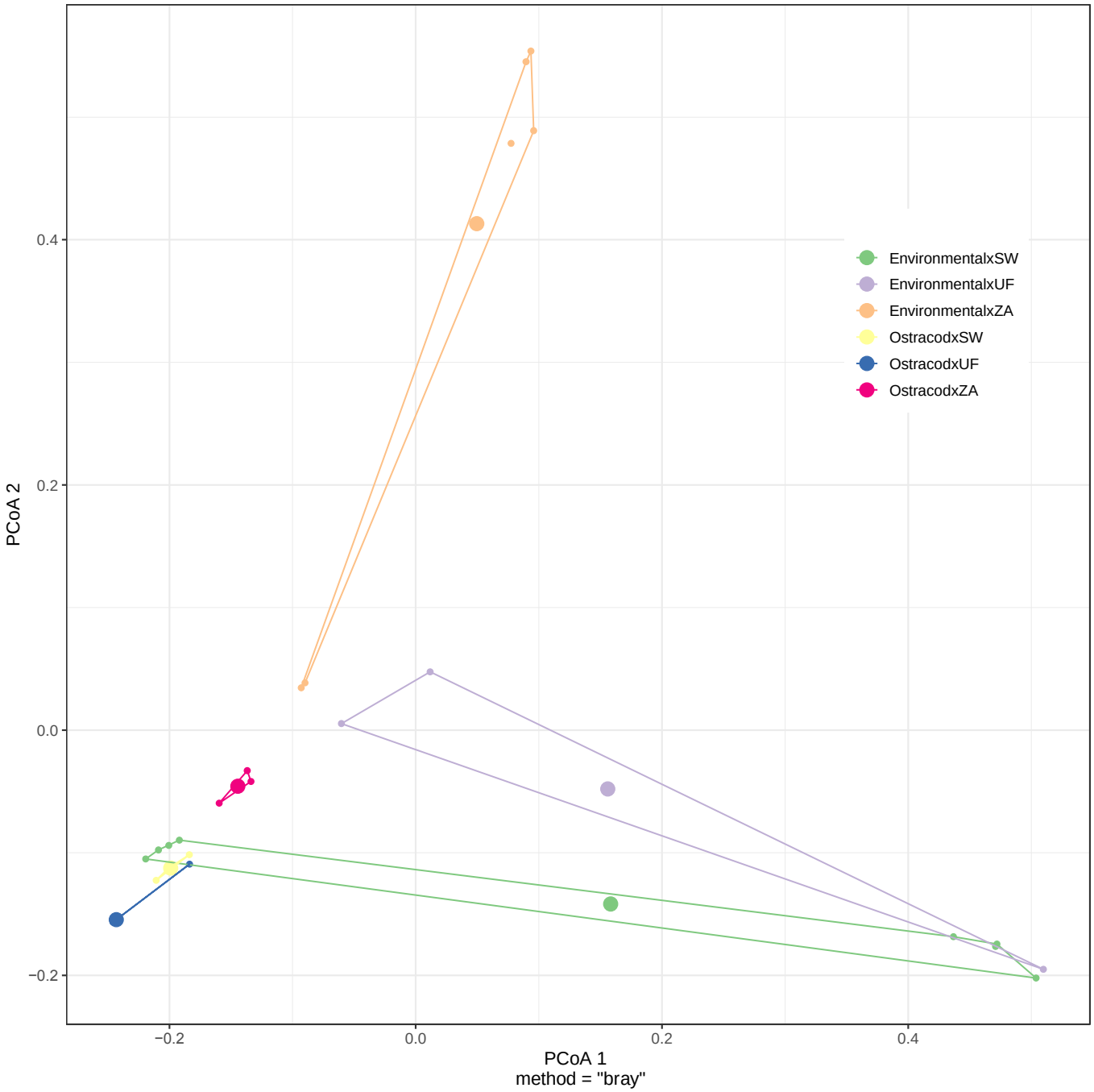

Figure 2c: PCoA dispersion plot of the composition of bacterial communities from ostracods based on V3 data.
SW = Semerwater; UF = Ullal Fosc; ZA = Zaventem**.**

