THE DIVERSITY AND COMMUNITY COMPOSITION OF MICROORGANISMS ASSOCIATED WITH ECHINODERMS

A Thesis

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Elliot Walter Jackson

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ABSTRACT

The field of animal-microorganism interactions has grown tremendously in recent decades as our understanding of how microorganisms influence host health and development has widened from the historical perspective as simply agents of disease. The knowledge gained from studying animal-microorganism interactions can bring about new ways for understanding the biology and ecology of the host of interest. Currently there has been little study of microorganisms associated with echinoderms despite the biological, ecological and economic interests that surround these organisms. Echinoderms are potentially interesting microbial environments due to their comparatively simple digestive systems and feeding habits, the lack of a specialized excretory organ and the influence disease has on population regulation for which there are very few described pathogens. This thesis focuses mostly on asteroids but aims to address three questions. 1) What microorganisms are associated with asteroids, 2) what variables shape the microbiome and 3) What are the potential relationships that can occur between the microorganisms found and the host. The third question cannot be fully addressed with the current data. Nonetheless, each chapter presents hypotheses on this subject in the discussion sections. Chapter 1 begins by examining the commonality and genotypic divergence of circular single-stranded DNA viruses among three echinoderm classes. Chapter 2 investigates the diversity and community composition of prokaryotes among asteroids and factors shaping the asteroid microbiome. The results presented herein provide the most comprehensive survey to date of the microbiome composition of echinoderms which provides an important baseline understanding for future work to elucidate the relationships for this host system.

To Dr. Holly Lutz

Your pursuit of knowledge and passion for adventure inspires me every day.

BIOGRAPHICAL SKETCH

Elliot was born on Saipan, a small island in the Northern Marianas Island Chain where he grew up catching hermit crabs on the beach with his brothers and sister and watching his dad windsurf along the shoreline. When he was four years old he moved to Harbor Springs in rural Northern Michigan. Growing up in Saipan and Harbor Springs fostered a deep passion and respect for the environment that instilled a responsibility to apply his education towards biology and ecology. After completing high school in 2008, Elliot pursued a degree in Environmental Science at the University of Michigan. During his undergraduate studies, he began working in research labs in the Earth and Atmospheric Science Department and the Ecology and Evolutionary Biology Department. It was during this period that Elliot became interested in aquatic ecology and soon after graduating college in 2012 he continued his interests through Cornell University working at the Cornell Biological Field Station with Dr. Lars Rudstam in 2013. Working as a research technician, Elliot had the opportunity to partake in field work across the Great Lakes Basin that allowed him to fully engage the research community both outside Cornell and within. In 2014 Elliot began his graduate studies in the Department of Natural Resources with Dr. Lars Rudstam and Dr. Ian Hewson.

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THESIS INTRODUCTION

Approximately 7,000 species make up the phylum Echinodermata that are globally distributed but found strictly in marine habitats. The phylum is divided into five extant classes that include Asteroidea (sea stars), Echinoidea (sea urchins and sand dollars), Holothuroidea (sea cucumbers), Ophiuroidea (brittle stars) and Crinoidea (sea lilies and feather stars). The interest in these organisms from the scientific community spans a wide range of sub-disciplines particularly embryology, physiology, ecology and evolution (1–3). These interests largely come from the facts that echinoderms exhibit deuterostome development making them valuable model organisms for studying human embryological development, but also because echinoderms have been recognized for their influence in marine community dynamics. In addition, certain species of sea urchins and sea cucumbers are harvested commercially. Currently there has been little study of microorganisms associated with echinoderms. The field of host-microorganism interactions has grown tremendously in recent decades as our understanding of how microorganisms influence host health and development has widened from the historical perspective as simply agents of disease (4). The term microorganism typically encompasses bacteria, archaea, viruses, fungi and other various microscopic eukaryotes while the study of the microbiome is the collection and interaction of these microorganisms on and within metazoans. Chapter 1 begins by examining the commonality and genotypic divergence of a circular single-stranded DNA virus group among three echinoderm classes. Chapter 2 investigates the diversity and community composition of prokaryotes among asteroids and factors shaping the asteroid microbiome. Potential animal-microorganism interactions are further discussed in chapter 2. The results presented herein provide the most comprehensive survey to date of the microbiome composition of echinoderms which provides an important baseline understanding knowledge for future work to elucidate the relationships for this host system.

The knowledge gained from studying animal-microorganism interactions can bring about new ways for understanding the biology and ecology of the host of interest. For example, the alphaproteobacteria, *Wolbachi*, is an endosymbiotic bacterium found in >65% of the insect species investigated to date and have the ability to manipulate the reproduction of the host in various ways including parthenogenetic induction and the killing of male progeny from infected females which inevitably causes a distortion of sex ratio in nature (5). Another unique animal-microorganism interaction studied are bioluminescent bacteria, *Vibrio* and *Photobacterium*, found within the light organs of marine invertebrates and fish (6). Bioluminescence can be performed by both bacteria and eukaryotic organisms though the symbiotic interaction in which bacteria perform this function for the host directly influences both the behavior and ecology of their host. Perhaps the most widely studied microorganism – host interaction is the way microbes supplement the diet of their eukaryotic hosts through metabolizing organic or inorganic material that the host cannot otherwise utilize. In the most extreme cases the reliance on microorganisms for diet supplementation is obligatory for the hosts; examples from marine invertebrates include tubeworms and mussels that live in hydrothermal vents or cold seeps. These invertebrates harbor methane or sulfur oxidizing bacteria that allow them to live in such extreme environments (7, 8). Another marine invertebrate system that is well studied is the microbiome of corals. Corals house a complex community of microorganism though among the most well studied is their symbiotic relationship with the photosynthetic algae, zooxanthellae. If these microorganisms are not present in the tissues for an extended period of time the corals will not survive (9). These are only a few examples of many in which microorganisms have been found to influence the biology, behavior or ecology of their associated host animals. Without considering the influence microorganisms may have on a host of interest, the study of that organism is incomplete.

Echinoderms contain potentially interesting microbial environments due to their comparatively simple digestive systems and feeding habits in addition to the lack of a specialized excretory organ. Asteroids arguably exhibit the most unique form of digestion within the phylum which generally occurs outside of the body by inverting the stomach on their food source. This method of feeding results in little indigestible material taken up in the animal which could reduce or completely bypass the need for microorganisms in the digestion process. The microbiome might also be involved in transforming metabolic waste. Metabolic waste, which is mostly in the form of soluble ammonia, is excreted directly into the coelom where it diffuses through the epidermis. The potential for toxic build-up of ammonia in echinoderms is not plausible given that these organisms are isotonic. However, it is not uncommon to find ammonium-oxidizing prokaryotes associated with marine invertebrates (10, 11). This subject is further examined and discussed in chapter 2. Aside from possible mutualistic or commensal relationships that could occur, parasitic interactions might arise under certain conditions which brings to question the role of the microbiome in the context of disease.

Echinoderms are prone to large population fluctuations that can be mediated by pervasive disease events for which there is often no described causative pathogen(s). The phylum has been suggested to be a "boom-bust" phylum due to the large density fluctuations events observed in the wild (12). These density variations can be outbreaks, die offs or a cyclical combination in which an outbreak is followed by a die off. These observed trends have been suggested to be due to the separation of adult and larvae ecology and are generally seen with species exhibiting planktotrophic as opposed to lecithotrophic larvae (12). Regardless of the organismal traits explaining these observed trends, the outcome of such events can cause dramatic ecosystem changes. For example, the mass mortality event of *Diadema antillarum*, black sea urchin, that

occurred between 1982-1984 throughout the Caribbean contributed greatly to the transformation of coral dominated communities to algal dominated communities (13, 14). *Acanthaster plancii,* the crown-of-thorns sea star, is a corallivorous tropical Indo-Pacific species that exhibits decadal scale population pulses that can significantly alter coral communities (15). More recently, a mass mortality event of >15 species of sea stars along the west coast of the United States due to a disease known as "Sea Star Wasting Disease" resulted in a precipitous decline for certain species in areas (16, 17). The community changes from such an event will likely result in a dramatic restructuring of the rocky intertidal until the sea star populations can recover. These are only a few examples of many, and the extreme population changes observed for these organisms are exceptional for two reasons. 1) The changes are not restricted to a specific species or even class but rather reported across most of the phylum and 2) the extreme population changes that are disease mediated are among, if not, the largest marine disease events observed. It is unclear how pathogens arise in these mass mortality events, but one possibility could be the consequence of a shifting microbiome under particular environmental stressors. Mutual or commensal relationships in the microbiome might shift to parasitic and if the host is immunosuppressed the result could be disease. To explore this hypothesis, it is crucial to understand how the microbiome is shaped, the potential relationships that can occur in the host and the organisms that define it. The ability to address these questions using culture-independent approaches has only recently become available through the advancements in the fields of genomics and bioinformatics.

The advancements in DNA sequencing technology and bioinformatics tools have given microbial ecologists the ability to explore, characterize and study microorganism relations with non-model organisms with no *a priori* knowledge. Paired with experimentation and microscopy,

researchers have the ability to tease apart these complex interactions between a microorganism and its host. This thesis utilizes two genomic approaches to study the microbial community associated with echinoderms. Chapter 1 uses viral metagenomics to examine a group of viruses found across three of the five extant classes and discusses the possible relationship they have to echinoderms. Viral metagenomics can be used as a culture-independent approach to identify candidate viral pathogens by providing molecular targets for further diagnostic studies. However, for this thesis, it is only used as a descriptive tool. Echinoderm viruses have only recently been explored and the discovery of a densovirus, a ssDNA virus, linked to a mass morality event of sea stars on the west coast of North America brings to question the role of viruses in echinoderm diseases (16, 18). Choosing a virus for further diagnostic assays from the wealth of viral diversity that can be found in viral metagenomic surveys can prove to be quite challenging especially since many viruses detected using genomic approaches are highly divergent from cultured representatives. It is important therefore to have a general knowledge of viruses associated with the host(s) of interest when such tools are used for diagnostic purposes as it can guide researchers in identifying potential viral pathogens more confidently. Chapter one investigates the presence of another type of ssDNA virus found in echinoderms to understand the genotypic divergence and commonality of these viruses across the phylum. This chapter shows the widespread nature of circular ssDNA viruses among echinoderms corroborating previous studies examining these viruses associated with other invertebrates (19). Chapter 2 uses high throughput amplicon sequencing of the universal 16s rRNA gene found in bacteria and archaea as a taxonomic tool to describe the community composition of prokaryotes associated with sea stars. Previous microscopy studies have demonstrated the presence of bacteria in adult asteroids in the subcuticular layer of the epidermis (20–22). The conclusion of these studies show the

widespread presence of gram-negative rod and spiral shaped bacteria among the subcuticular layer of asteroids. Chapter 2 shows the association of bacteria with four different tissues from 12 asteroid species which corroborates and significantly builds upon previous studies. These results provide a comprehensive analysis of the microbiome of healthy sea stars and shows that the community composition of bacterial taxa within the coelomic cavity is strict across asteroid species and habitat.

Echinoderms are unique organisms rich with opportunity for studying host-microorganism interactions in the context of physiology and disease. This thesis provides a basic knowledge of the microbial constituents associated with echinoderms to understand what microorganisms are found across different host taxa and geographic location and how variable the microbiome is across these factors. This baseline knowledge is valuable for further more targeted studies of the host-microbe relationships in echinoderms.

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Chapter 1

Novel circular single-stranded DNA viruses among an asteroid, echinoid and holothurian (Phylum Echinodermata)

Elliot Walter Jackson [1*], Kalia Sakaye-Irinaga Bistolas[1], Jason Benjamin Button[2] and Ian Hewson[1]

[1] Department of Microbiology, Cornell University, Wing Hall, 123 Wing Drive, Ithaca NY 14853, USA;

[2] Department of Oceanography, University of Delaware, 700 Pilot Town Road, Lewes DE 19958, USA; jb.button1@gmail.com

***** Correspondence: ewj34@cornell.edu; Tel.: +1-231-838-6042

Abstract:

Echinoderms are prone to large population fluctuations that can be mediated by pervasive disease events. For the majority of echinoderm disease events the causative pathogen is unknown. Viruses have only recently been explored as potential pathogens through culture-independent techniques though little information currently exists on echinoderm viruses. In this study ten circular ssDNA viruses were discovered in tissues among an asteroid (*Asterias forbesi)*, an echinoid (*Strongylocentrotus droebachiensis)* and a holothurian (*Parastichopus californicus)* using viral metagenomics. Genome architecture and sequence similarity place these viruses among the rapidly expanding circular *rep*-encoding single stranded (CRESS) DNA viral group. Multiple genomes from the same tissue were no more similar in sequence identity to each other than when compared to other known CRESS DNA viruses. The results from this study are the first to describe a virus from a holothurian and continue to show the ubiquity of these viruses among aquatic invertebrates.

Introduction:

Diseases of echinoderms, particularly echinoids (sea urchins) and asteroids (sea stars), have been extensively documented worldwide and include some of the largest marine epizootics known to date (1–4). For many of these disease events, a causative pathogen remains undescribed which severely limits the study of the ecology and evolution of infectious diseases of these animals. Of the cases in which a pathogenic agent has been identified or statistically associated with a disease, bacteria and eukaryotic parasites (amoebozoa) are the most well described (5, 6). To date, no fungi have been found associated with echinoderm disease and viruses have only recently been explored as potential pathogens (7). The discovery of a densovirus linked to a mass morality event of sea stars on the west coast of North America brings to question the role of viruses in other echinoderm diseases (4). More recently, the discovery of a circular *rep*-encoding single-stranded (CRESS) DNA virus was discovered from tissue of a *Asterias forbesi*, a common sea star on the east coast of North America, exhibiting symptoms similar to the sea star wasting disease observed in the Northeast Pacific. However, no significant correlation was found between viral load/prevalence and symptomatic vs asymptomatic individuals (8). We sought to further the investigation of CRESS DNA viruses among organisms within the phylum Echinodermata to understand the genotypic divergence and commonality of CRESS DNA viruses among these unique organisms.

Currently the study of echinoderm viruses requires culture-independent methods for the reason that no marine invertebrate cell lines exist. Metagenomics is a powerful tool that can be used to develop molecular diagnostic assays to explore suspect viruses found in animal tissues. However, choosing a virus for further diagnostic assays from the wealth of viral diversity that can be found in the viral metagenomic data can prove to be quite challenging especially since

many viruses detected using genomic approaches are highly divergent from cultured representatives. It is important therefore to have a general knowledge of viruses associated with the host of interest when such tools are used for diagnostic purposes as it can guide researchers in identifying potential viral pathogens more confidently. Here we report the findings from viral metagenomic data on a commonality of a viral group, CRESS DNA viruses, among echinoderms. Tissues from three organisms representing three of the five extant classes within the phylum Echinodermata (Asteroidea, Echinoidea and Holothuroidea) were examined for the presence of CRESS DNA viruses, and ten complete CRESS DNA viral genomes were discovered. Multiple CRESS DNA viral genomes were identified from each organism providing a unique opportunity to explore genotypic divergence within and between species. This study reports the first virus to be described from a holothurian and contributes to our knowledge of viruses associated with echinoderms.

