**Title:** Microniche sampling of the microbiome in the Starlet Sea Anemone, *Nematostella vectensis*, reveals a compartment-specific dominance of Spirochetes

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## Abstract

Microniche sampling can reveal highly specialized bacterial associations within the holobiont and also facilitate identification of core microbial symbionts that would otherwise be overlooked by bulk sampling methods. We characterized compartment-specific associations present within the model cnidarian *Nematostella vectensis* by dividing its morphology into three distinct body regions. This sampling design allowed us to uncover a compartment-specific dominance of spirochetes within the capitulum of *N. vectensis*. Bacteria from the Spirochaetaceae family made up 65.8% of the community in the capitulum, while only representing 1.2% and 0.1% of the communities in the mesenteries and physa, respectively. The capitulum community also exhibited significantly lower alpha-diversity compared to the other two compartments. A phylogenetic analysis of the predominate spirochete sequence recovered from *N. vectensis* showed a close relation to spirochetes previously recovered from wild *N. vectensis*. These sequences clustered closer to the recently described *Oceanispirochaeta*, rather than *Spirochaeta perfilievii*, supporting them as members of this genus. This suggests a consistent and potentially important association between *N. vectensis* and spirochetes from the Spirochaetales family.


## Introduction

The discovery of microniche specific microbial communities has become important for our understanding of microbial interactions and diversity (Ainsworth, Thurber and Gates 2010; Bourne, Morrow and Webster 2016). Microbial niches have been identified in many different organisms from an ecosystem scale, all the way down to the cellular level (Aagaard *et al.* 2014; Foster *et al.* 2017; Ricci *et al.* 2019). For example, in the Giant Clam, *Tridacna maxima*,



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different tissues harbor distinct microbial communities, with the gills harboring a high prevalence of *Endozoicomonas* bacteria. Compartmental evaluation of *T. maxima* helped uncover *Endozoicomonas*'s role in nitrogen-cycling within the holobiont (Rossbach 2019). The microniches within animal's is proposed to strongly influence the composition of the microbial community (Vaishnava *et al.* 2008; Hooper, Littman and Macpherson 2012). Consequently, the specific composition of each microniche can provide clues as to the community's functional contribution to host-physiology (Ainsworth, Thurber and Gates 2010).

In the Phylum Cnidaria (sea anemones, corals etc.) many species are known to associate with different symbiotic microbial partners, including bacteria, and these relationships are known to be important for the health of the cnidarian host (Rädecker *et al.* 2015; Bourne, Morrow and Webster 2016; Robbins *et al.* 2019). Previous studies have identified that whole organism sampling methods used for microbiome analysis can skew results (Sweet, Croquer and Bythell 2011). An advantage of separating an organism into microniches is that it can facilitate the identification of core microbial symbionts in different compartments (Apprill, Weber and Santoro 2016). Microorganisms inhabiting specific compartments within a cnidarian host, such as the epithelium and gastrodermal tissue, likely represent highly specialized symbiotic associations (Bourne, Morrow and Webster 2016). However, in the model sea anemone, *Nematostella vectensis* (Phylum: Cnidaria), the presence of microniches of microbial communities has not previously been identified.

*N. vectensis* is an estuarine sea anemone found in salt marshes along both the Atlantic and Pacific coastlines of North America (Har 2009). They are diploblastic (i.e., only two embryonic cell layers) and possess a single oral opening that is surrounded by 12 to 16 tentacles (Renfer *et al.* 2010). They have a clearly defined pharynx and mesenteries that run along the



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oral-aboral axis and a physa at the aboral end used for digging (Layden *et al.* 2010). *N. vectensis* has rapidly gained popularity as a model organism because it is easy to rear in the lab, simple to manipulate, and has a wide range of available genomic resources (Putnam *et al.* 2007; Reitzel, Ryan and Tarrant 2012).

Microbiome analyses through 16S rRNA high-throughput sequencing have increased our understanding of the microbial community diversity within many organisms including *N. vectensis.* In cnidarians, the microbiome is crucial for nutrient cycling, defense against diseases, thermoregulation, and the ability to rapidly adapt to environmental stressors (Rosenberg *et al.* 2007; Mortzfeld *et al.* 2016). A previous study conducted comprehensive 16S rRNA gene sequencing, gene expression analysis, and whole genome sequencing of associated bacteria from *N. vectensis* (Har *et al.* 2015). This work identified 11 bacterial phyla that were commonly associated in both wild caught and lab reared *N. vectensis*. These included Cytophaga-Flexibacter-Bacteroides, Chloroflexi, Cyanobacteria, Deferribacteres, Firmicutes, OD1, Planctomycetes, Proteobacteria, Spirochetes, Tenericutes and Verrucomicrobia (Har *et al.* 2015). The microbiome of *N. vectensis* can also be dynamic, with diel lighting and developmental stage heavily influence microbial community composition (Mortzfeld *et al.* 2016; Leach, Carrier and Reitzel 2019). However, the potential role of these bacteria within *N. vectensis* is still not fully understood. The current information on *N. vectensis* suggests that 1) the *N. vectensis* microbiome can be a highly dynamic and complex community, 2) there are external cues that may affect the microbiome community, and 3) the specific contribution of certain bacterial taxa may be overshadowed by bulk sampling methods.

In this study, we distinguished the microbial microniches present within the *N. vectensis* by using 16S rRNA amplicon sequencing of the three body compartments: the capitulum

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(pharynx + tentacles), mesenteries, and physa (Fig. 1). We found that the capitulum possesses significantly different bacterial community abundances compared with the mesenteries and physa and that spirochetes are the dominant bacteria phylum present in the capitulum. Spirochetes were previously identified in *N. vectensis*; however, their compartment specific nature was not understood. This work better clarifies our understanding of bacteria community diversity in cnidarians and sheds light on the importance of compartmental sampling in microbiome studies.

## Methods

### *Nematostella Vectensis Culture and Care*

The individuals used for this study are general cultured animals that have been housed at the Rosenstiel School of Marine and Atmospheric Science (RSMAS) Cnidarian Immunity Lab since 2016. All animals were kept in the dark in covered, glass dishes filled with non-circulating 11 parts per thousand (ppt), 2-micron filtered seawater (FSW) at room temperature (~23.8˚C). Two months prior to the beginning of this study, 30 *N. vectensis* adults were placed into a single glass dish and fed mussels 3 days a week, and *Artemia* 5 days a week for 2 months in order to increase their size. Water changes were conducted an hour after each mussel feeding. One week before beginning the trisection, 12 of the largest individuals were transferred individually into a 12-well plate with sterilized FSW and starved for seven days to minimize microbial carryover from food.

### *Trisection*

The 12 adult *N. vectensis* were anesthetized using 7% $MgCl_2$ solution and then aseptically trisected. The trisection resulted in the isolation of three tissue compartments from

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each anemone: (1) capitulum (pharynx and tentacles), (2) mesenteries, and (3) physa (Fig. 1) The scalpel and tweezers were both flame and ethanol sterilized between tissue divisions. Each cut was made as cleanly as possible to limit any cross-contamination of tissue compartments. The capitulum was removed first, followed by the mesenteries, then the physa. The cut for the capitulum and mesentery boundary was established as just below the visible pharynx. Some physa tissue was found in the mesentery compartment as the mesenteries were not directly removed from the body cavity. However, the physa compartment did not contain any mesentery material. Each sample was then stored in a 2 mL centrifuge tube with RNALater™ (Sigma Aldrich, St. Louis, MO) and placed in a -80°C freezer.