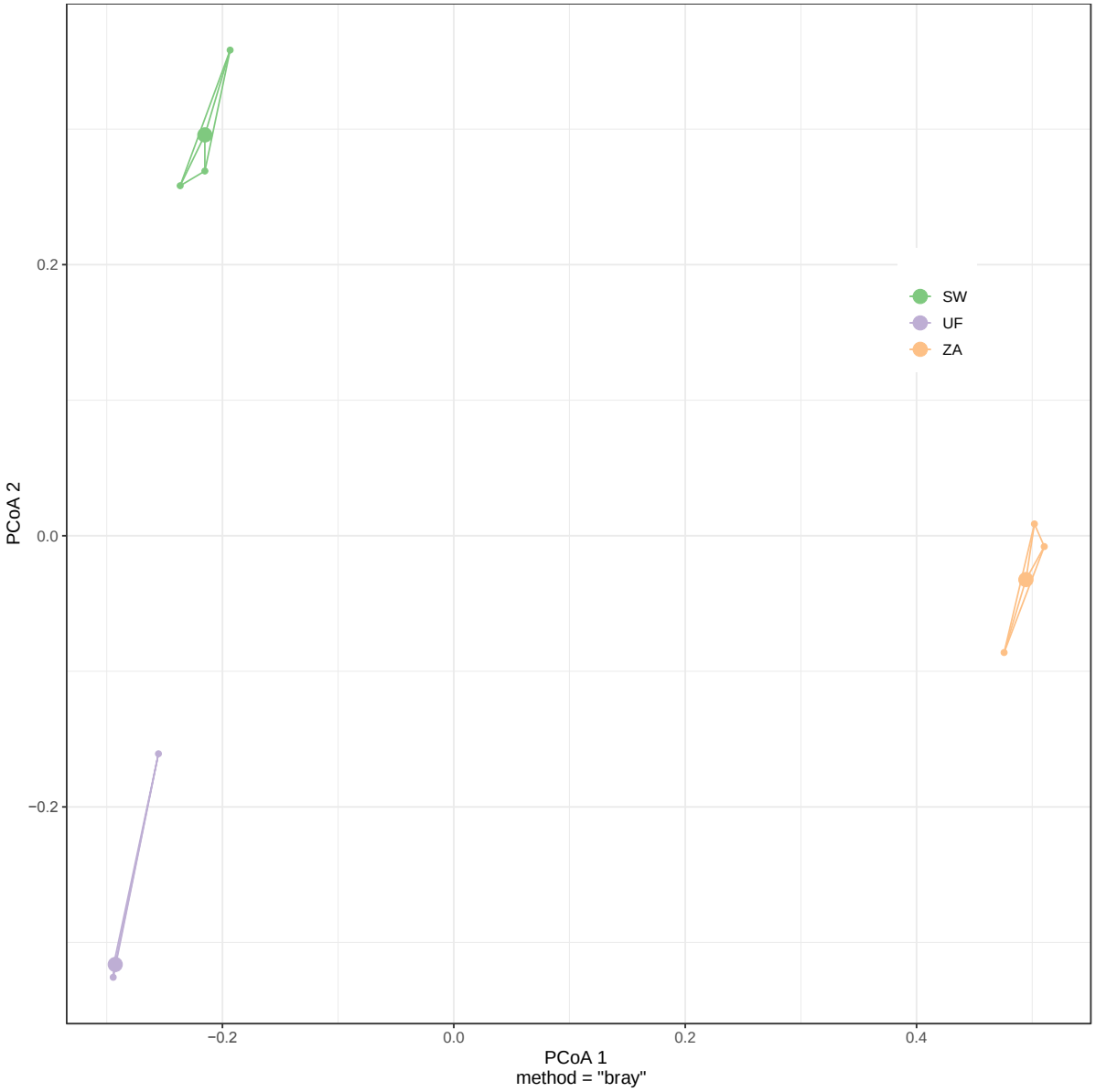

Figure 3a: Relative abundance and phylum identity of the top 20 bacterial species of all ostracod samples based on the V3 endosymbiotic data.
Different phyla are indicted by different colours.
OST = ostracod; SED = sediment; WAT = water; SW = Semerwater; UF = Ullal Fosc; ZA = Zaventem. Numbers indicate replicates from the same study site.