Methods:

Samples were collected from three separate locations spanning a broad geographic range. *Asterias forbesi* were collected from the intertidal from Nahant Bay, Massachusetts, USA (42.4208, -70.9064) in September of 2015 under the Ocean Genome Legacy's general scientific collection permit (#156386) issued by the Massachusetts Division Marine Fisheries (28). The *A. forebsi* used in the study had symptoms of disease similar to the sea star wasting disease reported on the west coast of the USA. *Strongylocentrotus droebachiensis* were collected from public display tanks at the Vancouver Aquarium, British Columbia, Canada in October 2014. *S. droebachiensis* were collected under permit XR 1 2014 issued from the Department of Fisheries and Ocean for Statistical Areas 28 and 29. *Parastichopus californicus* were collected from Ketchikan, Alaska, USA (55.3410, -131.6641) during March of 2015 through the Alaskan

Department of Fish and Game. Both *S. droebachiensis* and *P. californicus* were apparently healthy animals when collected i.e. they had no epidermal lesions or test balding that would be suggestive of disease. However, both *S. droebachiensis* and *P. californicus* were collected from populations with symptomatic individuals. Viral metagenomics libraries were generated from symptomatic species from these populations though no CRESS-DNA viruses were found among the sequence libraries. Upon collection, all samples were stored at either -20°C or -80°C prior to sample preparation. None of the animals collected for this study involved endangered or protected species.

Viral metagenomic libraries were generated for each specimen per protocols specified in (9). Samples were sent to the Cornell Institute of Biotechnology for Illumina MiSeq 500 bp sequencing v2 * (e.g. 2 x 250 bp). CLC Genomics Workbench 8.5.1 was used for read quality analysis and assembly. Reads with bases exceeding a quality score of 0.05 or containing ambiguities were discarded. Reads less than 249 nt or greater than 251 nt were also discarded. The remaining reads for each library were subjected to *de novo* assembly using CLC Genomics Workbench de Bruijn graph assembler with a minimum contig length of 500. Contigs were annotated by tBLASTx (10) against a curated in-house database of circular ssDNA virus genomes obtained from NCBI with an e-value cutoff $1x10^{-5}$. Contigs with significant (i.e. $e < 1x10^{-5}$) similarity to circular ssDNA virus genomes were isolated and imported back in CLC Genomics Workbench for read mapping. Reads from the respective libraries were mapped back to contigs using an overlap consensus sequence algorithm with a mis-match cost of 2, insertion cost of 3, deletion cost of 3, length fraction 0.8 and a similarity of 0.5. Contigs were updated based on mapping results. Open reading frames (ORFs) were obtained using CLC Genomic Workbench 8.5.1 by searching both strands for start codons with a minimum codon length of

100. Once the contigs were assessed for these criteria, a BLASTn analysis was conducted to compare the contigs to the closest respective genome to verify the independence of these viral genomes from endogenous viral elements. In addition, contigs were screened for sequence similarity to known laboratory contaminants (BLASTn, e-value $1x10^{-5}$). Laboratory contaminants were identified through a parallel metavirome preparation containing 0.02um filtered nuclease free water as a template (11).

After the contigs were finalized, the putative *rep* ORFs of each contig was translated using MUSCLE (12) and aligned with other putative *rep* genes from circular ssDNA viruses obtained from NCBI. Using the alignment as a guide, *rep* sequences were manually screened for the three rolling circle replication (RCR) and SF3 helicase motifs to verify homology to the vRep protein (13). The translated *rep* sequences were then imported into Sequence Demarcation Tool Version 1.2 (SDTv1.2) (14) for pairwise amino acid similarity analyses to sequences with strong similarity based on BLASTx results against the NCBI non-redundant database (S2 Table). Hydrophobicity of the amino acid sequences of the putative capsid protein were calculated using Kyte and Doolittle 1982 (15). Finally, contigs were screened for the conversed nonanucleotide motif and stem-loop structures were predicted using Mfold (16).

Results:

Ten complete circular ssDNA viral genomes were identified from metagenomic analysis of purified DNA from viral particles of tissue from *A. forbesi* (n = 4), *S. droebachiensis* (n = 2) and *P. californicus* (n = 4) (Fig1). After mapping the reads back to the contigs, the average coverage between the contigs was 572x ranging from 16x to 2,270x (Table 1). The size of the genomes spanned 1,704 to 3,192 nucleotides all encoding at least 2 ORFS exhibiting both unisense and ambisense orientation. All genomes contained a putative *rep* gene that had

significant similarity (BLASTx, e-value < $1x10^{-5}$) to a putative *rep* gene from another circular ssDNA virus genome. The amino acid sequence length of the putative *rep* genes ranged from 214aa to 318aa while the putative *cap* gene ranged from 215aa to 467aa. Nonanucleotide motifs were all found within a predicted stem-loop structure (Fig 1). All stem-loop structures predicted are energetically favorable ($\Delta G < 0$). TAGTATTAC and CAGTATTAC were the most represented nonanucleotide motifs, 4 of 10 each (Table 1). None of the viral genomes contained all conserved residues of motifs found in a vRep protein that would definitively place it into one of the four well defined eukaryotic circular ssDNA viral groups (S1 Table).

Figure 1.1 Genome illustrations of circular ssDNA viruses

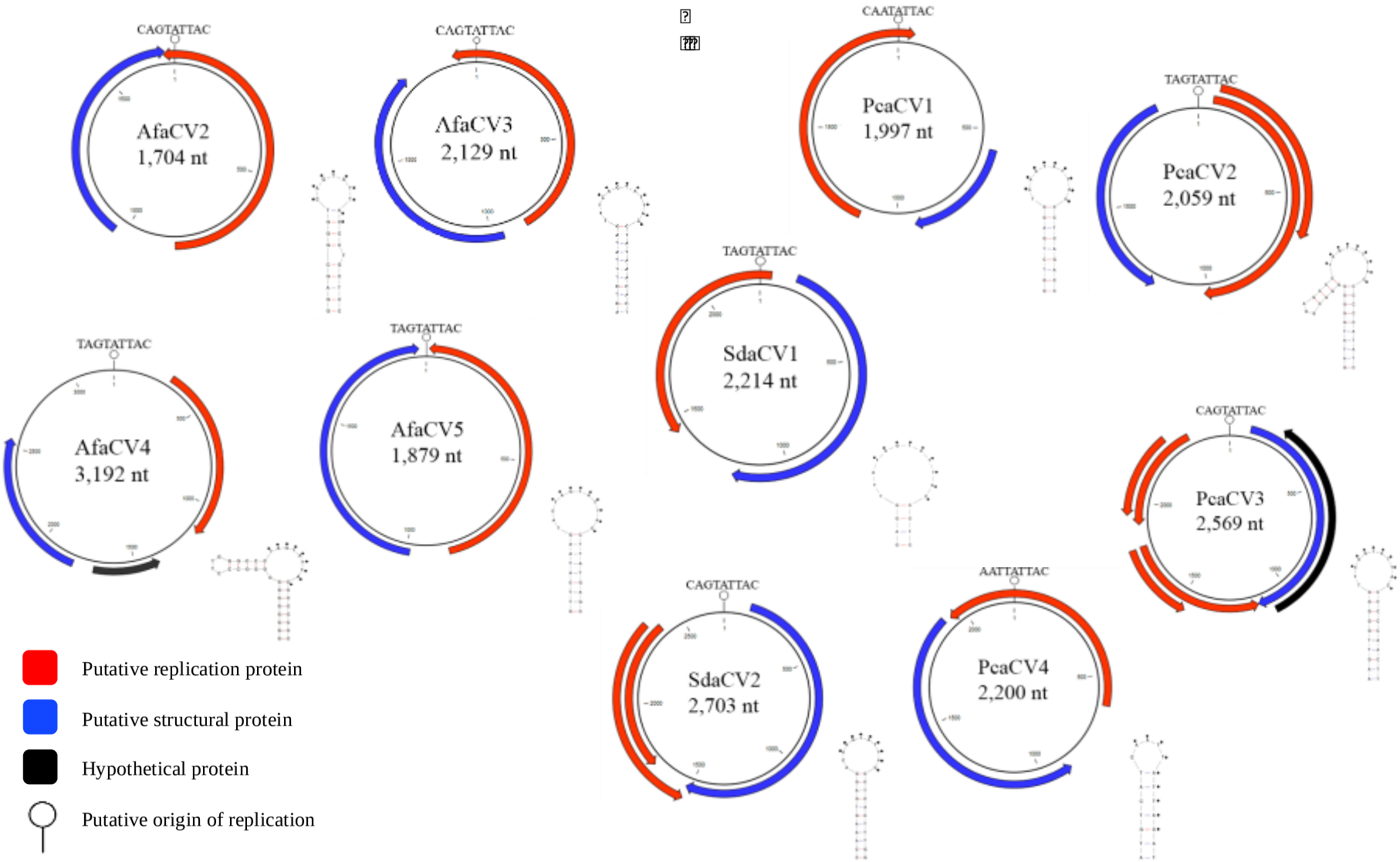

Table 1.1 Sample description, genome coverage and characteristics of ssDNA viruses based on genomic features

Host	Collection Site	Name	Genome Coverage	Genome Size (nt)	Putative Rep (aa)	Putative Cap (aa)	Nonanucleotide motif	Type	Orientation
Asterias forbesi (sea star)	Nahant, Massachusetts	AfaCV2	16	1,704	293	215	CAGTATTAC	IV	Ambisense
		AfaCV3	64	2,129	316	302*	CAGTATTAC	IV	Ambisense
		AfaCV4	205	3,192	285	245	TAGTATTAC	V	Unisense
		AfaCV5	44	1,879	286	289	TAGTATTAC	IV	Ambisense
Strongylocentrotus droebachiensis (sea urchin)	Vancouver, British Columbia	SdaCV1	349	2,214	270	356	TAGTATTAC	II	Ambisense
		SdaCV2	380	2,703	275†	467	CAGTATTAC	II	Ambisense
Parastichopus californicus (sea cucumber)	Ernest Sound, Alaska	PcaCV1	273	1,970	307	279	CAATATTAC	V	Unisense
		PcaCV2	2,270	2,059	318†	243	TAGTATTAC	I	Ambisense
		PcaCV3	131	2,569	214†	314*	CAGTATTAC	VI	Unisense
		PcaCV4	1,989	2,200	292	345	AATTATTAC	VI	Unisense

[1]Non-Rep encoding ORFs were identified as putative capsid proteins based on BLASTx results. Non-Rep-encoding ORFs that did not have significant similarity (evalue < $1e^{-5}$) are denoted (*)
[2] Genomes containing multiple ORFS encoding the same putative function are marked with (†). The ORF with the lowest evalue from the BLASTx results from NCBI non-redundant database are represented in this table

BLASTx analyses of the *rep* gene of each viral genome show strong homology by similarity to other replication genes of circular ssDNA viruses in the NCBI database. The majority of *rep* genes were most similar to CRESS DNA viral *rep* sequences identified through metagenomic surveys of environmental samples (Fig 2). AfaCV was not identified from the *A.forbesi* sample and viral genomes that were found did not show a stronger similarity to AfaCV when compared to other CRESS DNA viruses. Similar to most CRESS DNA viruses discovered through metagenomic analysis, the best amino acid percent identities for each of the *rep* genes ranged from 34-57% (Fig 1.2). 8 of the 10 putative capsid ORFs had significant similarities from the BLASTx analyses to other putative capsid proteins of circular ssDNA viruses. The lack of similarity in 2 of the 10 putative capsid ORFs is not unexpected because the capsid protein is

generally found to be less conserved on the amino acid level. In attempt to further verify the annotation of the putative capsid gene, hydrophobicity across the amino acid sequence was investigated. Circovirus capsid proteins are rich in basic amino acids in the N-terminus region which was generally observed along these putative capsid amino acid sequences (S1-11 Figures). Taken together, the sample preparation and thorough examination of both protein encoding and non-protein encoding genetic elements highly suggest the circular genomes identified in this study are viral and not another intracellular episomal element that replicates via a rolling circle mechanism such as a plasmid or helitron (37).

Figure 1.2. Pairwise amino acid sequence identity analysis of the putative Rep genes including sequences with strong similarity based on BLASTx results against the NCBI non-redundant database. Arrows indicate genomes obtained in this study.

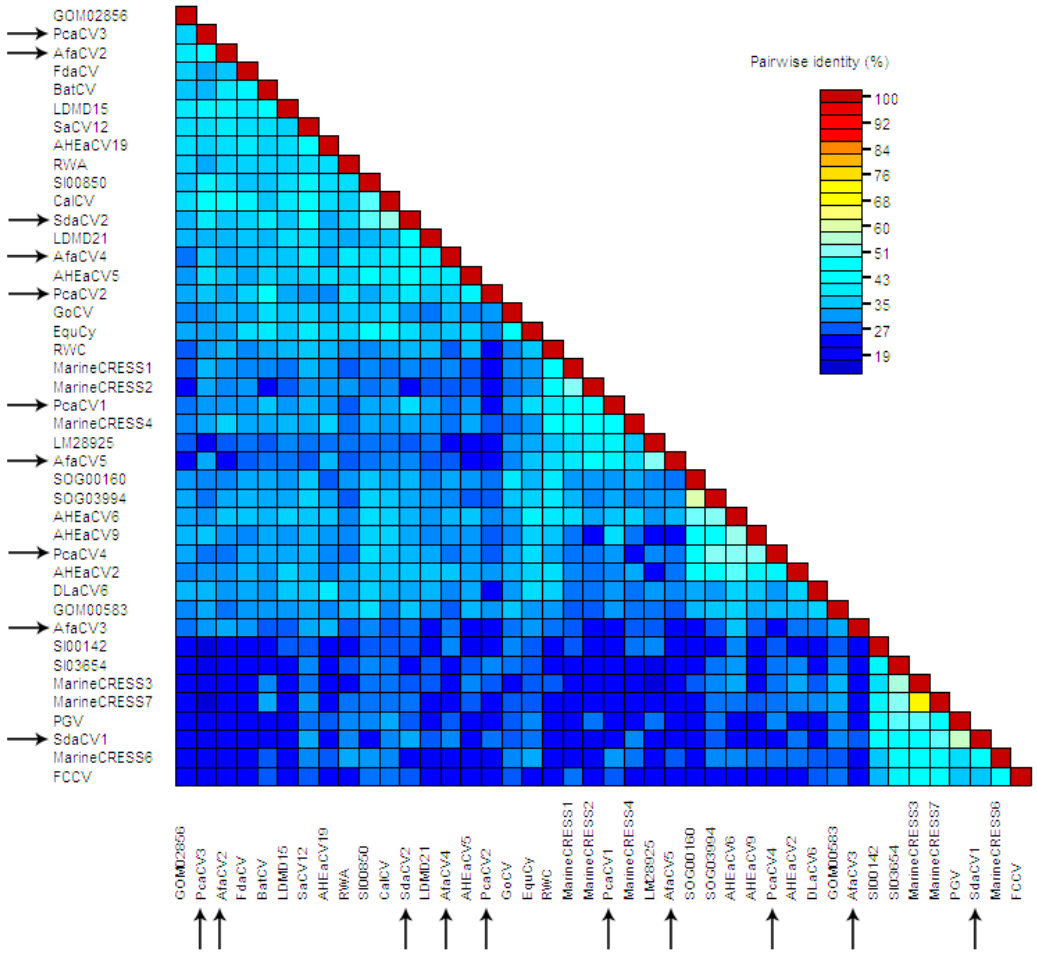

Table 1.2 RCR and SF3 Helicase motifs found in circular ssDNA viruses. Conserved amino acid motifs obtained from alignments of putative Rep gene using MUCLE.