*16S rRNA PCR and Sequence Library Preparation*

Total DNA was extracted from 36 samples using the Qiagen™ DNeasy Powersoil Kit. The concentration and quality of the DNA extracts were assessed using a Nanodrop™ spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). DNA was then stored in a -80°C freezer until library preparation. High-throughput 16s rRNA amplicon sequencing methods were adapted from the protocol developed by the Earth Microbiome Project (Thompson *et al.* 2017). In short, the V4 region of the 16S rRNA gene (515F-806R primers) was amplified using polymerase chain reaction (PCR) on 18 tissue samples (Apprill *et al.* 2015). A negative control was also amplified to account for extraction and PCR contamination. A total of 50 µL of PCR reaction mixtures were prepared according to the following recipe: 23 µL PCR-grade water, 20 µL PCR master mix (2x), 1 µL 806R primer (10 µM), 1 µL barcoded 515F primer (10µM), and 5 µL template DNA. PCR was performed with the following program: 1 x 3 min at 94°C, 35 x (45 s at 94°C, 60 s at 50°C, 90 s at 72°C), 1 x 10 min at 72°C. Amplified DNA was stored at -

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20°C for one week. The PCR product was run on a 1.5% agarose gel to check for correct size amplification. Following the gel, the 16S rRNA V4 amplicon was cleaned using AMPure XP Beads™ (Beckman Coulter Inc., Brea, CA). DNA concentrations were quantified using the Qubit dsDNA high sensitivity (HS) kit™ (Thermo Fisher Scientific Inc., Waltham, MA) and samples were diluted to 4 nM using PCR-grade water. Then 5 µL of each sample was pooled into a single 1.5 mL tube. The pooled DNA library was sent for sequencing on an Illumina MiSeq™ PE300 sequencer at the Center for Genome Technology (CGT) at the University of Miami's Miller School of Medicine for quality control and sequencing. Raw Illumina paired-end reads have been deposited in the NCBI Sequence Read Archive (SRA) database under BioProject PRJNA610635.

*Post-Sequencing Processing and Statistical Analysis*

Post-sequencing processing was conducted using the Quantitative Insights in Microbial Ecology 2 (QIIME2) v2019.1 pipeline (Bolyen *et al.* 2019). Demultiplexed paired-end reads were merged using DADA2 v1.10.0 and processed to yield amplicon sequence variants (ASVs) (Callahan *et al.* 2016). Within the DADA2 pipeline, forward sequences were truncated at 200 bp and reverse sequences were truncated at 180 bp. Taxonomy was assigned using the SILVA 132 99% classifier (https://data.qiime2.org/2019.10/common/silva-132-99-nb-classifier.qza) (Quast *et al.* 2013). Mitochondria and chloroplast sequences were filtered from the feature table. The Phyloseq R package v1.28.0 was used for data handling (McMurdie and Holmes 2013). Normalization across samples was conducted using a variance stabilizing transformation (VST) on the data using DESeq2 v1.24.0 (Love, Huber and Anders 2014). To account for abundance and evenness of microbial taxa within the VST dataset, the Shannon-Weiner Diversity Index and

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Simpson's Diversity Index were calculated for each compartment region using Phyloseq. DESeq2 v1.24.0 and the QIIME2 add-on, Analysis of Composition of Microbiomes (ANCOM), were used for microbial differential abundance analysis against tissue-compartments and differences were considered significant if they had a p-value < 0.05. Random forest classifier modeling was performed using randomForest in order to determine the main bacterial taxa driving tissue compartment composition (Liaw and Wiener 2002). For beta-diversity measurements, both Bray-Curtis and Aitchison (Euclidean distances of center-log ratio transformed counts) distance matrices were assessed as both are commonly used in microbial community studies. For Bray-Curtis distances, the VST-transformed data was used. For Aitchison distances, the R package Microbiome v1.6.0 was used to perform a center-log ratio transformation on the raw count data (Lahti *et al.* 2019). Vegan v2.5.6 was used to calculate Bray-Curtis and Euclidean distances and the *Adonis* function was used to perform PERMANOVA analyses in order to statistically assess dissimilarity in microbial community composition between tissue compartments (Oksanen *et al.* 2013). Ampvis2 v2.5.7 was used to generate a heat-map of the most abundant bacterial families according to tissue compartment (Andersen *et al.* 2018). All associated code can be found on Github: https://github.com/Abonacolta/Nvec_Compartmentalization.

*Mapping N. vectensis ASVs to a phylogenetic tree of Spirochetes*

Spirochaetae phylum 16s rRNA sequences were retrieved from NCBI for representatives from every major lineage in FASTA format. Using BLASTn, closely clustering (97%) sequences to the two spirochete ASVs recovered in this study were added to the FASTA file along with a Planctomycete sequence (Accession: X56305) as an outgroup, giving a total of 64 sequences.



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These sequences were aligned using MAFFT v7.450 (auto-setting) with the L-INS-i strategy (Katoh and Standley 2013). The alignment was trimmed to remove columns with gaps in more than 70% of the sequences or with a similarity score below 0.001 using TrimAl v1.2 (Capella-Gutiérrez, Silla-Martínez and Gabaldón 2009). A maximum-likelihood tree was constructed with RAxML v7.3.0 using the GTRCAT substitution model and 1000 sub-samplings to generate a best-fit tree (Stamatakis 2014). The final tree was visualized and refined in FigTree v 1.4.4 (Drummond *et al.* 2012).

## Results

*Spirochetes dominate the capitulum of the Nematostella vectensis microbiome*

The microbial composition of the capitulum (n= 6) is distinct from the mesenteries (n= 6) and physa (n= 6; Fig. 1). Spirochetes dominated the capitulum, while Proteobacteria dominated the mesenteries and physa (Fig. 1). In the mesenteries a larger prevalence of Gammaproteobacteria was found (Fig. S2). The physa of *N. vectensis* was dominated by the classes Gammaproteobacteria, Alphaproteobacteria, and Bacteroidetes (Fig. S2). Of the Gammaproteobacteria which dominated the mesenteries and physa compartments, Oceanospirillales and Pseudomonadales were the primary orders, with Pseudomonadales being more abundant than Oceanospirillales (Fig. 2). The relative abundance of the top nine most abundant bacterial families revealed a capitulum-specific dominance of Spirochaetaceae bacteria (~66%), with a high abundance of Pseudomonadaceae in the mesenteries and physa (~36% and ~38%, respectively) (Fig. 3). At the family-level, Pseudomonadaceae, Rhodobacteraceae, Alteromonadaceae, and Saccharospirillaceae bacteria made up the majority of amplicon

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sequence variants (ASVs) in the physa (Fig. S3). The mesenteries showed similar bacterial community composition as the physa, with the exclusion of Alteromonadaceae (only appearing in >5% abundance in just one individual) (Fig. S3). Overall the microbiomes were consistent between replicates, however, one individual harbored a different compartmental microbiome from the others in this study (Fig. S1).

Comparing the differential abundance of taxa across tissue compartments using a random forest classifier showed that the phyla Lentisphaerae, Proteobacteria, and Spirochetes were the strongest predictors of tissue compartments. According to differential abundance analysis, Spirochetes and Fusobacteria were significantly more abundant in the capitulum compared to the other compartments (DESeq2; p-adj=0.0000 and 0.0003, respectively). Lentisphaerae, Proteobacteria, and Chloroflexi were more abundant in the mesenteries compared to the capitulum (DESeq2; p-adj= 0.0000, 0.0058, and 0.0009, respectively). According to differential abundance analysis using ANCOM, Spirochetes and Bacteroidetes were the most differentially abundant phyla among tissue compartments (ANCOM; W = 16 & 14, respectively).