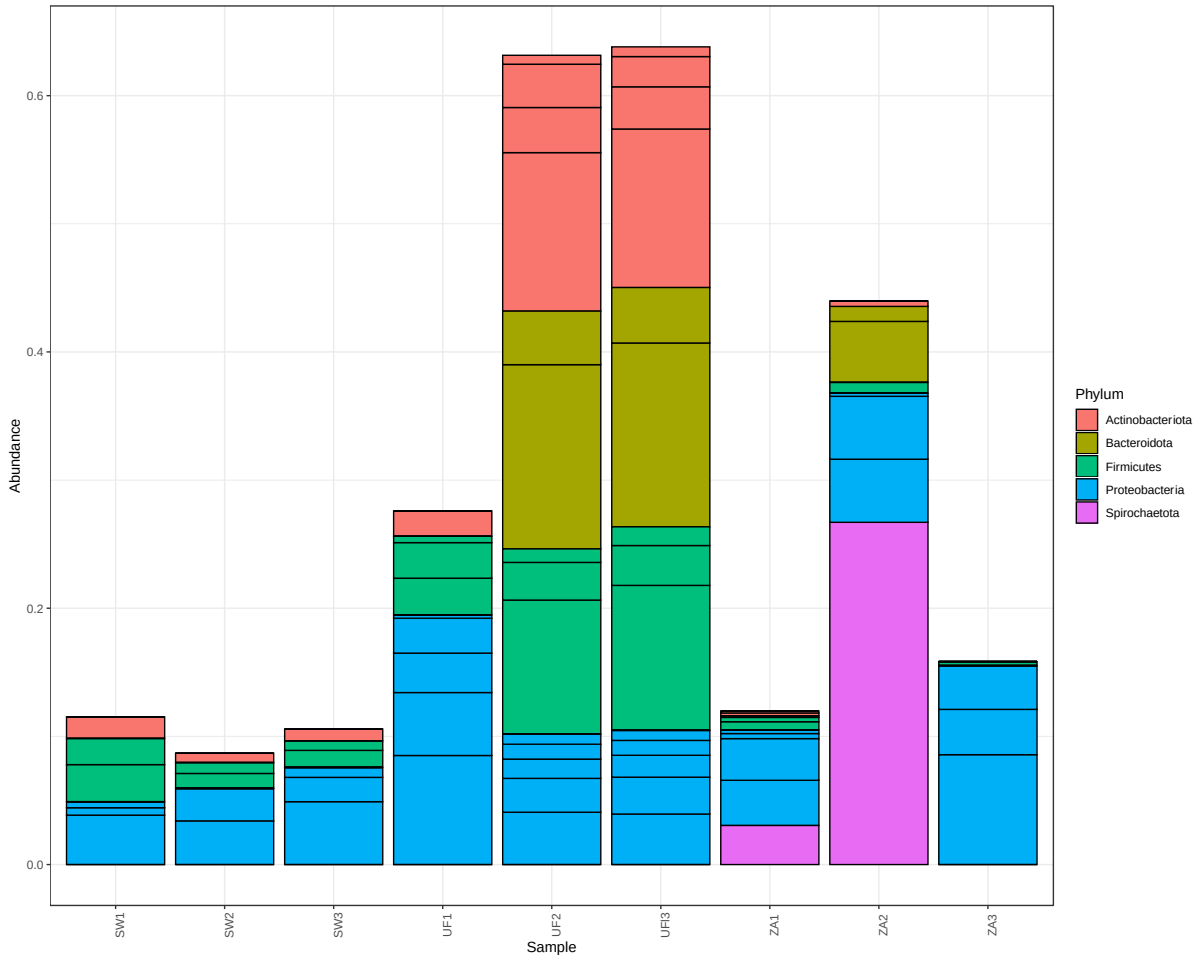

Figure 3b: Relative abundance and genus identity of the top 20 bacterial species of all ostracod samples based on the V3 endosymbiotic data.
Different genera are indicted by different colours.
OST = ostracod; SED = sediment; WAT = water; SW = Semerwater; UF = Ullal de Fosc; ZA = Zaventem. Numbers indicate replicates from the same study site.