	RCR Motifs			SF3 Helicase Motifs		
	I	II	III	Walker A	Walker B	Walker C
Circovirus	[CV]FT[LI]NN	PHLQG	YC[Sx]K	GP[Ps][Gc]xGKS	[VI][IML]DDF	UTSN
Cyclovirus	[CV]FT[WL]NN	[Px]HLQG	YC[Sx]K	G[Px][Pt]GxGKS	[IV][IU]DDF	UTS[Ne]
Geminivirus	FLTY[Ps]x	[Px]H[Lx]H[VAC]	Y[UAC]xK	Gx[ST]R[TI]GK[TS]	[VI][IV]DD[VI]	UL[Cx]N
Nanovirus	[VCx]FT[LI]N[FYN]	xHUQG	Y[CAS]xK	G[PS]xG[GN]EGK[TS]	[VIW][UAC][FIM]D[IVF]	V[FMI][AC]N
AfaCV2	MFTLFV	LHVQG	YCSK	GDPGSGKT	IFDDF	ITSN
AfaCV3	CFTDFK	KHNQG	YCSK	GKAGTGKT	LIDDF	ITSN
AfaCV4	CFTLNN	KHLQG	YACK	GPTGSGKT	LMDDF	ITSN
AfaCV5	VFTNYD	PHHQG	YCSK	GPSGAGKS	ILNEF	ITSV
SdaCV1	CFTLNN	PHLQG	YCSK	--------	----	----
SdaCV2	CWTLNN	PHLQG	YCKK	GPTGTGKT	LFDDF	ITSN
PcaCV1	VFTLNN	PHYQG	YCEK	GDTGVGKS	IINDF	ITSS
PcaCV2	VYTWNN	EHFQG	YSSK	GEPGTGKT	LLDDF	ITTN
PcaCV3	CFTWFA	PHVQG	----	GPTGVGKT	ILDDF	ITSN
PcaCV4	CLTINN	KHLQV	YCSK	GVTGSGKT	IIDDM	ITTT

U = I, L, V, M, F, Y, W

Discussion:

The use of culture-independent techniques (metagenomics and degenerate PCR assays) has illuminated the widespread nature and diversity of circular *rep*-encoding single-stranded (CRESS) DNA viruses (17, 18). In fact, no two same genotypes of CRESS DNA viruses have been found within or between environments sampled. The evolutionary breadth of eukaryotes that circular ssDNA viruses have now been associated with span the length of animal evolution from Ctenophora to Chordata (19, 20). Aquatic invertebrates, apart from insects, have been the most heavily surveyed and studied eukaryotic organisms for CRESS DNA viruses particularly organisms in the subphylum *Crustacea* notably amphipods (18, 21), cladocerans (22), decapods

(18) and copepods (18, 23). CRESS DNA viruses have also been found in other aquatic invertebrates including mollusks (phylum Mollusca) (18, 24, 25) and corals (phylum Cnidaria) (18). The majority of CRESS DNA viral genotypes however are biased towards arthropods. Recovering novel genotypes from a broader range of host organisms could clarify an evolutionary pattern of eukaryotic circular ssDNA viruses particularly within the family *Circoviridae.* The results from this study show that circular ssDNA viruses are commonly associated with other aquatic animals other than crustaceans and continued viral surveys of marine and freshwater fauna will most likely report similar results. Whether these viruses have the same or different relationships with hosts from disparate groups remains unknown.

The pathogenicity and ecological cost of infection of CRESS DNA viruses currently remains a mystery. The lack of immortal cell lines available for many freshwater and marine organisms severely limits the ability to establish virulence of their associated viruses. Additionally, it is often difficult to implicate these viruses in disease for the reason that these viruses are often associated with asymptomatic hosts. Therefore, correlation based studies have been used to infer the potential cost of infections by these viruses on host populations. The conclusions from these studies are mixed. CRESS DNA viruses have been correlated with mortality rates (22) and stressed host populations (21). Fashbender et al. 2015 reported an association of a CRESS DNA virus with disease symptoms of *A. forbesi*, but this correlation was not statistically significant. Nevertheless, a general observation from these correlation-based studies indicate that CRESS DNA viruses are highly prevalent and persistent among host populations (20, 22). These patterns of infection, in addition to the extreme diversity of viral genotypes, suggest that CRESS-DNA viruses are not strongly virulent and cost of infection may be cryptic or even mutualistic. The results from this study contribute to our understanding of the

nanobiome of echinoderms and further verify the widespread nature of CRESS DNA viruses among aquatic invertebrates.

Acknowledgments

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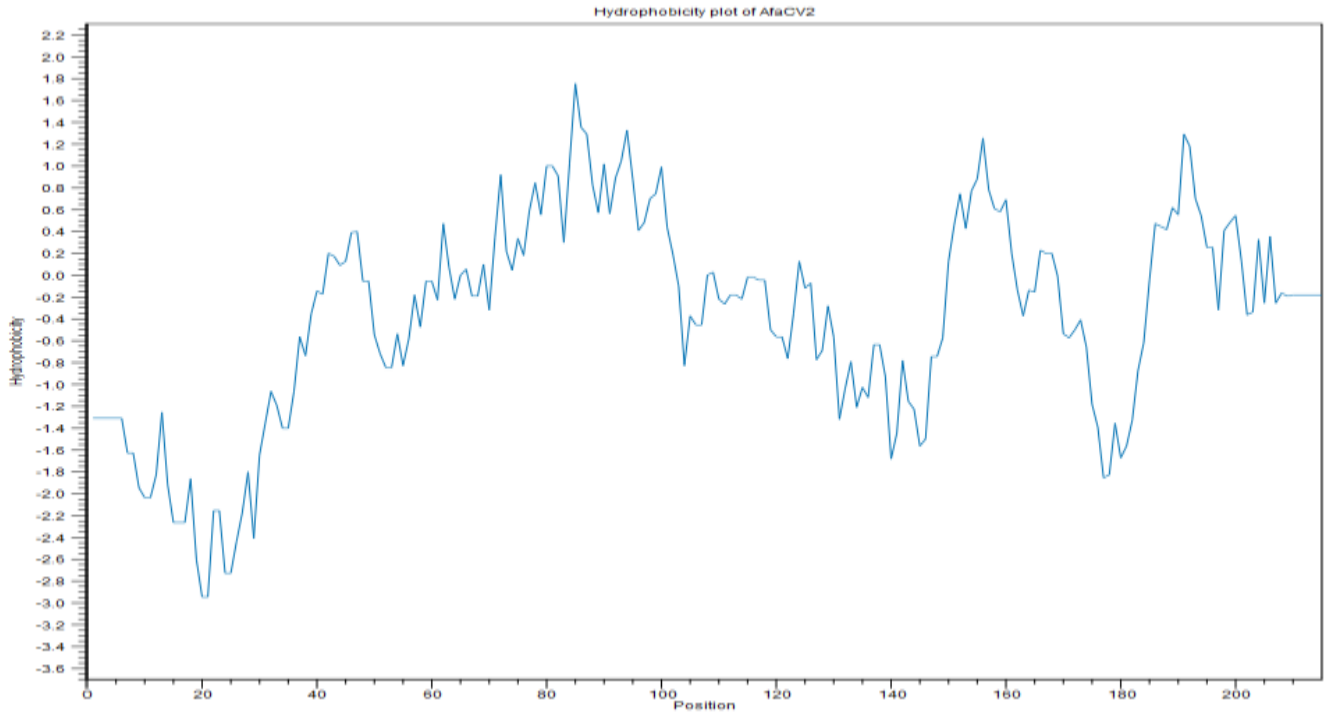
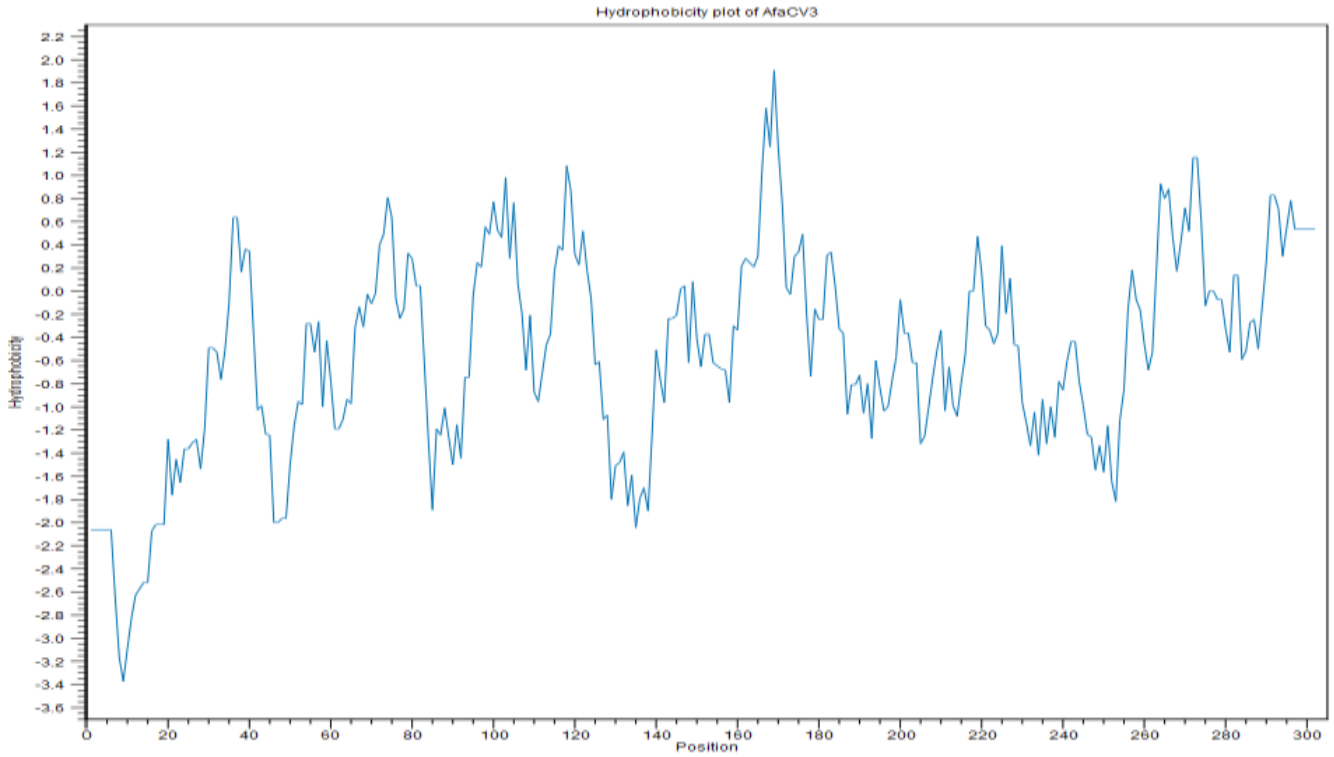
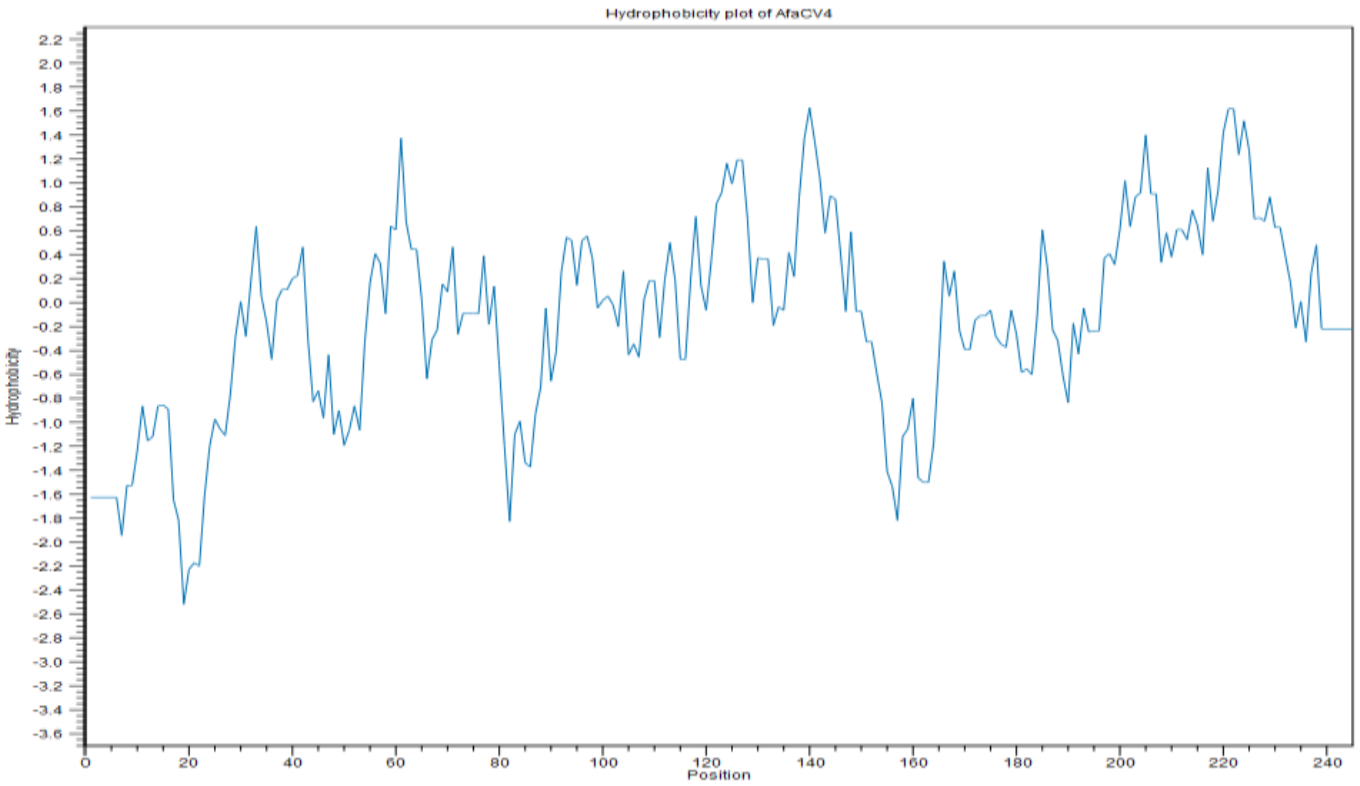
Figures 1.3-1.12. Hydrophobic plots of hypothetical capsid protein