*Alpha-diversity metrics differed significantly between the capitulum and other compartments*

Alpha-diversity metrics showed a significant difference in the biodiversity and species richness present in different tissue compartments. Species richness among the capitulum and the mesenteries and physa were significantly different (ANOVA; p = 0.023, p = 0.033, respectively; Fig. 4). The Simpson's Index between the capitulum and the mesenteries and physa was also significantly different (ANOVA; p = 0.039, p = 0.022, respectively; Fig. 4). However, the Shannon Diversity Index did not show a significant difference in alpha-diversity among body regions (Fig. 4). Overall, the capitulum of *N. vectensis* showed lower species richness compared

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to the mesenteries and physa, which had very similar species richness across individuals. A similar pattern was identified in the Simpson's Index, with the capitulum showing a lower species diversity, but with a higher variation between individuals.

*Beta diversity metrics inconclusive regarding the significance between tissue compartments*

Both Bray-Curtis Dissimilarity and Aitchison distance were used to assess the differences in the microbial communities between tissue compartments (Fig. 5). A multivariate homogeneity of groups dispersions analysis confirmed that an ANOVA test could appropriately be performed on both datasets (Koleff, Gaston and Lennon 2003). Bacterial communities between tissue compartments showed significant dissimilarity (PERMANOVA; permutations = 9999, p = 0.0315) according to the Bray-Curtis Dissimilarity Index. However, the same PERMANOVA test on the Aitchison distances found insignificant dissimilarity between tissue compartments (PERMANOVA; permutations = 9999, p = 0.3294).

*Phylogenetic analysis of the Spirochete ASVs*

The majority of spirochetes found in the capitulum was from a single ASV (Spirochete 1) within the Spirochaetales family. This ASV along with the other spirochete ASV (Spirochete 2) recovered in this study were mapped to a maximum-likelihood tree of representative Spirochaetae sequences. Strong bootstrap support (93% bootstrap) placed Spirochete 1 as closely related to four spirochete ASVs recovered from wild *N. vectensis* from Har *et al.* 2015 and to a spirochete ASV recovered from the cold-water coral, *Lophelia pertusa* (Fig. 6). Together, these sequences formed a strongly supported (90%) monophyletic clade with the two representative species of the recently described *Oceanispirocheata* genus. Spirochete 2, along with several

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closely clustering wastewater ASVs, showed strong relation (100% bootstrap) to *Turneriella parva*. Interestingly spirochetes associated with a diverse group of corals, including *Corallium rubrum, Orbicella faveolata, and Lophelia pertusa,* did form a well-supported (100% bootstrap) monophyletic clade within the *Leptospira* genus.

**Discussion**

In this study, spirochetes (predominantly from 1 ASV) within the Spirochaetaceae family made up over ~65% of the bacterial community within the capitulum of *N. vectensis*, but only ~1% of the community in the mesenteries and physa (Figs. 1 & 3). Although spirochetes are known to be one of the most abundant phyla within the microbiome of *N. vectensis* (Mortzfeld *et al.* 2016), their compartment-specific distribution within the microbiome was masked by whole organism sampling and the high prevalence of Proteobacteria. The differences in dominant taxa between the capitulum and the other two tissue compartments in *N. vectensis* highlight the need to use compartmentalization to better understand the roles of the microbiome within the holobiont- be that in metabolism, defense, or nutrient cycling. This finding is similar to another microbiome compartmentalization study of the Crown-of-thorns Starfish, *Acanthaster planci*, which found a high relative abundance (43-64%) of spirochetes (predominantly from 1 OTU) within body wall samples. Prior to this Alphaproteobacteria was thought to be the most abundant phylum of bacteria across the sea stars (Høj *et al.* 2018).

Spirochetes are gram-negative, organo-chemoheterotrophs known to attach to each other and to other live cells, commonly as epibionts (Wier *et al.* 2010). They have been identified as symbionts in bivalves, corals, oligochaetes, and termites (as epibionts on intestinal protists)


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where they perform reductive acetogenesis and nitrogen fixation (Margulis, Nault and Sieburth 1991; Lilburn, Schmidt and Breznak 1999; Campbell and Cary 2001; Ohkuma *et al.* 2015). In cnidarians, spirochetes are the predominant bacteria of the red coral, *Corallium rubrum*, and the octocoral, *Lobophytum pauciflorum,* where they are hypothesized to play a significant role in nitrogen fixation and even microbial community structuring through antibiotic secretion (Van De Water *et al.* 2016; Wessels *et al.* 2017). They have also been identified in *Hydra vulgaris,* predominately in transparent vacuoles at the apical end of epitheliomuscular cells (Hufnagel and Myhal 1977). The predominance of spirochetes as symbionts in some cnidarians suggests a potential conserved benefit to the Cnidaria hosts. While we do not know why there is a consistent, and highly specific compartment abundance of spirochetes in the capitulum of *N. vectensis,* we hypothesize that it may play a significant role in the host physiology. In the future, testing the capabilities of spirochetes to perform nitrogen fixation in the capitulum of *Nematostella* may inform our understanding of these potential symbionts.

In order to better understand the phylogenetic placement of the spirochetes within *N. vectensis,* we conducted a phylogenetic analysis of our ASVs with representative sequences from every major lineage within Spirochaetae. The predominant spirochete ASV (Spirochete 1) recovered from the capitulum of *N. vectensis* is part of a strongly supported monophyletic clade of spirochete sequences recovered from wild *N. vectensis* supporting this spirochete as a consistent associate of the anemone (Fig. 6). The potential role of the spirochetes within the capitulum of *N. vectensis* should be investigated further as this relationship appears to be conserved, regardless of environment. These *N. vectensis* ASVs also showed a strong relation to a spirochete recovered from the cold-water coral, *Lophelia pertusa*. Together, these sequences clustered closer to the recently described *Oceanispirochaeta*, rather than *Spirochaeta perfilievii*,



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supporting them as members of this genus. The *Oceanispirochaeta* consists solely of two representatives, *O. litoralis* and *O. sediminicola*, which are gram-negative, moderately halophilic, and obligately anaerobic (Subhash and Lee 2017). Spirochete 2, along with various wastewater associated spirochetes, formed a monophyletic clade with *Turneriella parva* (Fig. 6). *Turneriella parva* is a gram-negative, obligately aerobic spirochete with morphological similarities to *Leptospira* (Stackebrandt *et al.* 2013). Interestingly, a clade of cnidarian-associated spirochetes clustered closely within the *Leptospira* genus (Fig. 6). Despite the broad geographical ranges of these coral species (*O. faveolata* is a Caribbean species*, C. rubrum* is a Mediterranean species, & *L. pertusa* is a deep sea species), they all share an association with *Leptospira*. The potential role of these cnidarian associated *Leptospira* species should be investigated further.

Other abundant phyla of the *N. vectensis* microbiome revealed in this study include Proteobacteria, Actinobacteria, Firmicutes, and Tenericutes (Fig 1). In previous studies on the microbiome of lab-raised *N. vectensis*, Proteobacteria was found to be the most abundant bacterial phylum, with Firmicutes, Planctomycetes, Tenericutes, Verrucomicrobia, and Spirochetes having lower, but still significant, abundances (Har *et al.* 2015; Mortzfeld *et al.* 2016; Leach, Carrier and Reitzel 2019). In particular, Gammaproteobacteria have been shown to play an important role in microbial community structuring in juvenile *N. vectensis* (Domin *et al.* 2018). Additionally, fluorescent *in situ* hybridization revealed that Proteobacteria and Pseudomonas occur predominantly in aggregates along the walls of the mesenteries (Har 2009). We found a similar pattern of compartmentalization in our study, where only the physa and mesentery tissue compartments had a high abundance of Proteobacteria (Orders: Pseudomonas and Oceanospirillales). These two compartments also showed significantly higher alpha-diversity


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compared to the capitulum, most likely due to the dominance of the singular Spirochete ASV in the capitulum. One of our beta-diversity analyses also supported our hypothesis that microbial communities differed significantly between tissue compartments, with the capitulum harboring a notably distinct microbial community from the mesenteries and physa.