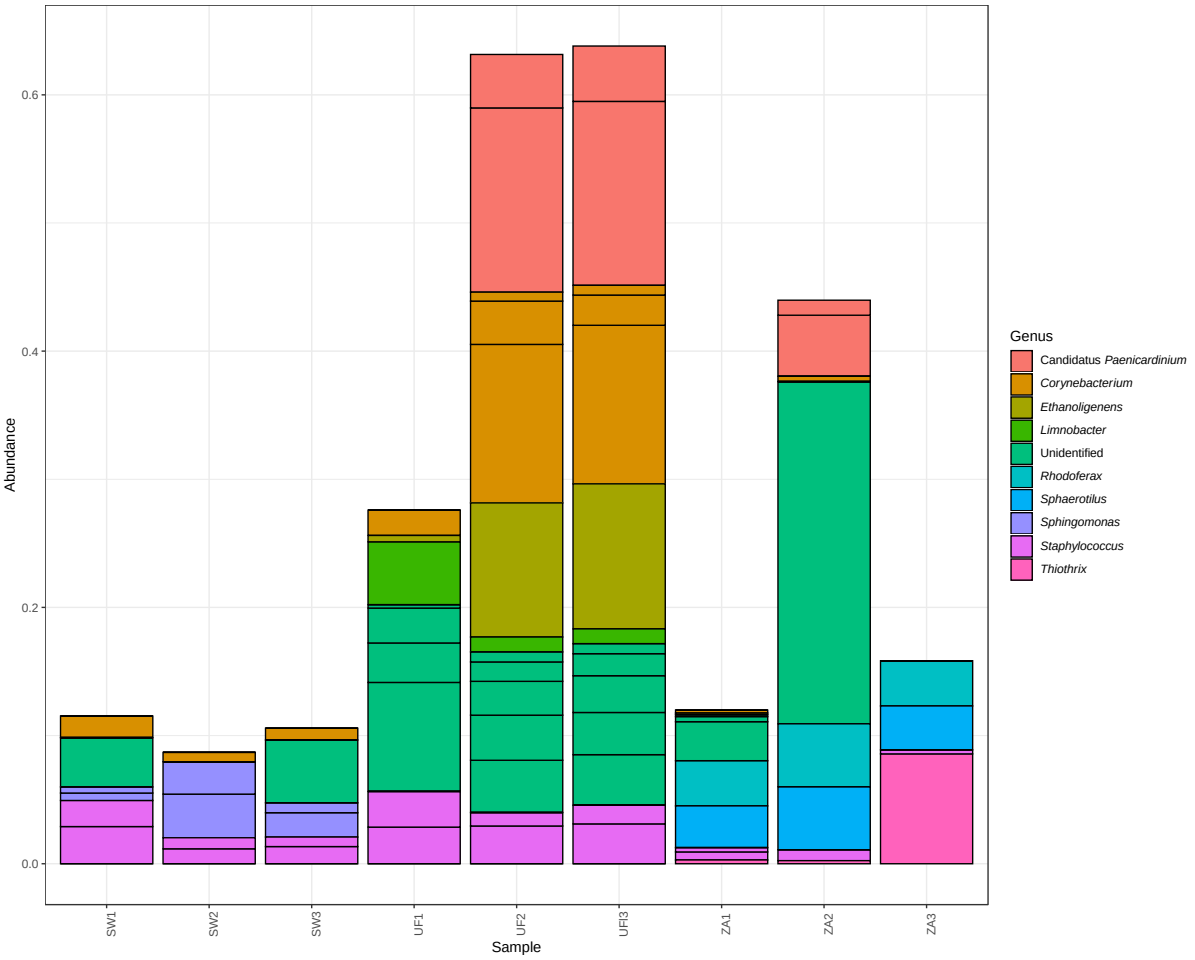

Table 1a: Overview of sequencing reads with quality score >30 of different regions of the bacterial 16S gene.

V3 = region V3-V4 of bacterial 16S gene. V3 endosymbionts = V3 region of bacterial 16S gene with specific primers for ostracod endosymbionts. Nr, nr = number. reads refers to sequencing reads. Min = minimum; Max = maximum; Avg = average. ASV = Amplicon Sequence Variant

SampleType	Nr of samples	Total nr of reads	Min nr of reads	Max nr of reads	Median nr of reads	Avg nr of reads	Total nr of ASVs
V3 all samples	33	932,172	1,311	114,038	17,328	28,248	13,122
V3_sediment	12	422,927	1,286	112,002	21,369	35,243	5,791
V3_water	12	304,853	5,931	88,195	11,648	25,404	5,799
V3_ostracods	9	195,830	11,197	44,278	21,169	21,759	3,635
V3_endosymbionts	9	107,714	8,993	17,734	10,839	11,968	1,779
V3_ostracods from Ullal Fosc	12	211,865	11,136	32,182	15,353	17,655	1,783

Table 1b: Overview of ASVs and their taxonomy as identified with dada2.
V3 = region V3-V4 of bacterial 16S gen. V3 endosymbionts = V3 region of bacterial 16S gene with specific primers for ostracod endosymbionts. ASV = Amplicon Sequence Variant. Nr, nr = number. % are calculated from total nr of ASVs.