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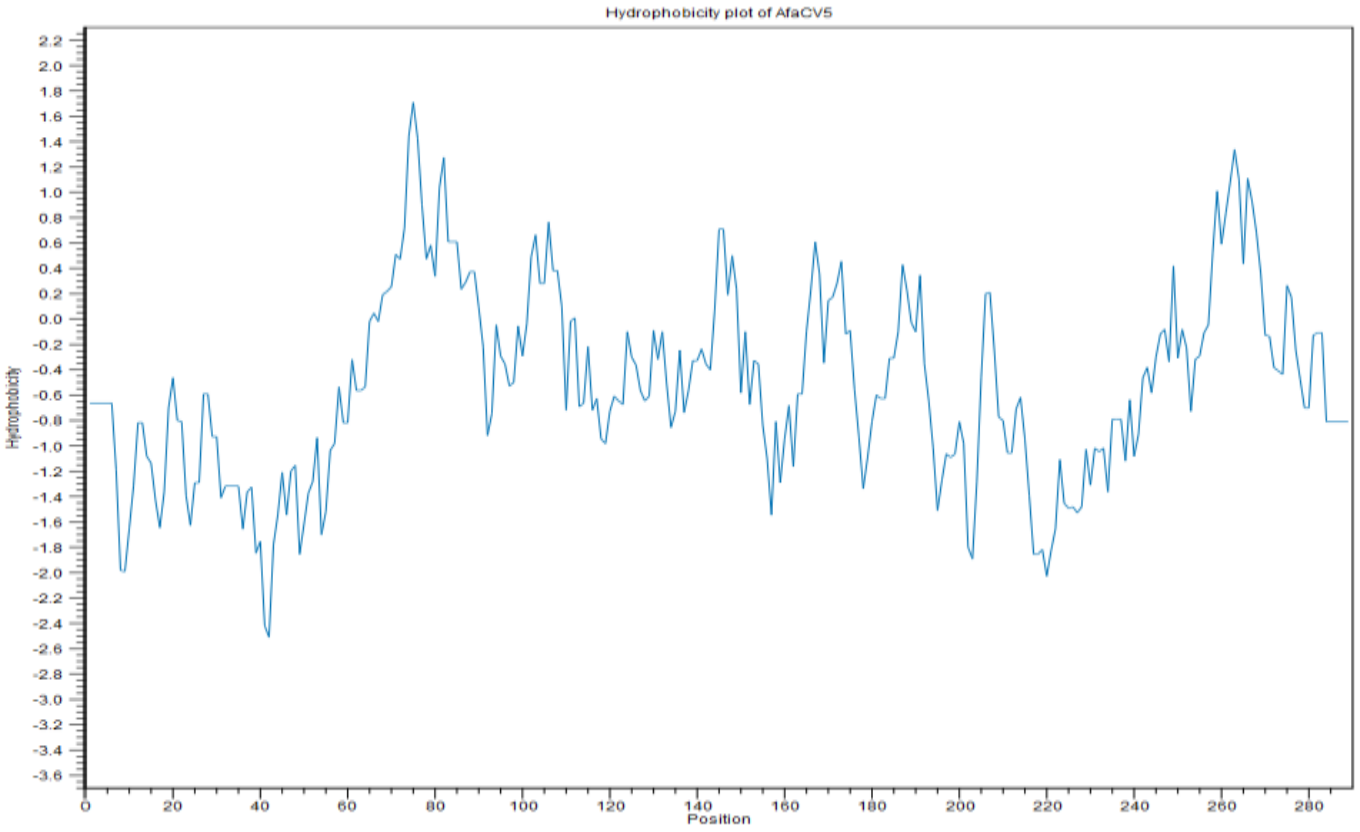
Hydrophobicity
Position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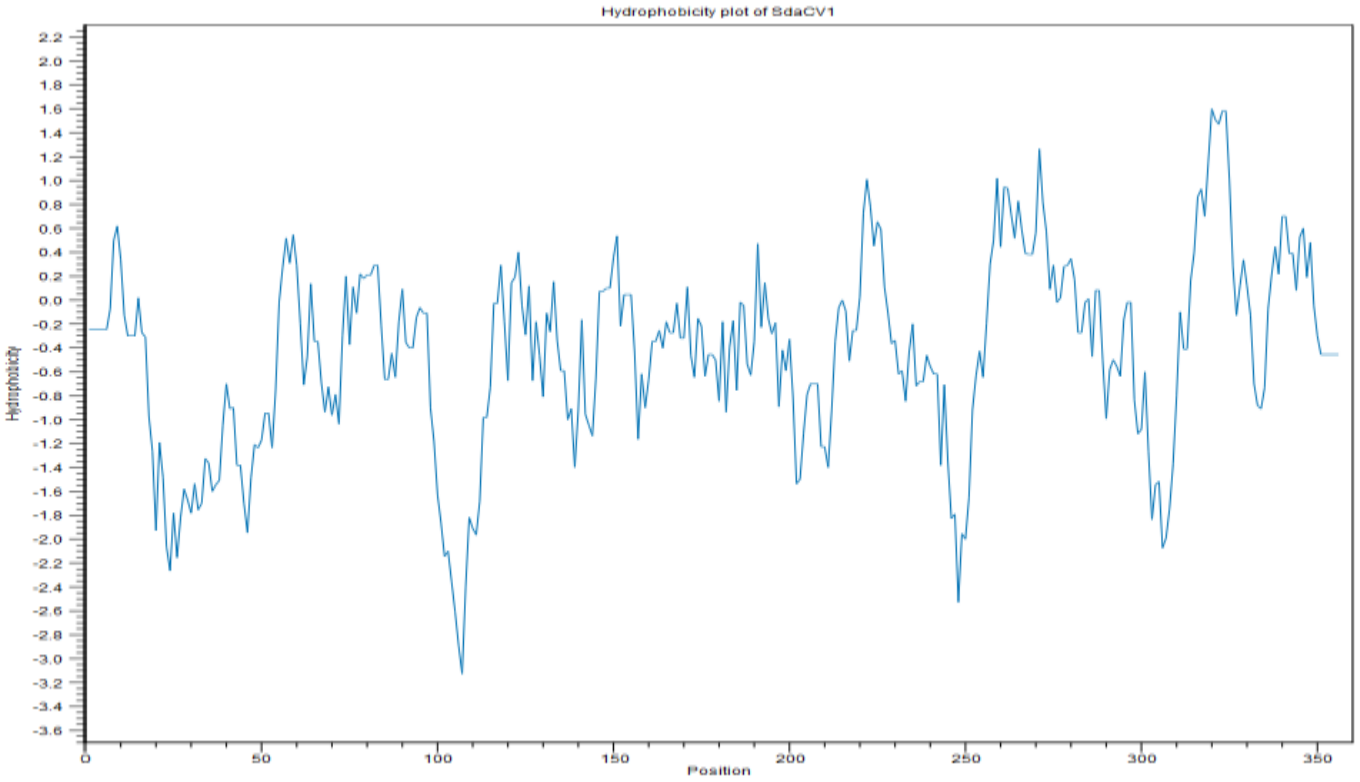
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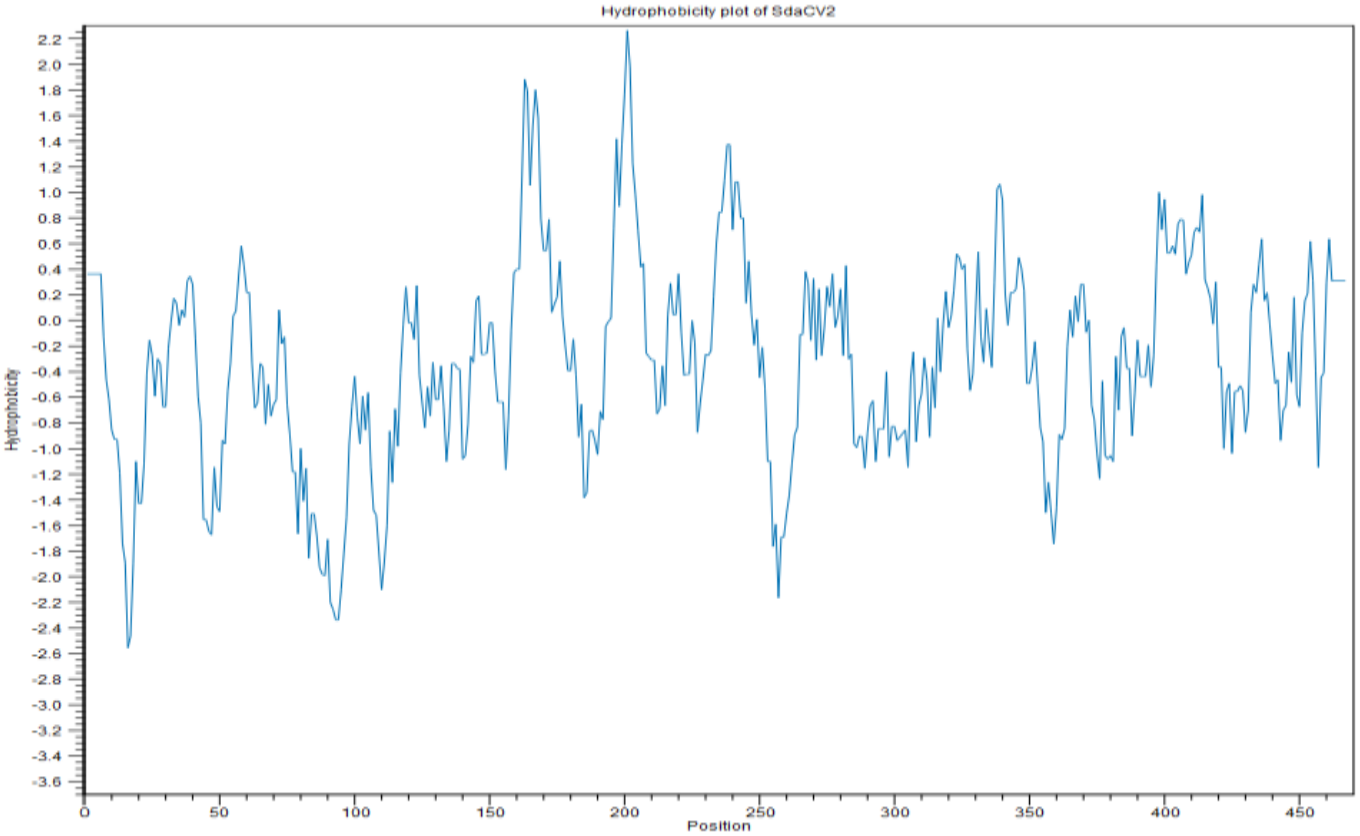
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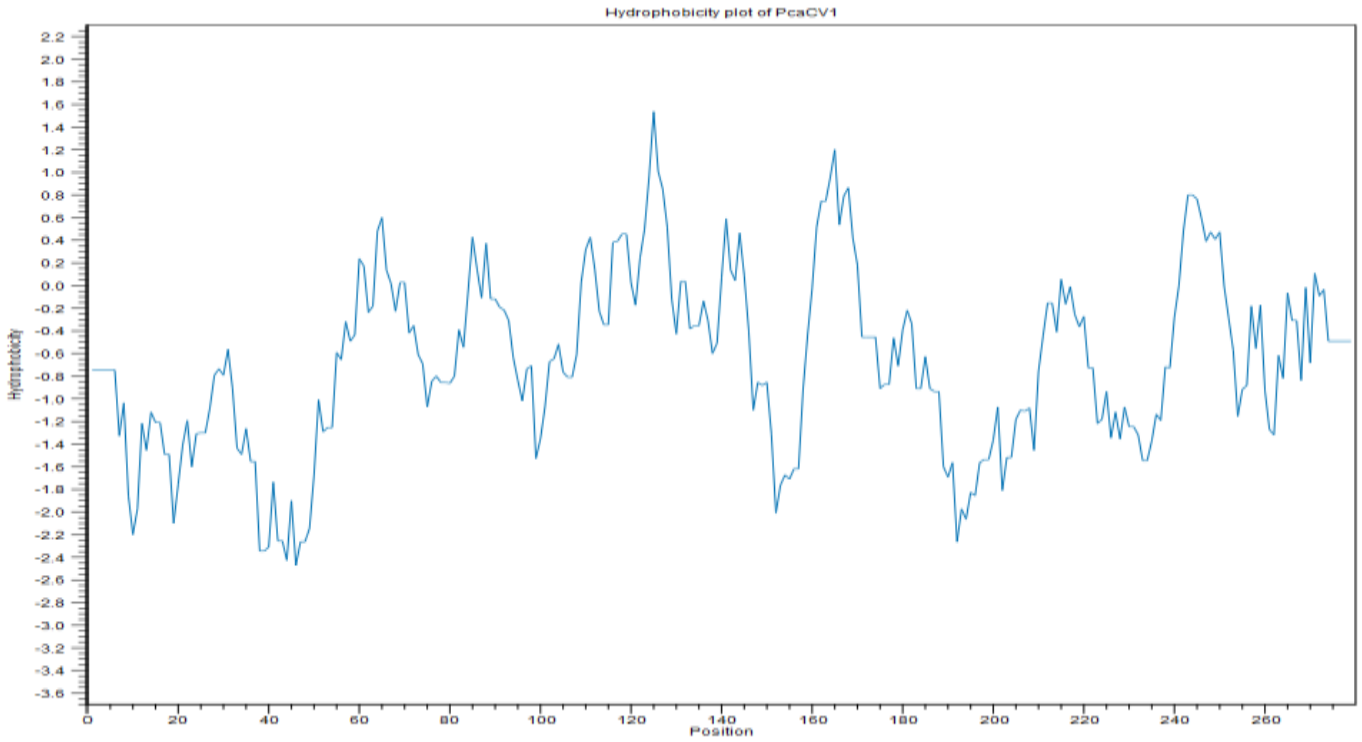
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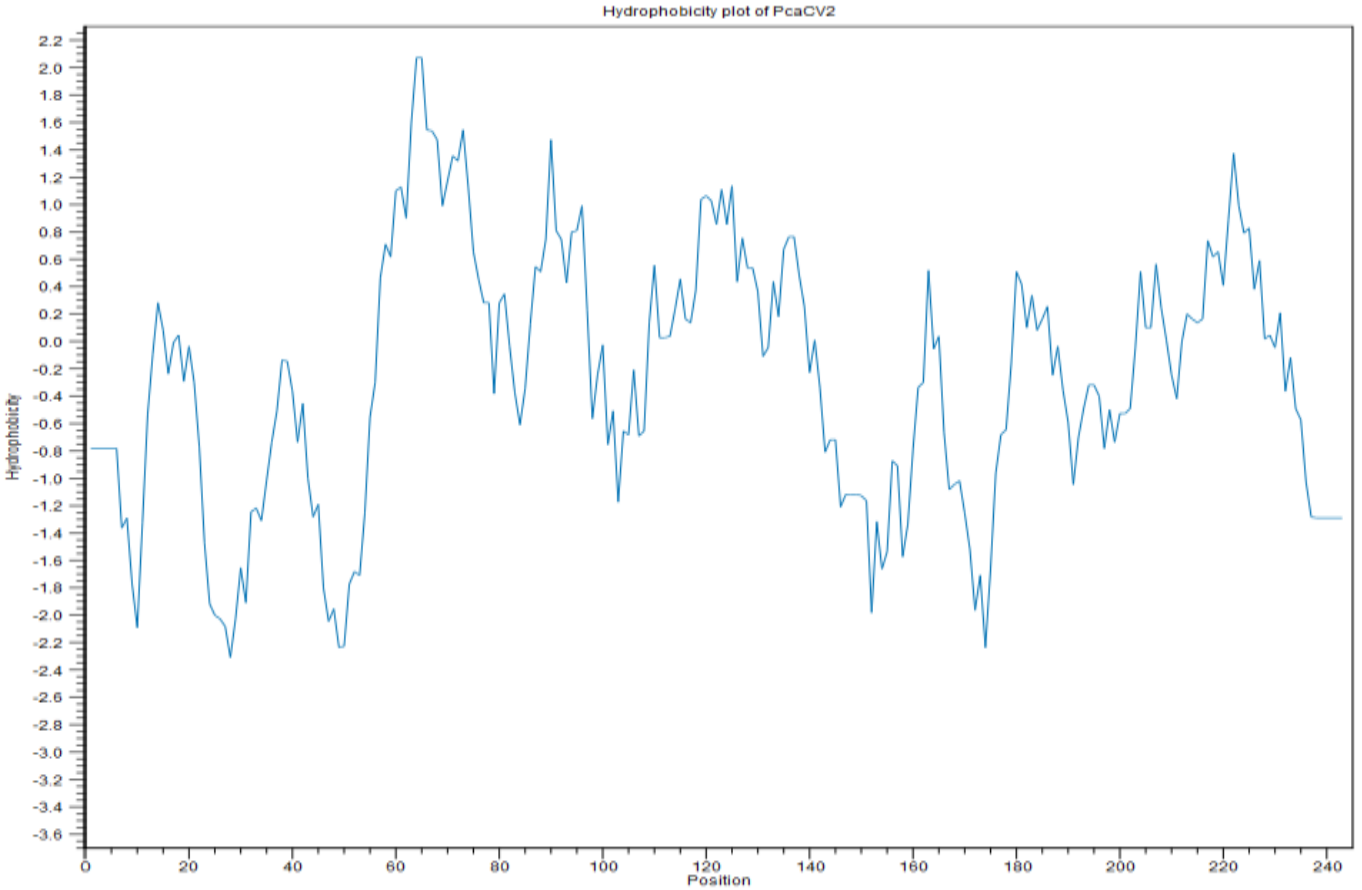
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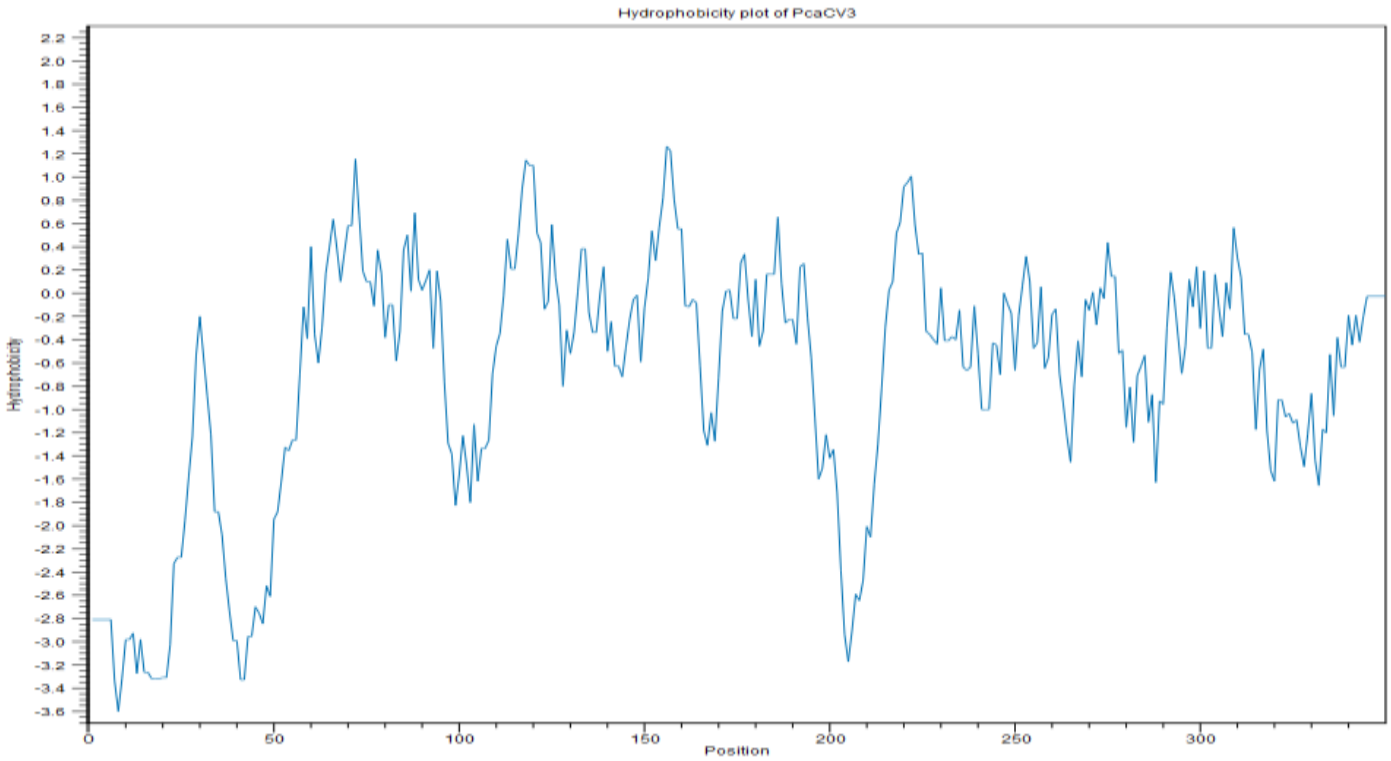
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0.0
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-0.4
-0.6
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-1.6
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-2.2
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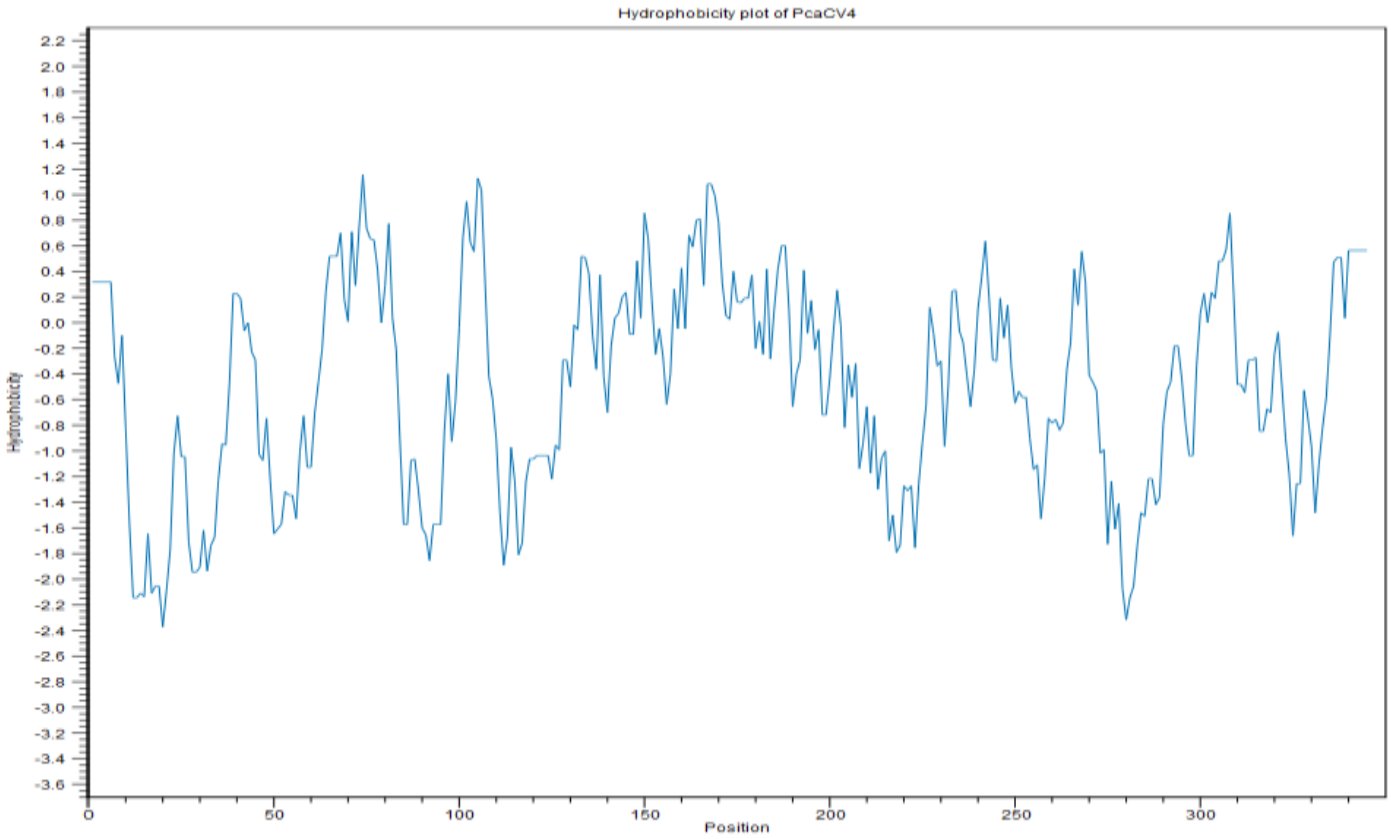
Hydrophobicity plot of PcaCV4
Hydrophobicity
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Chapter 2