Interestingly, one study found that as *N. vectensis* develops, Betaproteobacteria, Actinobacteria, and Bacteroidetes decrease, while Spirochetes become one of the most abundant phyla in the adult organism (Mortzfeld *et al.* 2016). The development and growth of the capitulum during this time span may explain the increase in abundance of spirochetes in the adult organism compared to its earlier developmental stages. Spirochetes may play an active role in the capitulum development of *N. vectensis* or may preferentially thrive in this tissue compartment over other bacteria. Their ability to establish dominance over the Proteobacteria present throughout the rest of *N. vectensis* implies a competitive advantage on the part of the Spirochete.

In this study we characterized the microbiome of three distinct body compartments within *N. vectensis*. A capitulum-specific dominance of spirochetes was uncovered highlighting the possible importance of this bacteria in *N. vectensis* physiology. Previous bulk-sampling techniques could not untangle compartment-specific bacterial associations, which should be taken into consideration in future cnidarian studies. Specifically, the role of spirochetes within the capitulum of *N. vectensis* should be further considered in subsequent studies. Overall, our findings further establish spirochetes as consistent bacteria associates within *N. vectensis* and suggests that the functional role of this bacteria in the capitulum may be important for the anemone.

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**References**

Aagaard K, Ma J, Antony KM *et al.* The Placenta Harbors a Unique Microbiome. *Sci Transl Med* 2014;**6**, DOI: 10.1126/scitranslmed.3009864.

Ainsworth TD, Thurber RV, Gates RD. The future of coral reefs: a microbial perspective. *Trends Ecol Evol* 2010;**25**:233–40.

Apprill A, Mcnally S, Parsons R *et al.* Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat Microb Ecol* 2015;**75**:129–37.

Apprill A, Weber LG, Santoro AE. Distinguishing between Microbial Habitats Unravels Ecological Complexity in Coral Microbiomes. *mSystems* 2016;**1**:e00143-16.

Bolyen E, Rideout JR, Dillon MR *et al.* Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 2019;**37**:852–7.

Bourne DG, Morrow KM, Webster NS. Insights into the Coral Microbiome: Underpinning the Health and Resilience of Reef Ecosystems. *Annu Rev Microbiol* 2016;**70**:317–40.

Callahan BJ, Mcmurdie PJ, Rosen MJ *et al.* DADA2: High resolution sample inference from Illumina amplicon data. *Nat Methods* 2016;**13**:581–3.

For Peer Review

Campbell BJ, Cary SC. Characterization of a Novel Spirochete Associated with the Hydrothermal Vent Polychaete Annelid, Alvinella pompejana. *Appl Environ Microbiol* 2001;**67**:110–7.

Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T. trimAl: A tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* 2009;**25**:1972–3.

Domin H, Zurita-Gutiérrez YH, Scotti M *et al.* Predicted bacterial interactions affect in vivo microbial colonization dynamics in Nematostella. *Front Microbiol* 2018;**9**:1–12.

Drummond AJ, Suchard MA, Xie D *et al.* Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Mol Biol Evol* 2012;**29**:1969–73.

Foster KR, Schluter J, Coyte KZ *et al.* The evolution of the host microbiome as an ecosystem on a leash. *Nature* 2017;**548**:43–51.

Har JY. Introducing the starlet sea anemone Nematostella vectensis as a model for investigating. 2009.

Har JY, Helbig T, Lim JH *et al.* Microbial diversity and activity in the Nematostella vectensis holobiont: Insights from 16S rRNA gene sequencing, isolate genomes, and a pilot-scale survey of gene expression. *Front Microbiol* 2015;**6**, DOI: 10.3389/fmicb.2015.00818.

Høj L, Levy N, Baillie B *et al.* Crown-of-Thorns Sea Star Acanthaster cf. solaris Has Tissue-Characteristic Microbiomes with Potential Roles in Health and Reproduction. *Appl Environ Microbiol* 2018;**84**:1–18.

Hooper L V., Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science (80- )* 2012;**336**:1268–73.

Hufnagel LA, Myhal ML. Observations on a Spirochaete Symbiotic in Hydra. *Trans Am Microsc Soc* 1977;**96**:406–11.

For Peer Review

Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol Biol Evol* 2013;**30**:772–80.

Koleff P, Gaston KJ, Lennon JJ. Measuring beta diversity for presence-absence data. *J Anim Ecol* 2003;**72**:367–82.

Layden MJ, Meyer NP, Pang K *et al.* Expression and phylogenetic analysis of the zic gene family in the evolution and development of metazoans. *Evodevo* 2010;**1**:1–16.

Leach WB, Carrier TJ, Reitzel AM. Diel patterning in the bacterial community associated with the sea anemone Nematostella vectensis. *Ecol Evol* 2019;**9**:9935–47.

Liaw A, Wiener M. Classification and Regression by randomForest. *R News* 2002;**2**:18–22.

Lilburn TG, Schmidt TM, Breznak JA. Phylogenetic diversity of termite gut spirochaetes. *Environ Microbiol* 1999;**1**:331–45.

Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;**15**:1–21.

Mandal S, Van Treuren W, White RA *et al.* Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb Ecol Heal Dis* 2015;**26**, DOI: 10.3402/mehd.v26.27663.

Margulis L, Nault L, Sieburth JM. Cristispira from oyster styles: complex morphology of large symbiotic spirochetes. *Symbiosis* 1991;**11**:1–17.

McMurdie PJ, Holmes S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS One* 2013;**8**, DOI: 10.1371/journal.pone.0061217.

Mortzfeld BM, Urbanski S, Reitzel AM *et al.* Response of bacterial colonization in Nematostella vectensis to development, environment and biogeography. *Environ Microbiol*

For Peer Review

2016;**18**:1764–81.

Ohkuma M, Noda S, Hattori S *et al.* Acetogenesis from H2 plus CO2and nitrogen fixation by an endosymbiotic spirochete of a termite-gut cellulolytic protist. *Proc Natl Acad Sci U S A* 2015;**112**:10224–30.

Price MN, Dehal PS, Arkin AP. FastTree 2 - Approximately maximum-likelihood trees for large alignments. *PLoS One* 2010;**5**, DOI: 10.1371/journal.pone.0009490.

Putnam NH, Srivastava M, Hellsten U *et al.* Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. *Science (80- )* 2007;**317**:86–94.

Quast C, Pruesse E, Yilmaz P *et al.* The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res* 2013;**41**:590–6.

Rädecker N, Pogoreutz C, Voolstra CR *et al.* Nitrogen cycling in corals: The key to understanding holobiont functioning? *Trends Microbiol* 2015;**23**:490–7.

Reitzel AM, Ryan JF, Tarrant AM. Establishing a model organism: A report from the first annual Nematostella meeting. *BioEssays* 2012;**34**:158–61.

Renfer E, Amon-Hassenzahl A, Steinmetz PRH *et al.* A muscle-specific transgenic reporter line of the sea anemone, Nematostella vectensis. *Proc Natl Acad Sci U S A* 2010;**107**:104–8.

Ricci F, Rossetto Marcelino V, Blackall LL *et al.* Beneath the surface: Community assembly and functions of the coral skeleton microbiome. *Microbiome* 2019;**7**:1–10.

Robbins SJ, Singleton CM, Chan CX *et al.* A genomic view of the reef-building coral Porites lutea and its microbial symbionts. *Nat Microbiol* 2019:1–11.