SampleType	Nr of samples	Total nr of ASVs	ASVs identified as chloroplasts	ASVs identified as mitochondria
V3 all samples	33	13,122	1.60%	0.06%
V3_sediment	12	5,791	2.28%	0.03%
V3_water	12	5,799	1.35%	0.05%
V3_ostracods	9	3,635	2.14%	0.18%
V3_endosymbionts	9	1,779	2.47%	0.16%
V3_ostracods Ullal Fosc	12	1,783	2.30%	0.22%

Table 2: Results of the PERMANOVA analyses after data normalization with Total Sum Scaling.
V3 = V3 data set; V3 (endosymbionts) = V3 endosymbiotic data set; ZA=Zaventem; UF = Ullal Fosc; SW= Semerwater; df = degrees of freedom. SS = Sum of Squares; MS = Mean Sum of Squares; R^2 = R^2 statistic. p-value_bonf_corr= p value after Bonferroni correction.

V3 (all)	**df**	**SS**	**MS**	**R^2**	**Pseudo-F**	**p-value**
SampleType	1	1.2322	1.2322	0.1072	3.5209	0.001
Locality	2	1.7708	0.8854	0.1541	2.5301	0.001
SampleType:Locality	2	1.4906	0.7453	0.1297	2.1297	0.001
Residual	20	6.9991	0.3491	0.6090		
Total	25	11.4927	0.4597	1		

V3 (only ostracods)	**df**	**SS**	**MS**	**R^2**	**Pseudo-F**	**p-value**
Locality	2	1.6054	0.8027	0.5664	3.9186	0.004
Residual	6	1.2291	0.2048	0.4336		
Total	8	2.8345	0.3543	1		

V3 endo

group1	**group2**	**R^2**	**Pseudo-F**	**df1**	**df2**	**p-value**	**p-value_bonf_corr**
Semerwater	Ullal Fosc	0.505	3.059	1	3	0.1	0.2
Semerwater	Zaventem	0.562	2.570	1	2	0.333	0.666
Ullal Fosc	Zaventem	0.605	4.591	1	3	0.1	0.2

Table 3a: Results of the IndVal analyses testing for indicative taxa of all sample types based on the V3 dataset.
Seq numbers can be linked to ASVs with the information in Tables S2 and S3 (supplementary material). group 1 = Semerwater; group 2 = Ullal Fosc; group 3 = Zaventem. pvalue_fdr = corrected p values for multiple comparisons with fdr method. "none" means that the sequence cannot be identified to this taxonomic level.

	group	indval	pvalue	pvalue_fdr	freq	Phylum	Class	Order	Family	Genus	Species
seq18	1	0.636363636363636	0.004	0.005	7	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	Bradyrhizobium	none
seq66	1	0.636363636363636	0.003	0.005	7	Proteobacteria	Gammaproteobacteria	Burkholderiales	Chitinibacteraceae	Deefgea	none
seq86	1	0.636363636363636	0.004	0.005	7	Proteobacteria	Gammaproteobacteria	Burkholderiales	Chitinibacteraceae	Deefgea	none
seq105	2	0.491167192429022	0.005	0.005	4	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiales Incertae Sedis	none	none
seq133	1	0.616314536527302	0.005	0.005	8	Proteobacteria	Gammaproteobacteria	Burkholderiales	Chitinibacteraceae	Deefgea	chitinilytica
seq145	1	0.636363636363636	0.004	0.005	7	Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	none	none
seq393	3	0.666666666666667	0.003	0.005	6	Proteobacteria	Gammaproteobacteria	Burkholderiales	none	none	none
seq577	2	0.5	0.004	0.005	3	Spirochaetota	Spirochaetia	Spirochaetales	Spirochaetaceae	Treponema	none
seq686	3	0.555555555555556	0.004	0.005	5	Proteobacteria	Gammaproteobacteria	Burkholderiales	Rhodocyclaceae	Dechloromonas	none
seq1395	3	0.555555555555556	0.005	0.005	5	Spirochaetota	Leptospirae	Leptospirales	Leptospiraceae	RBG-16-49-21	none