Diversity and community composition of prokaryotes associated with Asteroids

Elliot Walter Jackson [1*], Charles Pepe-Renney[2], Spencer Debenport[2], Dan Buckley[1,2] and Ian Hewson[1]

[1] Department of Microbiology, Cornell University, Wing Hall, 123 Wing Drive, Ithaca NY 14853, USA

[2] School of Integrative Plant Science, Cornell University, Ithaca NY 14853, USA

***** Correspondence: ewj34@cornell.edu; Tel.: +1-231-838-6042

Abstract:

Microorganisms can have dramatic impacts on the biology and ecology of their associated host though our knowledge of these interactions in marine organisms is limited to a few well studied systems. Asteroids (sea stars) have a highly reduced digestive tract, a water vascular system and a complex innate immune system making them a potentially unique microbial host system. We characterize the microbiome of asteroids using 16S rRNA gene amplicon sequencing by comparing four different potential microbial environments within an asteroid– epidermis, gonads, pyloric caeca and coelomic fluid – and compare the microbiome across 12 asteroid species and to their surrounding environment. We found that the microbial community associated with sea stars was distinct though not unrelated from their ambient environment which suggests selection of the microbial community by the host despite the intake and distribution of sea water throughout the body. Proteobacteria (α-,β-,γ-proteobacteria) consistently dominate the community composition making up to 70-80% of the relative proportion in sample libraries and overall community composition of Proteobacteria was highly

similar between host species. Spirochaetaes and Tenericutes were also found in relatively high proportions though were highly variable across sample libraries of different tissues types. Variability between microbial communities was significantly lower in coelomic fluid compared to pyloric caeca, gonads and epidermis tissues of the animal. Overall, the bacterial community of asteroids is highly strict across host taxa and geographic location and does not mimic the microbial community composition in the surrounding environment.

Introduction:

The knowledge gained from studying animal-microorganism interactions can bring about new ways for understanding the biology and ecology of the host of interest. Among the most well-known roles of microorganisms is diet supplementation of their associated eukaryotic hosts through metabolizing organic or inorganic material that the host cannot otherwise utilize. Microorganisms can influence the behavior of their host which is shown from examples of symbiotic bioluminescent bacteria in marine invertebrates and fish (1, 2). Non-intimate symbioses of bacteria are also significant and are important for the development of many planktonic marine invertebrates that require a microbial cue from bacteria biofilms in order to settle and metamorphose (3). Without considering the influence of microorganisms on host biology and ecology the study of the larger organism is incomplete. For this study we characterize the bacterial diversity and community composition within and between asteroids and discuss the role microorganisms might play in regards to asteroid biology.

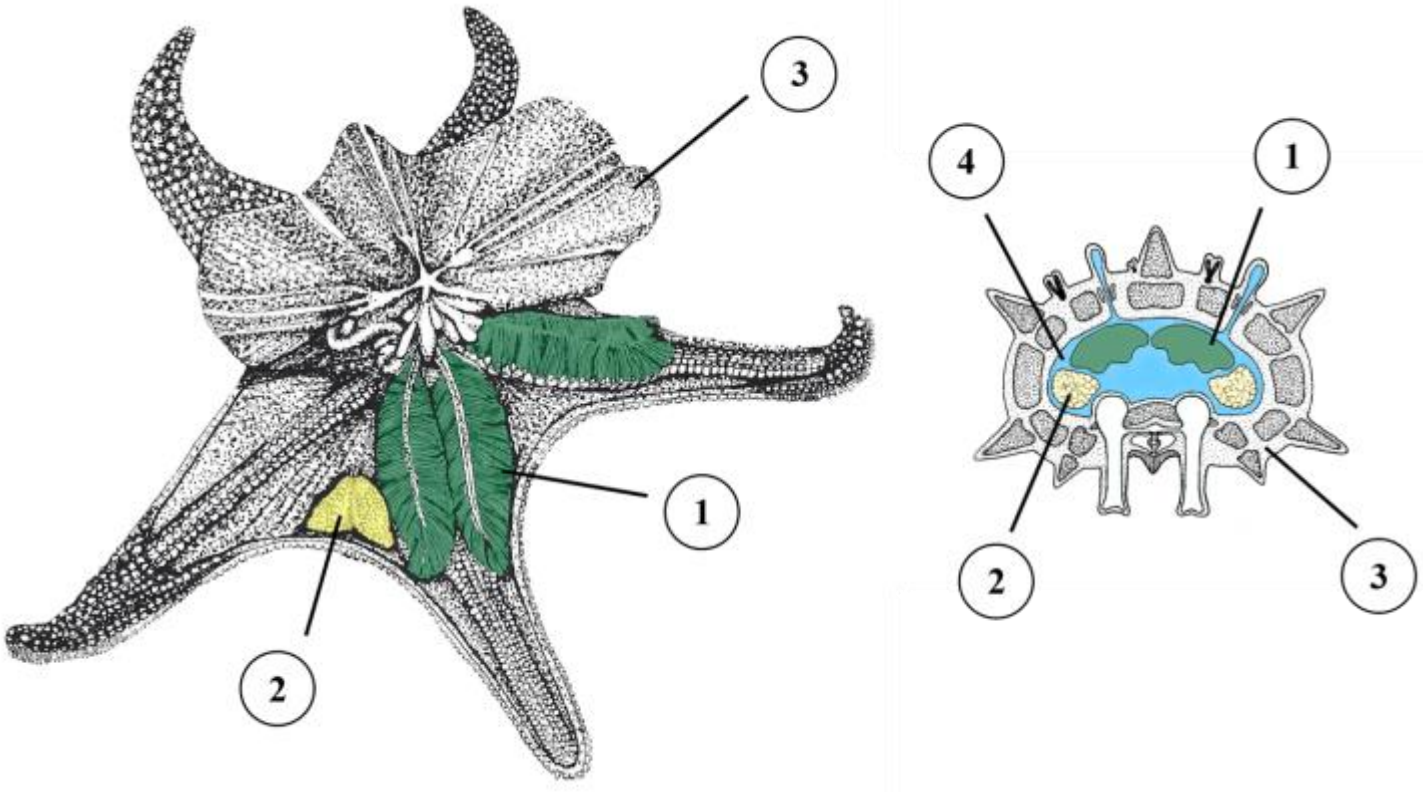
Asteroids are strictly marine organisms that are found globally in a variety of different benthic environments including the rocky intertidal, coral reefs, abyssal plains and polar waters where they commonly occupy the top trophic level as predators. In rocky intertidal environments asteroids, notably *Pisaster ochraceus*, and *Stichaster australis*, have dramatic impacts of the

community composition through the regulation of bivalves and are identified as keystone species meaning they have a disproportionate impact relative to their abundance (4–6). Like many marine invertebrates, asteroids have a planktonic larval stage that is different in both the biology and ecology from their benthic adult stage. We focus on the adult asteroid microbiome for this study.

We defined the microbiome for each asteroid by comparing four different potential microbial environments– body wall (epidermis), gonads, pyloric caeca and coelomic fluid - using high throughput 16S rRNA gene amplicon sequencing of the V4 region (Figure 2.1). These sample types from the animal were chosen for the reason that they might represent different microbial niches within the animal. Asteroids are relatively simplistic anatomically by having a perivisceral coelom that contains a network of fluid-filled canals that make up the water vascular system and only two major organs, the pyloric caeca and gonads, that are suspended by the perivisceral fluid i.e. coelomic fluid. Digestion for asteroids generally occurs outside of the body by inverting the stomach on their food source. This method of feeding results in little indigestible material be taken up in the animal. The pyloric caeca is an extension to the cardiac stomach where final absorption of nutrients is thought to take place (7). The coelomic fluid is produced through osmosis of water from the water vascular system and body wall into the coelomic cavity. The water vascular system is unique to the phylum Echinodermata and functions by bringing water from their surrounding environment and distributing it through a network of canals to the tube feet for locomotion. The direct intake of sea water into the animal might then highly mimic the microbial community composition of its surrounding environment if the host is not selective. We therefore compared the microbiome composition of the sea star to the microbial community composition of the animal's surrounding environments (both sea water and sediments) to gauge

the degree of selection by the host. Finally, we compare the microbiome composition of 12 asteroid species, spanning 4 orders and 7 families in the class *Asteroidea*, to examine the variability across host taxa. We provide a detailed analysis of the healthy microbiota of adult asteroids which shows little variability across taxonomy of the host but is distinct from the microbial community of sea water.