Rosenberg E, Koren O, Reshef L *et al.* The role of microorganisms in coral health , disease and evolution. *Nat Rev Microbiol* 2007;**5**:355–62.

Rossbach S. Tissue-Specific Microbiomes of the Red Sea Giant Clam Tridacna maxima

For Peer Review

Highlight Differential Abundance of Endozoicomonadaceae. 2019;**10**, DOI: 10.3389/fmicb.2019.02661.

Stackebrandt E, Chertkov O, Lapidus A *et al.* Genome sequence of the free-living aerobic spirochete Turneriella parva type strain (HT), and emendation of the species Turneriella parva. *Stand Genomic Sci* 2013;**8**:228–38.

Stamatakis A. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 2014;**30**:1312–3.

Subhash Y, Lee SS. Description of oceanispirochaeta sediminicola gen. Nov., sp. nov., an obligately anaerobic bacterium isolated from coastal marine sediments, and reclassification of spirochaeta litoralis as oceanispirochaeta litoralis comb. nov. *Int J Syst Evol Microbiol* 2017;**67**:3403–9.

Sweet MJ, Croquer A, Bythell JC. Bacterial assemblages differ between compartments within the coral holobiont. *Coral Reefs* 2011;**30**:39–52.

Thompson LR, Sanders JG, McDonald D *et al.* A communal catalogue reveals Earth's multiscale microbial diversity. *Nature* 2017;**551**:457–63.

Vaishnava S, Behrendt CL, Ismail AS *et al.* Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proc Natl Acad Sci U S A* 2008;**105**:20858–63.

Van De Water JAJM, Melkonian R, Junca H *et al.* Spirochaetes dominate the microbial community associated with the red coral Corallium rubrum on a broad geographic scale. *Sci Rep* 2016;**6**:1–7.

Wessels W, Sprungala S, Watson SA *et al.* The microbiome of the octocoral Lobophytum pauciflorum: Minor differences between sexes and resilience to short-term stress. *FEMS*

For Peer Review

*Microbiol Ecol* 2017;**93**:1–13.

Wier AM, Sacchi L, Dolan MF *et al.* Spirochete Attachment Ultrastructure: Implications for the Origin and Evolution of Cilia. *Biol Bull* 2010;**218**:25–35.

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**Fig 1. Compartmentalization of the microbiome in *N. vectensis*.** The microbiome of *N. vectensis* was divided into three tissue compartments for this study: the capitulum, mesenteries, and physa. The capitulum had a bacterial community dominated by Spirochetes. The mesenteries and physa had a bacterial community dominated by Gammaproteobacteria and Alphaproteobacteria, with very little presence of Spirochetes.

**Fig 2. The relative abundance of bacterial orders recovered from *N. vectensis* in individuals and tissue compartments.** Only taxonomic orders with a relative abundance above 2% were incorporated. Spirochaetales dominated the bacterial communities in the majority of capitulum samples (Individual 1 being an outlier). The mesentery and physa body regions showed more variety in their order composition with Pseudomonadales, Alteromonadales, and Oceanospirillales making up the majority of most samples.

**Fig 3. Heat-map of the percent read abundance for top bacterial families found in *N. vectensis* separated by tissue compartment.** Bacteria of the family Pseudomonadaceae were found to be the most prevalent in the mesenteries and physa tissue of *N. vectensis* (37.9% and 36.3% abundance, respectively). In contrast, Pseudomonadaceae only had a 7.3% abundance in capitulum tissue. Likewise, Spirochaetaceae were found to make up 65.8% of the community within the capitulum tissue of *N. vectensis*, while only being 1.2% and 0.1% abundant in the mesentery and physa tissues, respectively. Overall, the capitulum tissue appears to have a very distinct community from the mesenteries and physa.

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**Fig 4. Bacterial community Alpha diversity measures between tissue regions.** Species richness between the capitulum, mesenteries, and physa was significantly different (ANOVA; p=0.023, p=0.033, respectively). The Simpson Alpha Diversity Index between the capitulum, mesenteries and physa was significantly different (ANOVA; p=0.039, p=0.022, respectively). The Shannon Diversity Index did not show a significant difference in the bacterial communities between body regions.

**Fig 5. Beta diversity measures between tissue regions of *N. vectensis.*** (A) Bray-Curtis PCoA of *N. vectensis* bacterial communities after a variance stabilizing transformation. Capitulum samples clustered tightly, while physa and mesentery communities clustered closely together. Individual 1 appeared to be an outlier. (B) Aitchison PCA (Euclidean distance of center-log ratio transformed sample counts) of *N. vectensis* bacterial communities. Again, most capitulum samples clustered closely together, however they were also clustered with mesenteries and physa samples.

**Fig 6. Best-fit maximum likelihood tree based on 16S rRNA sequences depicting the phylogenetic relationship of the spirochetes recovered from the capitulum of *Nematostella vectensis.*** This tree was constructed using RAxML v7.3.0 with 1000 sub samplings. Bootstrap values (> 50%) are shown. Nodes with 100% bootstrap support are indicated by open circles. GenBank accession numbers are listed before each sequence's name. The host/habitat of uncultured species is in parentheses. Spirochaetales associated with cnidarians are in bold. *Gemmata obscuriglobus* was used as an outgroup to root the tree. The *N. vectensis* spirochetes from this study are indicated by an asterisk. The predominant spirochete ASV from this study

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(Spirochete 1) forms a strongly supported (100% bootstrap) monophyletic clade with spirochete sequences recovered from wild *N. vectensis*. Spirochete 2 clusters within the *Turneriella* genus.

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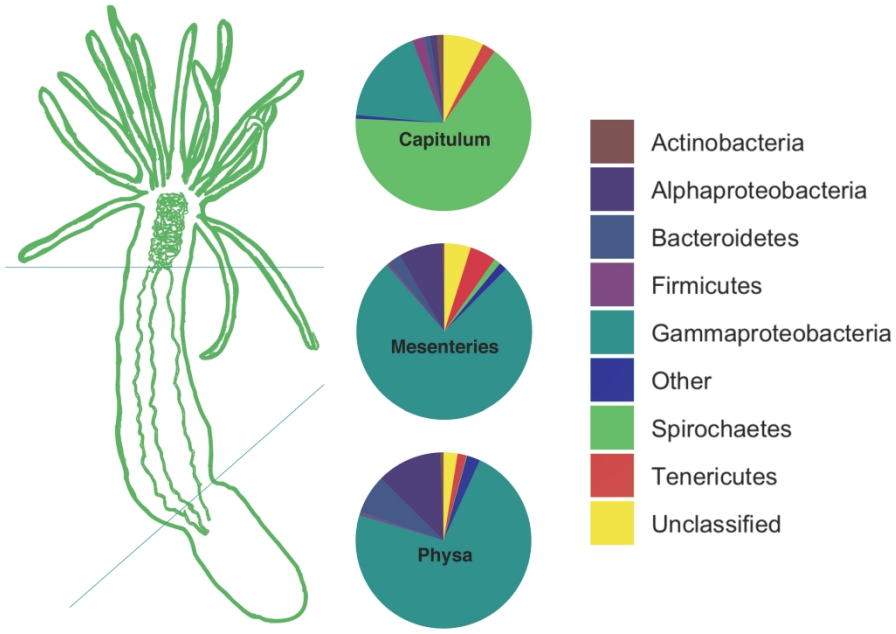