Table 3b: Results of the IndVal analyses testing for indicative bacterial taxa of ostracods based on the V3 ostracod data.
Seq numbers can be linked to ASVs with the information in Tables S2 and S3 (supplementary material). group 1 = Semerwater; group 2 = Ullal Fosc; group 3 = Zaventem. pvalue_fdr = corrected p values for multiple comparisons with fdr method. "none" means that the sequence cannot be identified to this taxonomic level.

	group	indval	pvalue	pvalue_fdr	freq	Phylum	Class	Order	Family	Genus	Species
seq28	2	0.978187919463087	0.023	0.032	4	Proteobacteria	Gammaproteobacteria	Burkholderiales	Chitinibacteraceae	Deefgea	chitinilytica
seq36	3	1	0.032	0.032	3	Bacteroidota	Bacteroidia	Chitinophagales	Chitinophagaceae	Asinibacterium	none
seq37	3	1	0.03	0.032	3	Proteobacteria	Gammaproteobacteria	Burkholderiales	none	none	none
seq43	2	0.831649831649832	0.022	0.032	5	Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	none	none
seq55	1	1	0.031	0.032	3	Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	none	none
seq66	3	1	0.029	0.032	3	Proteobacteria	Gammaproteobacteria	Burkholderiales	Burkholderiaceae	Polynucleobacter	asymbioticus
seq81	3	1	0.031	0.032	3	Proteobacteria	Gammaproteobacteria	Burkholderiales	Burkholderiaceae	Polynucleobacter	none
seq388	1	1	0.028	0.032	3	Bacteroidota	Bacteroidia	Cytophagales	Spirosomaceae	Lacihabitans	soyangensis
seq483	3	1	0.024	0.032	3	Firmicutes	Clostridia	Peptostreptococcales-Tissierellales	Fusibacteraceae	Fusibacter	none
seq499	3	1	0.029	0.032	3	Bacteroidota	Bacteroidia	Bacteroidales	Dysgonomonadaceae	none	none

Table 3c: Results of the IndVal analyses testing for indicative bacterial taxa of ostracods based on the V3 endosymbiotic data.
Seq numbers can be linked to ASVs with the information in Tables S2 and S3 (supplementary material). group 1 = Semerwater; group 2 = Ullal Fosc; group 3 = Zaventem. pvalue_fdr = corrected p values for multiple comparisons with fdr method "none" means that the sequence cannot be identified to this taxonomic level.

	group	indval	pvalue	pvalue_fdr	freq	Phylum	Class	Order	Family	Genus	Species
seq4	2	0.991725015913431	0.028	0.028	4	Proteobacteria	Gammaproteobacteria	Burkholderiales	Oxalobacteraceae	none	none
seq5	2	1	0.026	0.028	3	Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	none	none
seq7	2	0.678571428571429	0.025	0.028	6	Proteobacteria	Gammaproteobacteria	Burkholderiales	Burkholderiaceae	Limnobacter	thiooxidans
seq28	2	1	0.026	0.028	3	Bacteroidota	Bacteroidia	Flavobacteriales	Weeksellaceae	none	none
seq32	2	1	0.02	0.028	3	Proteobacteria	Alphaproteobacteria	Azospirillales	Azospirillaceae	Azospirillum	none
seq34	2	0.954430379746835	0.022	0.028	4	Fusobacteriota	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	none	none
seq91	2	1	0.025	0.028	3	Firmicutes	Clostridia	Peptostreptococcales-Tissierellales	Family XI	Anaerococcus	vaginalis
seq191	2	1	0.025	0.028	3	Firmicutes	Clostridia	Oscillospirales	Ethanoligenenaceae	Ethanoligenens	none
seq254	2	1	0.025	0.028	3	Fusobacteriota	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	none	none
seq339	2	1	0.027	0.028	3	Firmicutes	Bacilli	Staphylococcales	Staphylococcaceae	Staphylococcus	none

Table 4: Summary of predicted functions of ostracod microbiomes according to the KEGG pathway database.
The top 100 predicted pathways were ranked by their maximal means in the heatmap of the V3 data set (Figure S7a).

Function	% of top 100 pathways
Amino acid metabolism	25
Nucleotide and ribonucleotide metabolism	25
Carbohydrate sugar metabolism	17
Lipid metabolism	15
Metabolism of co-factors and vitamins	10
Energy metabolism	2
Secondary metabolisms	1
Carbon fixation	1
Glycan metabolism	1