Figure 2.1 A) Basic anatomy of an asteroid modified and reproduced with permission from J.S. Pearse in: *Starfish: Biology and Ecology of the Asteroidea* 2013. (**B**) Cross section of a ray modified and reproduced with permission from BIODIDAC. Samples used for microbial community analysis are colored and labeled as followed: **1**) pyloric caeca (green), **2**) gonads (yellow), **3**) epidermis (grey) and **4**) coelomic fluid (blue).



Methods:

Sample collection:

Samples used in this study were collected from various locations in the Salish Sea off the coast of Washington State, United States and two locations off the coast of Queensland, Australia (Table 2.1). Asteroids used in this study were photographed for taxonomical verification and inspected for signs of disease and abnormalities. All asteroids used in this study appeared completely asymptomatic upon collection. Asteroids in the Salish Sea were collected onboard the R/V Clifford Barnes by dredging from depths between 24 to 50 meters. Sediment and surface water samples were taken from the same locations where asteroids were collected as well as from several locations in the surrounding area. Sediment samples were collected using a Van Veen Grab. Immediately after retrieval, the sediment was cored using a sterile 3mL syringe and the 0-1 cm sediment surface layer was used for DNA extraction. 20L of surface water was collected that was first prefiltered through a 150mm GF/A [Cat.No 1820-150] then collected on a 142-mm, 0.22 um Durapore membrane filter [Cat. No GVWP14250]. Asteroids collected in Queensland, Australia were from Moreton and Heron Island. Animals were collected during low tide from 1 to 2 meter depths. Surface water and sediment were collected at the same locations of asteroids. 1-2 L of surface water was collected by filtering through a 0.22 um sterivex filter [Cat. No SVGP01015] and surface sediment was collected by scooping sediment into a sterile 15mL centrifuge tube. Asteroids collected were processed immediately upon collection. Coelomic fluid was extracted first using a 25Gx1½ (0.5mm x 25mm) needle [Cat. No 305127] attached to a 3mL syringe [Cat. No 309657]. Pyloric caeca and gonads were collected next using sterile forceps from the coelomic cavity after making a small insertion into the epidermis. Tube feet and epidermal tissue along the ambulacral groove were collected last by vivisection. All samples

were frozen in liquid nitrogen upon collection in sterile 15mL tubes and kept at -80°C until processing. Tissue and fluid samples from three adult *Evasterias troschelii* kept in aquaria containing artificial sea water at Cornell University for 6 months were also used in this study. These *Evasterias troschelii* were collected from Dutch Harbor, Alaska in September of 2015.

DNA extraction and sequencing

DNA extractions were performed following the manufactures protocols for each sample using Zymo Research Fungal/Bacterial DNA Miniprep kits [Cat. No D6005] except for sediment samples in which the Zymo Research Soil Microbe DNA MiniPrep kits [Cat. No D6001] were used. 250 mg of sediment was used for DNA extraction. Roughly ¼ of the 142-mm, 0.22 um Durapore membrane filter was used for DNA extraction while the whole sterivex filter was used for DNA extraction. Approximately 100 mg of animal tissue and 1-2 mL of coelomic fluid were used for DNA extraction (Table 1.3). The coelomic fluid was first spun at 15,000 x g for 5 min then resuspended in 200µl of nuclease-free water prior to DNA extraction. Following DNA extraction, samples were held at – 20 °C prior to PCR amplification.

PCR reactions were carried out in 96-well plates using dual-indexed barcoded primers of the V4 region of the 16s rRNA gene (8). For each sample 5ng of DNA template was amplified in triplicate 25µl total volume PCR reactions using the 515f (5' – GTGCCAGCMGCCGCGGTAA – 3') and 806r (5' – GGACTACHVGGGTWTCTAAT – 3') primers at 50°C annealing temperature for 30 cycles. Triplicate samples were pooled after PCR amplification, purified and normalized at 25 ng DNA/sample. All amplicon products were pooled in equal concentrations for sequencing using Illumina MiSeq 500 bp sequencing v2 * (e.g. 2 x 250 bp) at the Cornell Institute of Biotechnology. Duplicate blanks consisting of elution solution as template were performed with each PCR run but no amplification was observed so therefore they were not

included for any downstream analysis. The elution solution used for blanks however were not run through the DNA extraction kits.

16s community sequence analysis

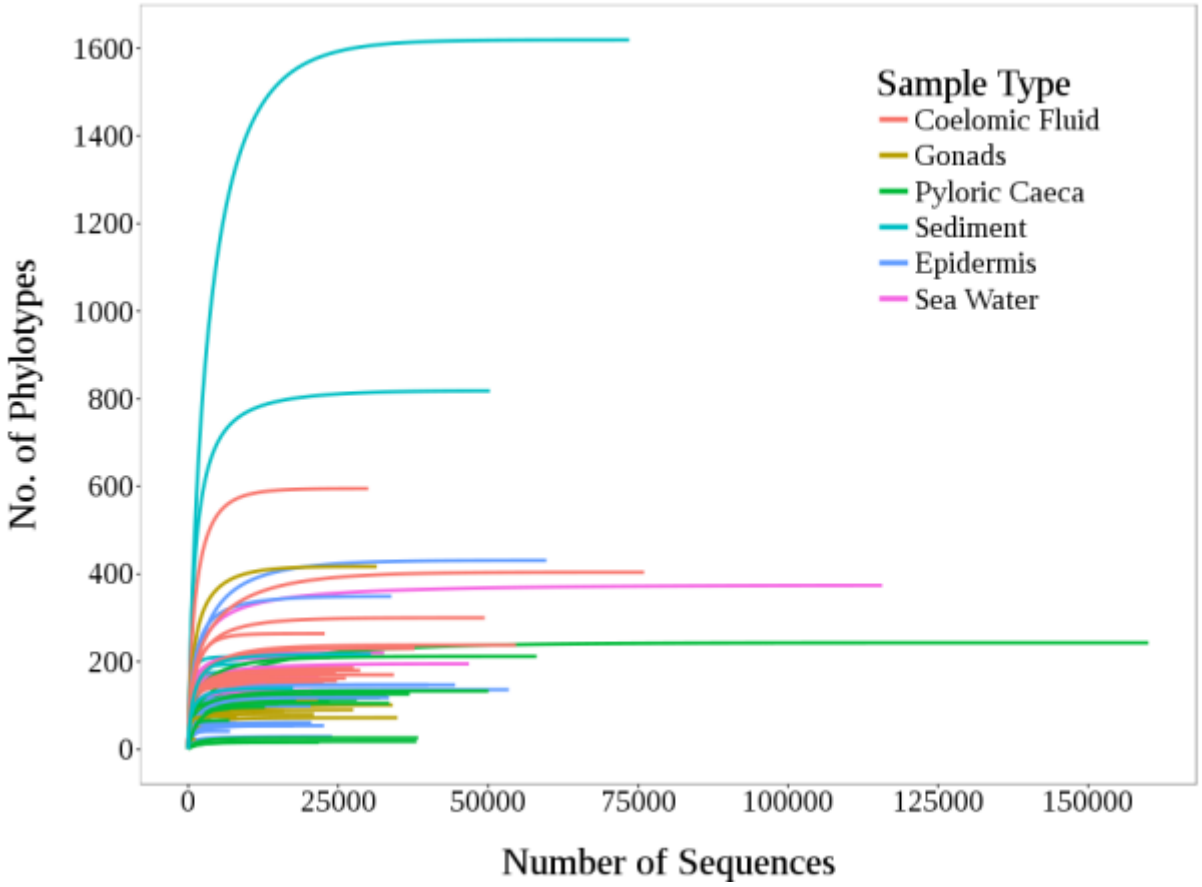
Reads were first demultiplexed then analyzed for quality to determine trimming parameters. Reads were inspected by the average quality per base of the forward and reverse reads separately. The first 10 nucleotides for each read was trimmed and the total length of reads were truncated to 150 nucleotides due to the decrease in quality score observed after 150 nucleotides in both the forward and reverse reads. Reads containing any ambiguities were removed as well as reads exceeding the probabilistic estimated error of 2 nucleotides. Quality parameters were enforced on both paired-end reads and if one of the reads did not pass the filtering parameters both reads were removed. Total amplicon length after trimming and filtering was 232 nucleotides with a 60 bp overlap between the paired reads**.** After quality screening and trimming, sequence variants were determined using DADA2 as well as chimeric variants which were subsequently removed from further analysis (9). The sum of reads for all libraries passing the quality control parameters mentioned above (n=103) totaled 2,428,987 with a mean of reads per library of 23,580. Only libraries containing at least 1000 reads were used for analysis which resulted in 93 libraries (n=75 for asteroids and n=18 for environmental samples). The SILVA 123 database was used for taxonomical assignment. Reference sequences in the SILVA 123 database were first trimmed to the V4 region with the 515f-806r primers used in the PCR reactions. Taxonomy assignments were performed using UCLUST with a minimum confidence threshold of 80% (10). Sequences identified as chloroplast or mitochondrial were removed from libraries prior to analysis. Diversity measurements (α and β) were analyzed using the phyloseq R

package (11). The function *betadisper* in the vegan R package was used to calculate mean dissimilarity distances of sample types based on Unifrac distance metrics (12).

Results:

The read depth of the libraries was sufficient to capture the total richness in the samples as seen by the saturation for all libraries in the rarefaction curve (Figure 2.2). The total number of sequence variants found among the asteroid samples were 3,485 which spread across 45 phyla. The total number of sequence variants found among the environmental samples were 3,578 which spread across 49 phyla. The number of sequence variants found within the

Figure 2.2 Rarefaction curve of microbial richness for each library. Colors correspond to tissue type and environmental samples.



environmental samples are not as great as reports of previous work that found 29,457 OTUs in seawater based on a 97% sequence similarity which is most likely due to the limited sampling locations in this study and the sequencing depth per library which is considerably lower than others (13). If a mean filter parameter of $>10^{-3}$ (i.e. sequence variants making up less than 0.1% of total community composition are excluded) is applied separately to the asteroids and environmental libraries the number of sequence variants dropped to 110 and 174 respectively. This shows that the majority of sequence variants found in libraries have low relative proportions which is not unusual for high-throughput 16s studies.

Within-community diversity indices (α) rank the environmental samples and coelomic fluid as having the highest richness and lowest evenness among communities with the coelomic fluid showing the least variation among samples. The gonads, pyloric caeca and epidermis samples all have a lower median α diversity index value with greater variation among samples (Figure 2.3, Figure 2.4).

Figure 2.3 Within-community diversity (α) measure. Shannon Diversity Index of tissue type and environmental samples

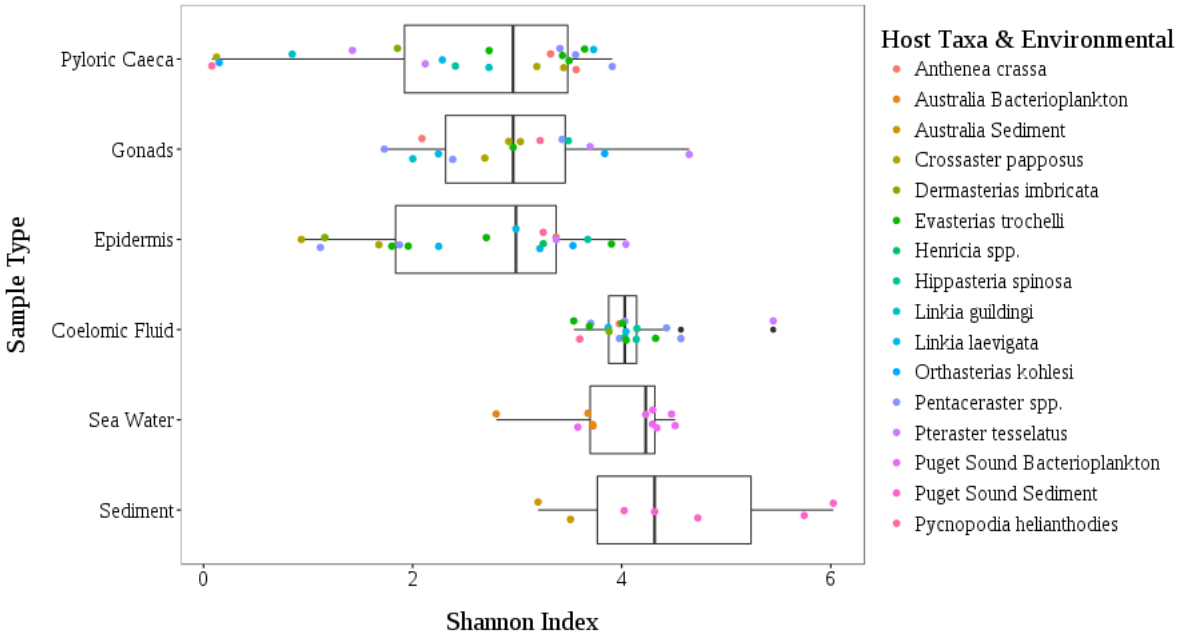
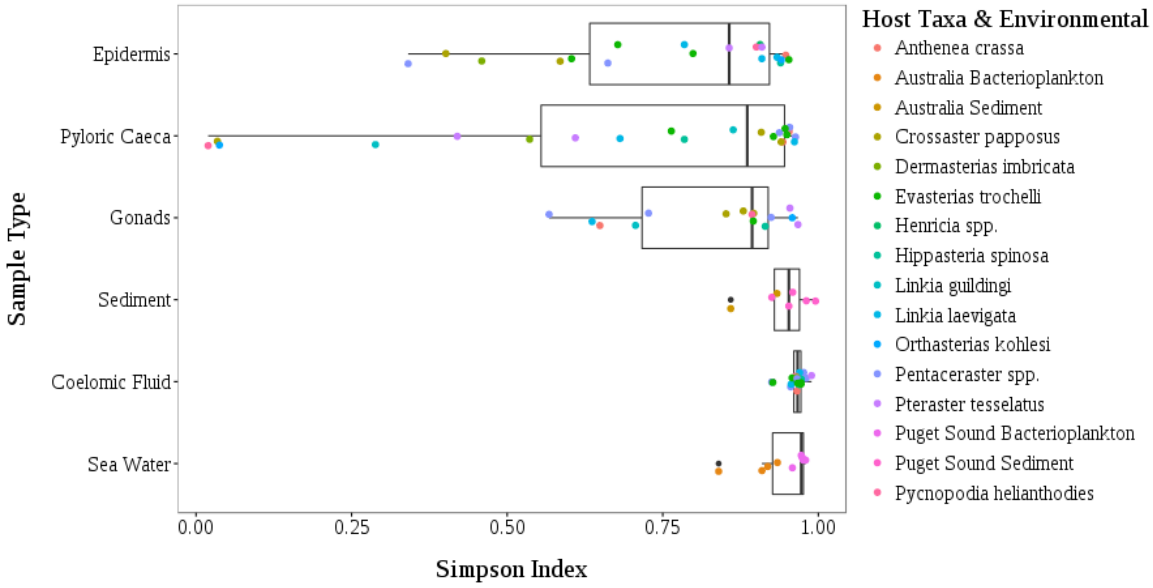


Figure 2.4 Within-community diversity (α) measure. Simpson Diversity Index of tissue type and environmental samples



Non-metric multidimensional scaling (NMDS) based on the weighted Unifrac distance values show that the asteroid associated microbial communities are distinct from microbial communities in their surrounding environment but share similarities (Figure 2.5). This suggests that the bacterial community composition is selected by the host despite the intake and distribution of sea water through the body via the water vascular system. The clustering of asteroid samples shows a similarity of community composition between the tissue types. The gonads, pyloric caeca and epidermis samples have much greater variations compared to the coelomic fluid (Figure 2.5, Figure 2.6). The weighted Unifrac distance values show a similar variation seen in the α diversity measures in that the coelomic fluid samples show the least dissimilarity between samples followed by the gonads, pyloric caeca and epidermis (Figure 2.6). No clear clustering pattern was observed between host species or geographic location of host species (data not shown).