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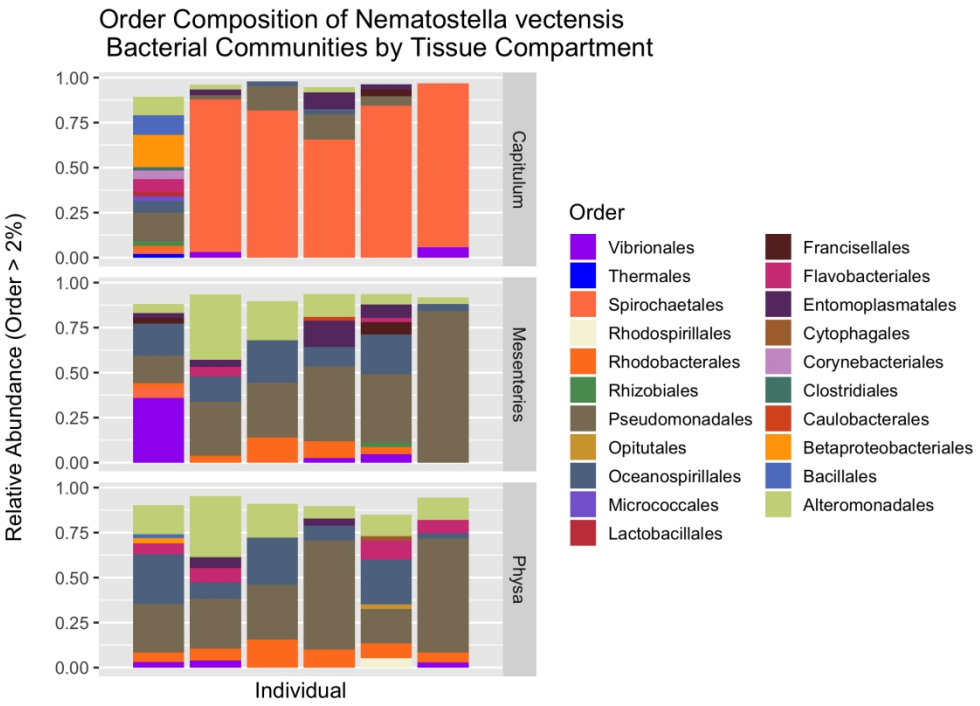


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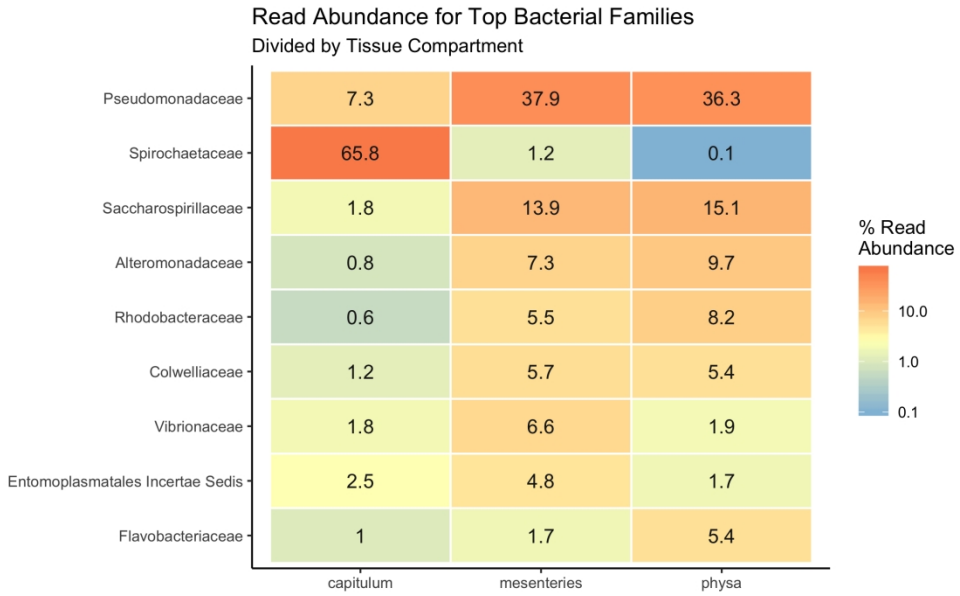


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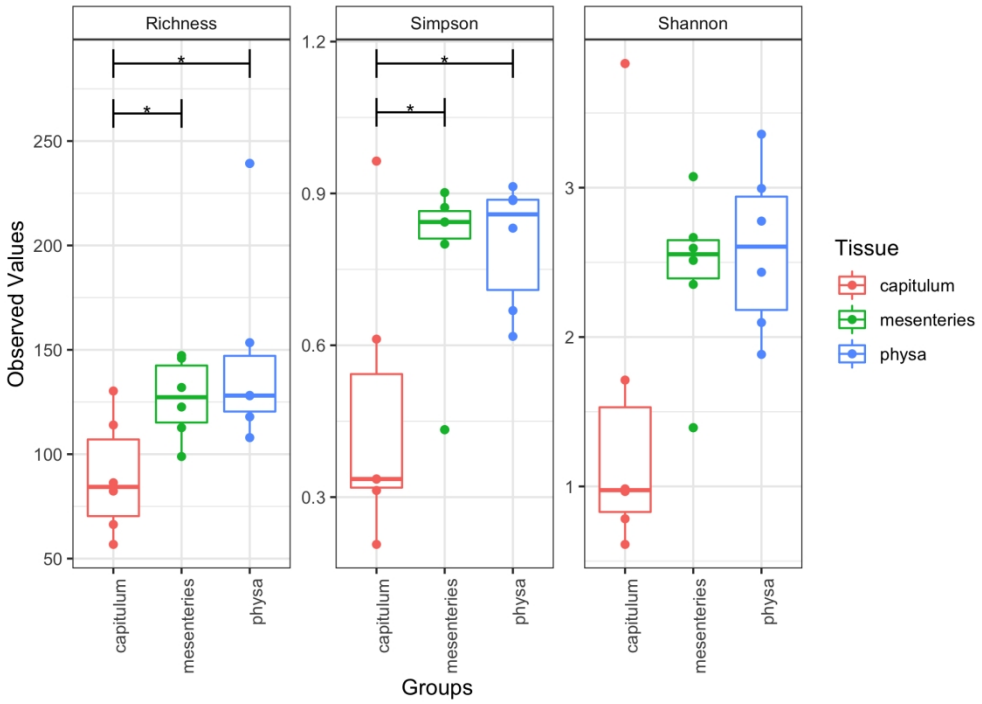


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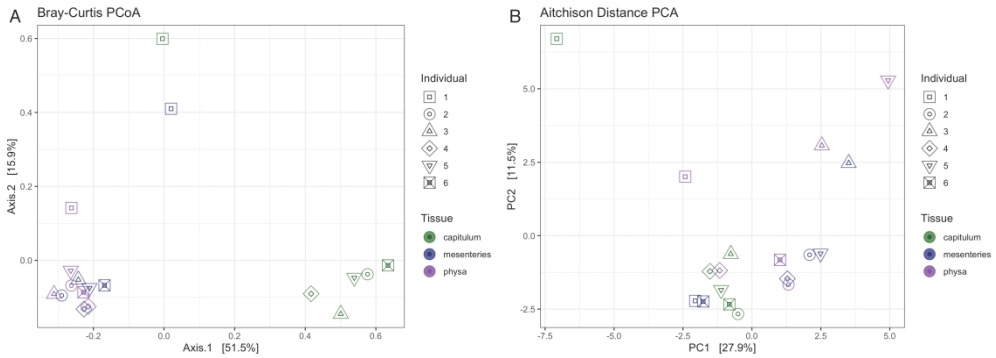