Figure 2.5 Community similarity analysis. Non-metric multidimensional scaling (NMDS) of weighted Unifrac distances. Asteroid samples are shown in red boxes and environmental samples are shown in blue circles. Ellipses represent 95% confidence intervals of the respective

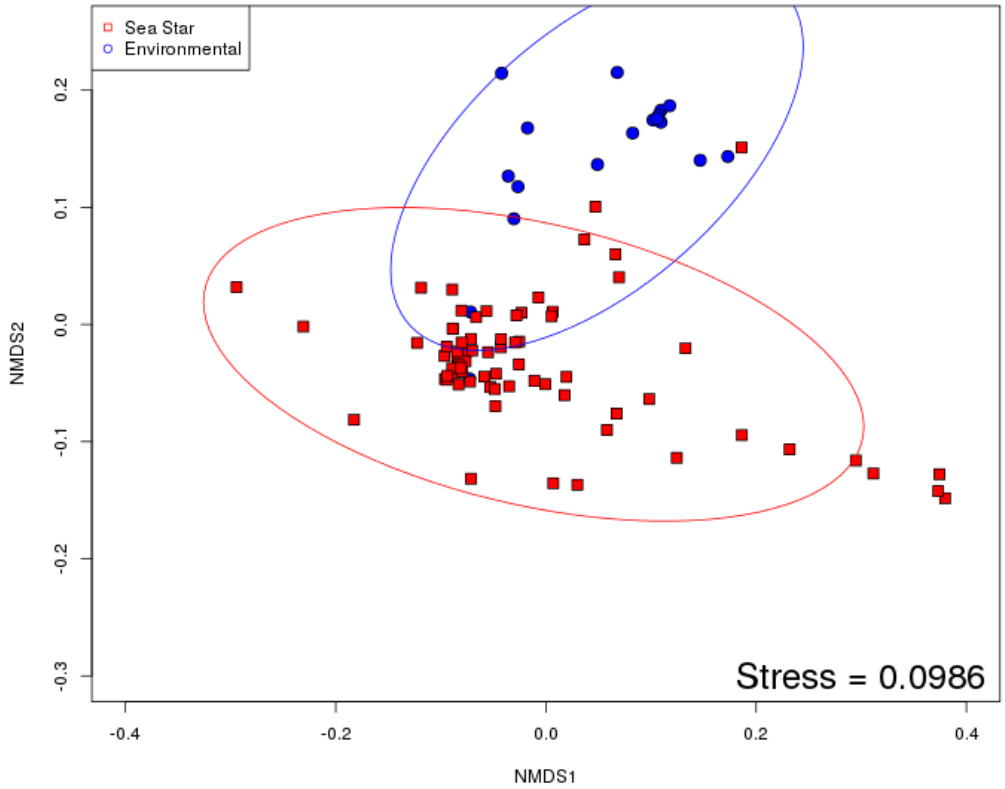


Figure 2.6 Intraspecific community dissimilarity measured as distance of samples to group centroid from weighted Unifrac distances. Vertical bar represents the median, the box represents the first to third quartiles and whiskers show the lowest or highest datum within 1.5 times the interquartile range of the lowest and upper quartile, respectively.

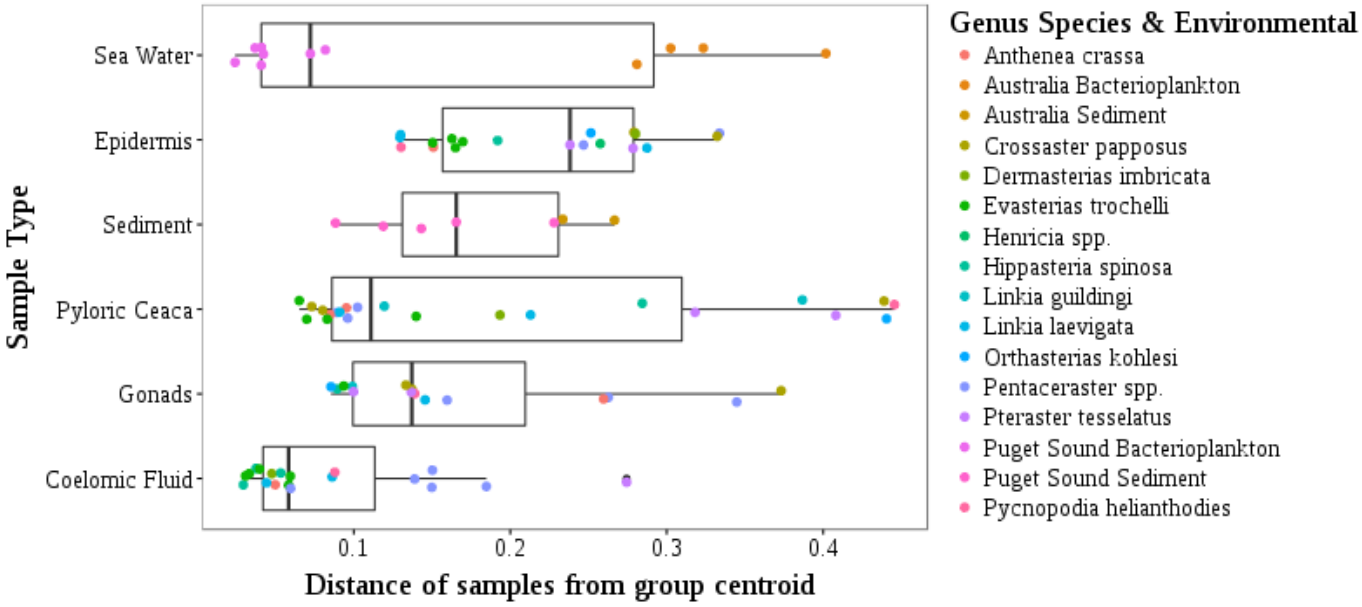
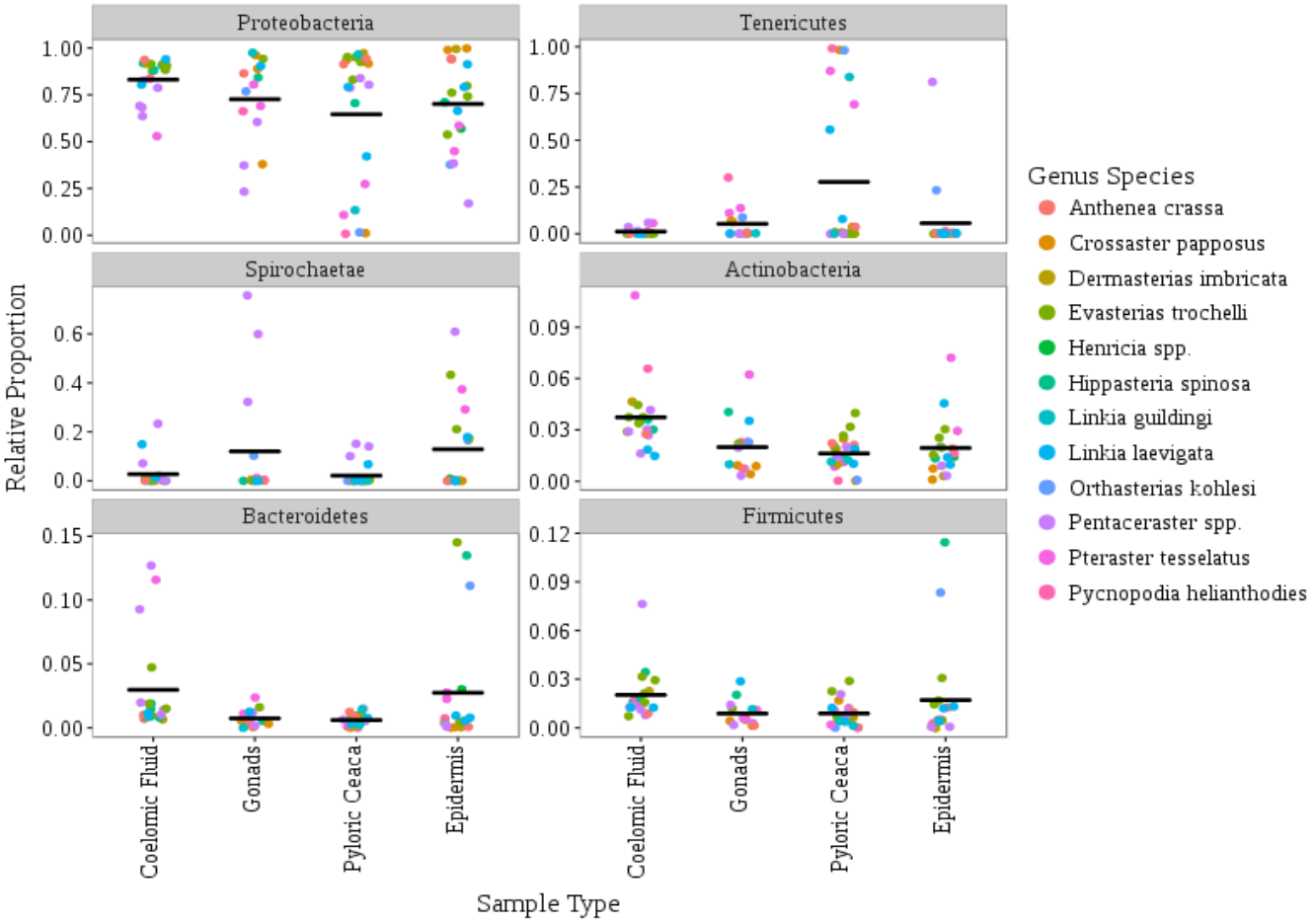


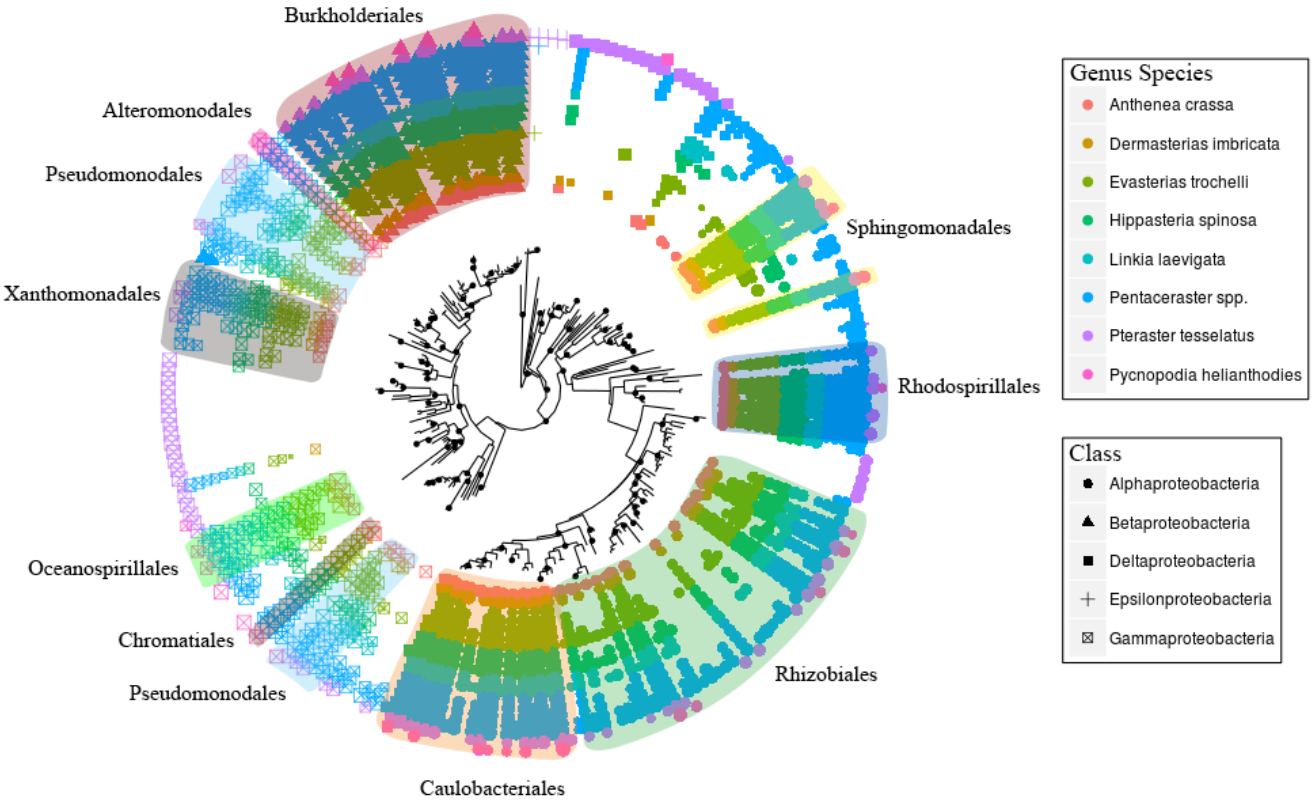
Figure 2.7 Relative proportions of top 6 bacterial phyla in asteroid tissue types. Horizontal black bar represents the mean value for the phyla associated with the respective tissue type.



Six bacterial phyla collectively make up the majority of asteroid libraries (Figure 2.7) These phylums include: Actinobacteria, Bacteroides, Firmicutes, Proteobacteria, Spirochaeata and Tenericutes. Actinobacteria, Bacteroides and Firmicutes are consistently found across the libraries but make up a small relative proportion collectively (<15%) (Figure 2.8). Cyanobacteria and Thaumarchaetoa were also found intermittently in the asteroid libraries but the relative proportion is generally low (<5%) although the relative proportion of Thaumarchaetoa in one library was approximately 50% (data not shown). Proteobacteria consistently dominate the

community composition making up to 70-80% of the relative proportion in libraries (Figure 2.7).

Figure 2.8 Unrooted phylogenetic tree of the Proteobacteria composition of coelomic fluid samples. Nodes labels shown represent ≥ 90% support. Colors correspond to the genus species of asteroid. Shape of points represent different classes within the phylum Proteobacteria. Orders within Proteobacteria that are well represented among libraries are highlighted and labeled.



The relative proportions of α-,β-,γ-proteobacteria are nearly equal and collectively make up the majority of Protebacteria composition. δ-, ε-proteobacteria were also found but at had significantly lower relative proportions compared to the α-,β-,γ-proteobacteria (Figure 2.9). The greatest richness of sequence variants within Proteobacteria were in the α followed by the γ, β, δ and ε (Figure 2.8). The Spirochaetaes and Tenericutes were found in greater relative proportions in the hard tissues (pylorica caeca, gonads and epidermis) compared to the coelomic fluid.

(Figure 2.7). The confidence threshold of 80% used for taxonomic assignment was set to this level otherwise sequence variants in Spirochaetae and Tenericute would not have been identified. The relative proportion of Tenericutes in 7 of the 22 pyloric caeca libraries and one of the epidermis libraries was 50% to > 90% of the community composition and the relative proportion of Spirochaetea contributed approximately 20% or more in 11 libraries.