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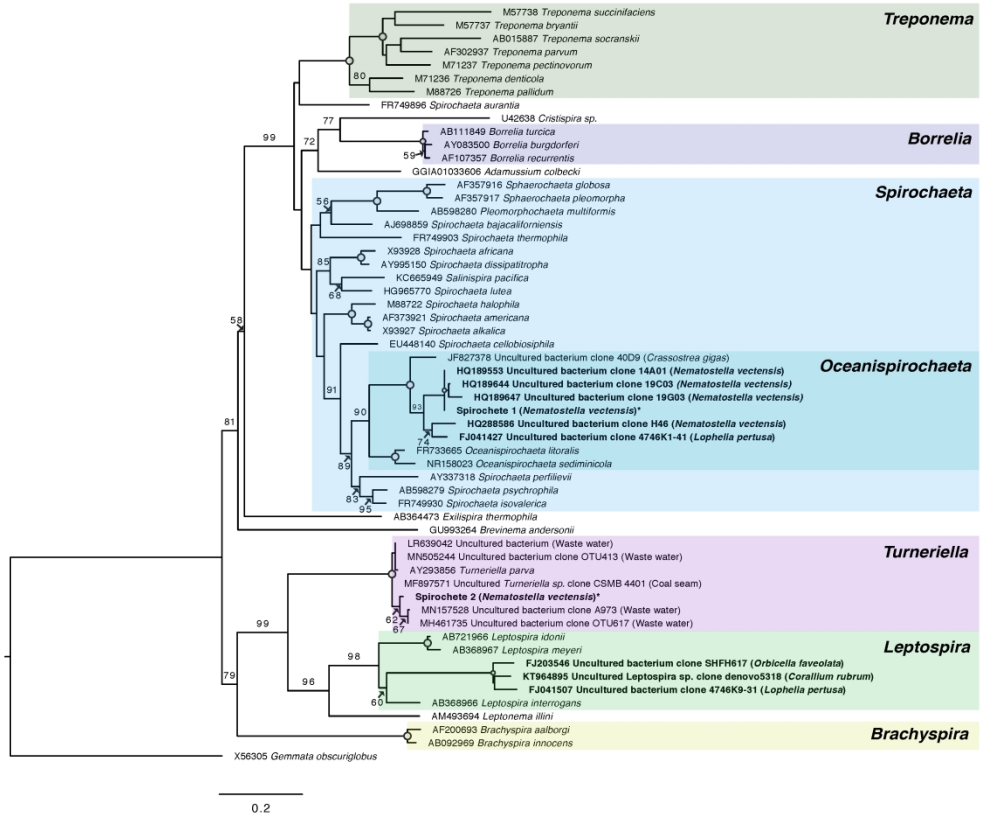


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**Title:** Microniche sampling of the microbiome in the Starlet Sea Anemone, *Nematostella vectensis*, reveals a compartment-specific dominance of Spirochetes

**Authors:** A. M. Bonacolta, M. T. Connelly, S. Rosales, J. del Campo, N. Traylor-Knowles

**Supplemental Material**

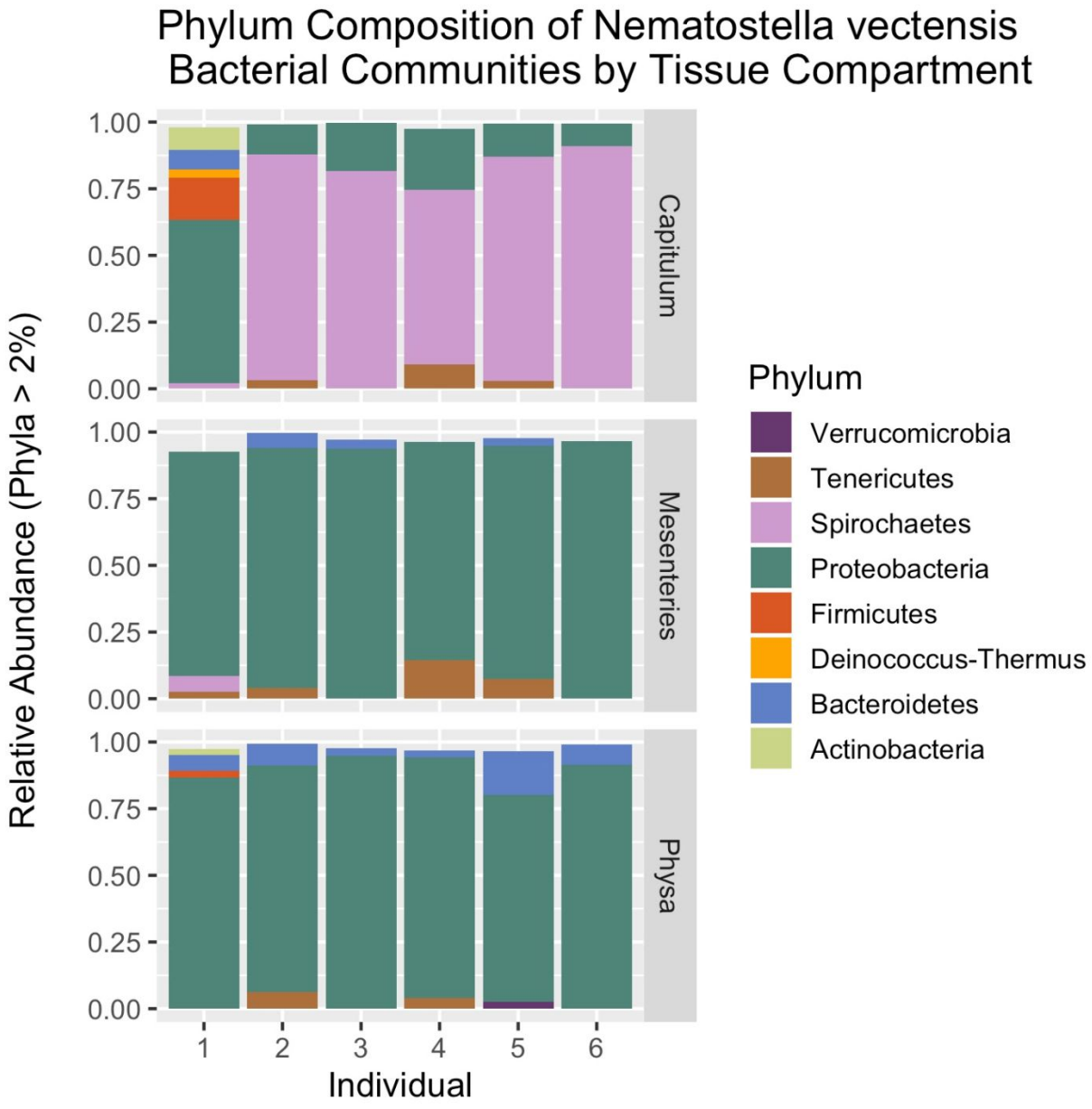




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**Fig S1. The relative abundance of bacterial phyla recovered from *N. vectensis* in individuals and tissue compartments** Only taxonomic phyla with a relative abundance above 2% were incorporated. Spirochetes dominated the bacterial communities in the majority of capitulum samples (Individual 1 again being an outlier). Proteobacteria dominated the bacterial communities of mesentery and physa samples.

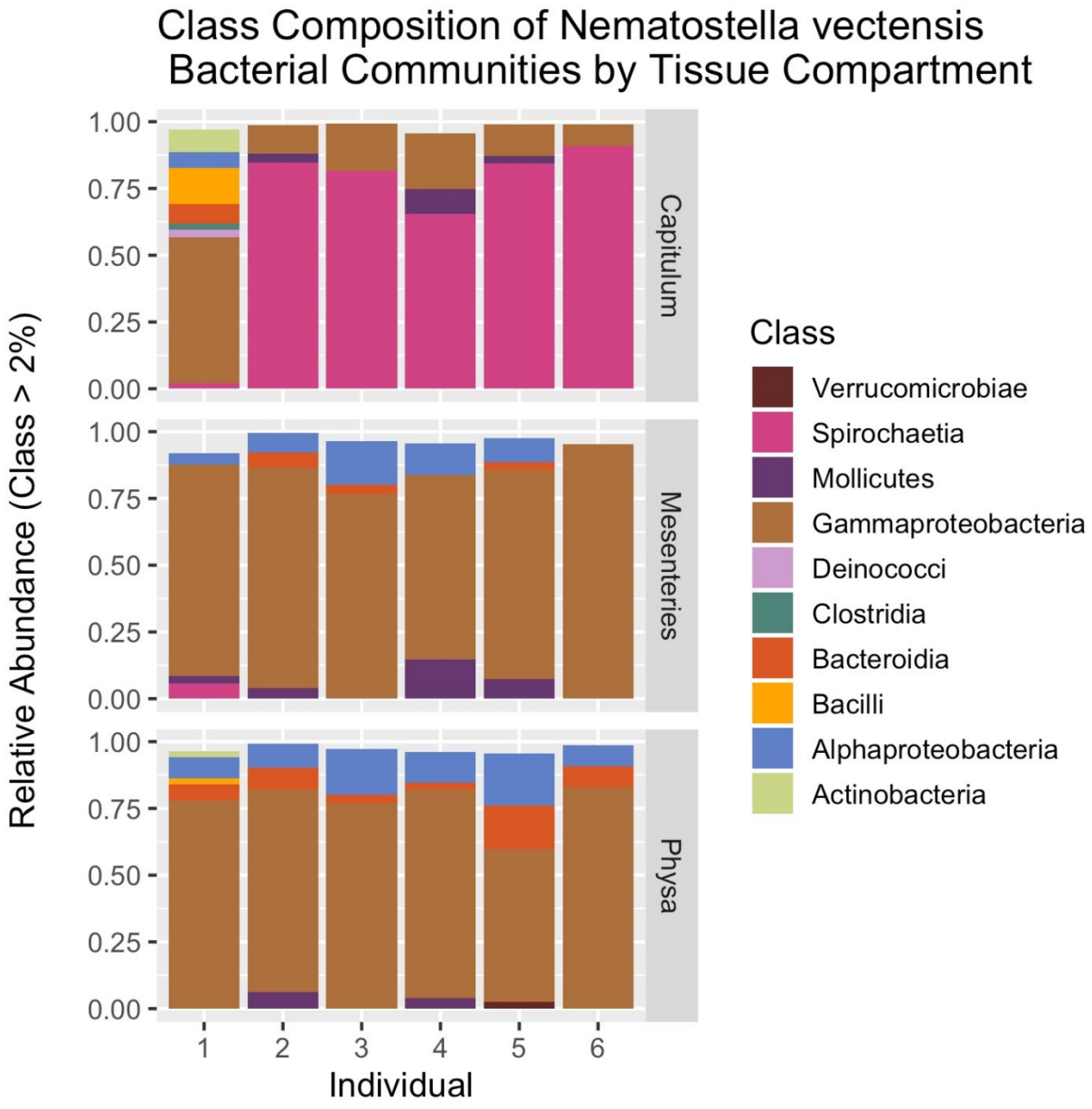




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**Fig S2. The relative abundance of bacterial classes recovered from *N. vectensis* in individuals and tissue compartments** Only taxonomic classes with a relative abundance above 2% were incorporated. Spirochaetia dominated the bacterial communities in the majority of capitulum samples (Individual 1 again being an outlier). Gammaproteobacteria dominated the bacterial communities of mesentery and physa samples.

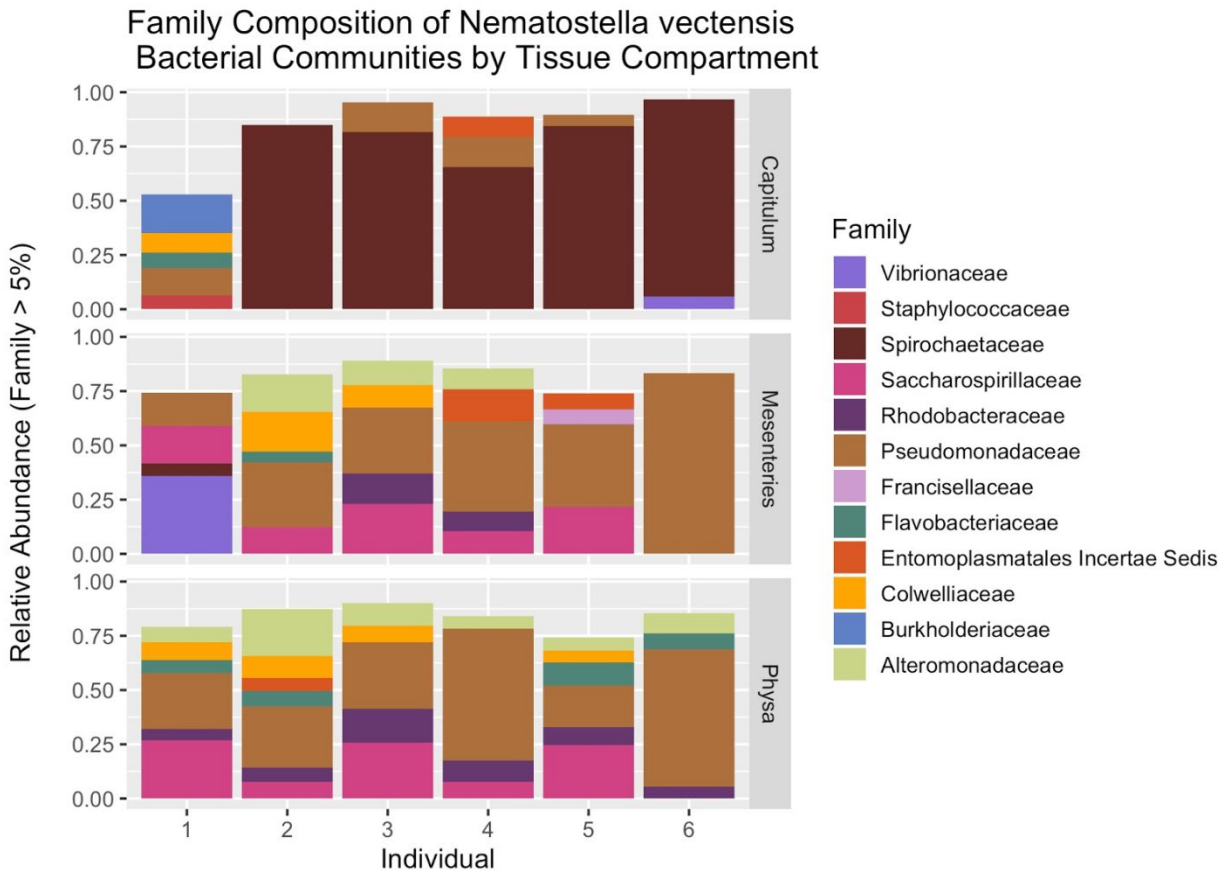


**Fig S3. The relative abundance of bacterial families recovered from *N. vectensis* in individuals and tissue compartments.** Only taxonomic families with a relative abundance above 5% were incorporated. Spirocheataceae dominated the bacterial communities in the majority of capitulum samples (Individual 1 again being an outlier). The mesentery and physa body regions showed more variety in their bacterial family composition with



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Pseudomonadaceae, Saccharospirillaceae, Colwelliaceae, and Alteromonadaceae making up the majority of most samples.

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