Discussion:

Microscopy studies have demonstrated the presence of bacteria in adult asteroids in the subcuticular layer of the epidermis (14–16). The conclusion of these studies show the widespread presence of gram-negative rod and spiral shaped bacteria among the subcuticular layer of asteroids. Here we show the association of bacteria with four different tissues within asteroids which corroborates and significantly builds upon previous work. The microbial communities we found among the tissue types are similar to each other suggesting that the microbial community composition in the perivisceral coelom is relatively uniform in bacteria community composition. The relative proportion of sequence variants assigned to Spirochaetea and Tenericutes was greater in the hard tissues compared to the coelomic fluid although the variability between libraries was large. This would explain the greater variation in the α and β diversity estimates of the hard tissues compared to the coelomic fluid. The small variation in community composition among the coelomic fluids from species that were collected from two completely different geographic locations and habitats was striking. Furthermore, species sampled in this study represent carnivores and detritivores. This suggests that host phylogeny and animal diet does not play a large role in microbial community selection. It also suggests that the selective properties of the coelomic fluid (nutritional or host immune related) are uniform across species.

Apart from the hard tissues, this study is the first to report the presence of bacteria in the coelomic fluid of asteroids. Whether the bacterial cells in the coelomic fluid are free living in the fluid or are present due to sloughing of cells from biofilms formed on the coelomic epithelium or organs is unknown. The coelomic fluid was thought to be a sterile medium for the reason that coelomic cells which freely circulate the coelom are found in the fluid and are immune cells that have phagocytic abilities. Future studies should investigate bacterial cell abundance in the coelomic fluid using microscope techniques and examine if bacterial cell abundance fluctuates through feeding, reproduction, environmental patterns and disease progression.

The lack of a clear distinction between the microbial community of the pyloric caeca and other areas in the animal was unexpected given the general specificity of microbial communities found among digestive tissues in metazoans. This relationship is seen in ruminants, shipworms, insects, and humans among others (17–20)**.** Because digestion does not occur primarily in the pyloric caeca could be a reason for the lack of a specialized microbial community. The finding of Tenericutes in greater relative proportion within the pyloric caeca samples is in agreement to similar studies that have investigated the microbial consortium of the stomach or digestive tissues (22–24). The common occurrence of these bacteria in digestive organs goes unexplained but also have been found associated with terrestrial and aquatic organisms across the metazoan evolutionary tree from sponges to chordates (24, 25). The fact that the pyloric caeca does not harbor a unique microbial community in comparison to other tissues suggests that the role microorganisms play within the perivisceral coelom is not different between tissues.

The perivisceral coelom is a spacious cavity filled mostly by gonads and pyloric caeca that are surrounded by the coelomic fluid, a fluid that accounts for 23-27% of the body weight in *Pisaster ochraceus* (26). Asteroids lack any type of specialized excretory organ for waste

removal so metabolic waste which is mostly in the form of ammonia (NH_3) is passed into the coelomic fluid and diffused through the body wall. The coelomic cells were once hypothesized to be involved in nitrification but this was not supported by experimental evidence (27). We suggest an alternative – that bacteria fill this role in asteroids**.** This hypothesis is plausible given that many of the well-studied marine symbioses include microorganisms that carry out nitrogen fixation and/or nitrogen transformations (nitrification, denitrification, ANAMMOX) for their host (28). Nitrogen transforming prokaryotes have been reported in sponges (29) and corals (30) while nitrogen fixing prokaryotes are more common symbionts that are found in sponges (31), corals (32), sea urchins (33) and a variety of protists (34)**.** Future investigations should employ metagenomics or genomic analysis of cultured isolates to functionally define the microbial community found in asteroids.

Finally, invertebrate models have been of particular interests for studying animal-microorganism interactions in the context of the innate immune system and the mechanisms for which the host can differentiate between a beneficial microbe and a pathogen (35). Echinoderm model systems like *Patiria miniata*, the bat star, or *Stronglylocentrotus purpuratus,* the purple urchin, would be exceptional invertebrate organisms for studying the early evolution of these mechanisms in deuterostomes. *S.purpuratus* encodes for 222 toll-like receptors (TLRs) making the complexity of innate immune system much greater in comparison to other model systems such as *Drosophila melanogaster* which encodes for 9 TLRs or *Ciona intestinalis,* sea squirt, which encodes for 2 TLRs (36–38). Continued study in this avenue could reveal novel and ancient immunological pathways for dealing with microbial relations.

Microorganisms can have dramatic impacts on the biology and ecology of their host though our knowledge of these intimate relationships for marine organisms comes mostly from

just a few well studied systems. Here we report the diversity and community composition of microorganisms associated with asteroids in comparison to their environment (sea water and sediment), across animal tissue types and between species. We found that the bacterial community composition of asteroids is distinct from their surrounding sea water but share similarities. The microbial community associated with tissues types within the perivisceral coelom are relatively similar though hard tissues (gonads, pyloric caeca and epidermis) vary more in community composition (driven by taxa in Spirochaetea and Tenericutes) than the coelomic fluid. Lastly, we find that the coleomic cavity is a highly strict environment within the animal in regards to Proteobacteria community composition. Our results provide a baseline knowledge of the asteroid microbiome that will facilitate further work in understanding the degree of reliance of asteroids to their associated microorganisms.

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Figure 2.9 Relative proportions of top 5 Proteobacteria classes in asteroid tissue types. Horizontal black bar represents the mean value for the phyla associated with the respective tissue type.

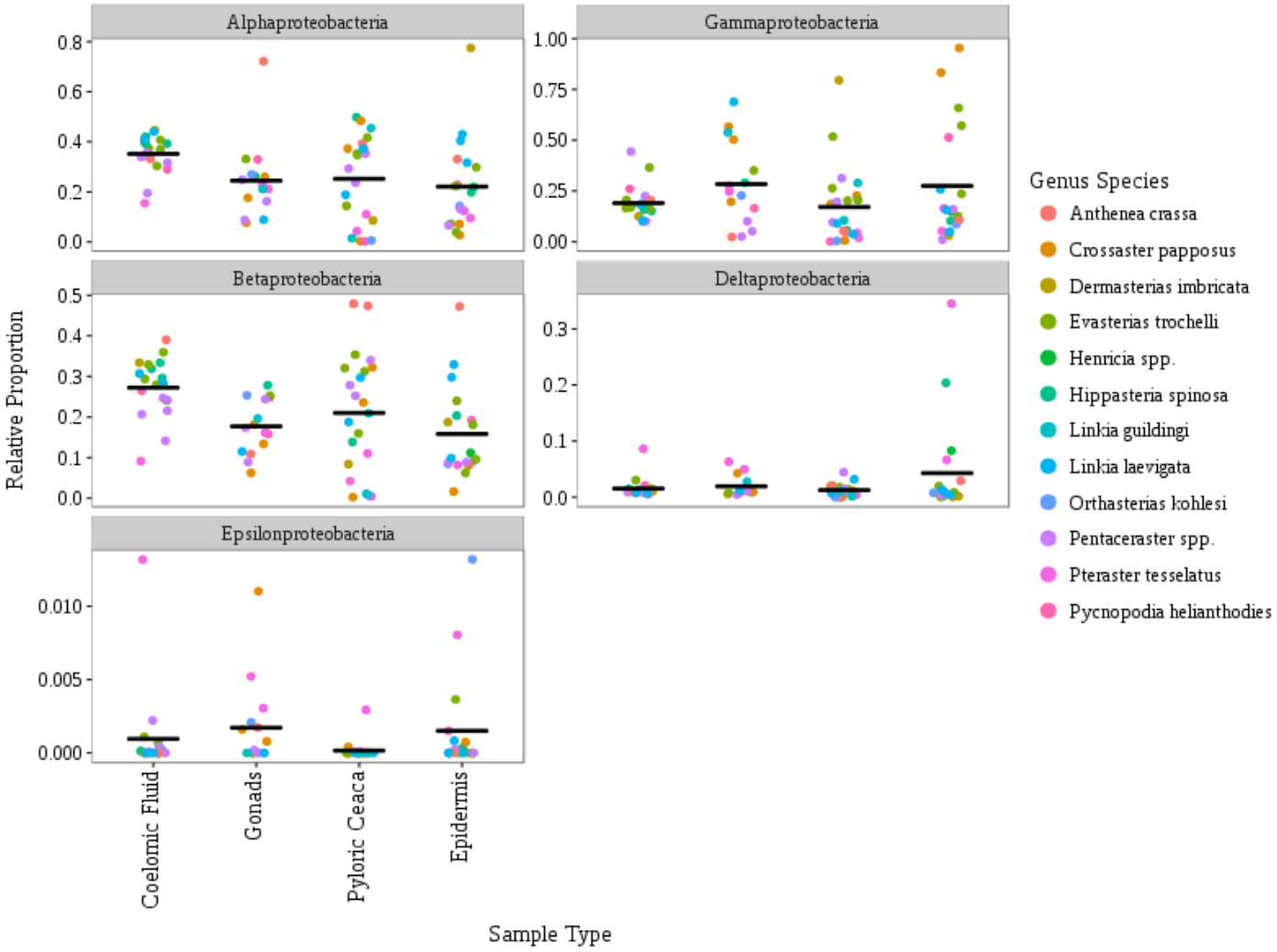

Figure 3.0 Community similarity analysis. Non-metric multidimensional scaling (NMDS) of weighted Unifrac distances calculated only from asteroid libraries. Top figure depicts community similarity between tissues types. Bottom figure separates community analysis shown in top figure by the top 6 bacterial phyla found in all libraries.

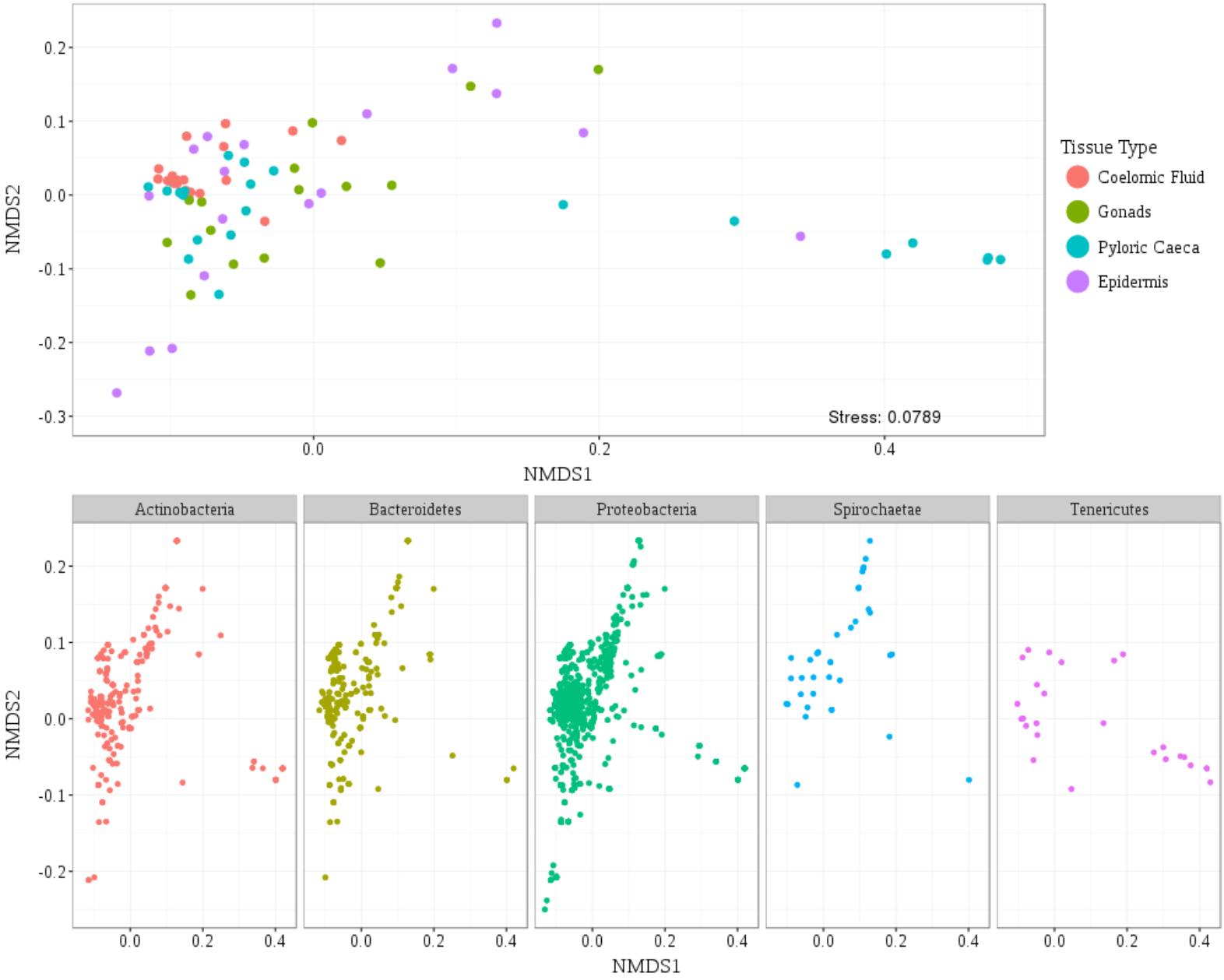

Table 2.1

Sample collection information and library size used for this study

Collection Location	Depth	Collection GPS	Sample Amount	Genus Species	Sample Type	Reads in Library
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	1 mL	Pentaceraster spp.	Coelomic Fluid	37736
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	1 mL	Pentaceraster spp.	Coelomic Fluid	27580
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	0.1145 g	Anthenea crassa	Pyloric Caeca	15022
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	2 mL	Anthenea crassa	Coelomic Fluid	24620
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	0.109 g	Anthenea crassa	Epidermis	4083
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	0.1306 g	Anthenea crassa	Gonads	27518
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	0.1013 g	Anthenea crassa	Pyloric Caeca	6302
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	2 mL	Pentaceraster spp.	Coelomic Fluid	75964
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	0.1078 g	Pentaceraster spp.	Epidermis	20498
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	0.1069 g	Pentaceraster spp.	Gonads	21600
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	0.1133 g	Pentaceraster spp.	Pyloric Caeca	12792
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	2 mL	Pentaceraster spp.	Coelomic Fluid	49399
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	0.1118 g	Pentaceraster spp.	Gonads	20942
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	0.1107 g	Pentaceraster spp.	Pyloric Caeca	6938
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	2 mL	Pentaceraster spp.	Coelomic Fluid	54647
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	0.1083 g	Pentaceraster spp.	Epidermis	6972
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	0.1247 g	Pentaceraster spp.	Gonads	34869
Moreton Bay, Australia	Subtidal	[-27.4802, 153.4034]	0.1160 g	Pentaceraster spp.	Pyloric Caeca	58076
Heron Island, Australia	Subtidal	[-23.4431, 151.9118]	2 mL	Linckia laevigata	Coelomic Fluid	34279
Heron Island, Australia	Subtidal	[-23.4431, 151.9118]	0.0452 g	Linckia laevigata	Epidermis	8051
Heron Island, Australia	Subtidal	[-23.4431, 151.9118]	0.1282 g	Linckia laevigata	Pyloric Caeca	17972
Heron Island, Australia	Subtidal	[-23.4431, 151.9118]	2 mL	Linckia laevigata	Coelomic Fluid	28676
Heron Island, Australia	Subtidal	[-23.4431, 151.9118]	0.1007 g	Linckia laevigata	Pyloric Caeca	50047
Heron Island, Australia	Subtidal	[-23.4431, 151.9118]	0.0992 g	Linckia laevigata	Epidermis	2656
Heron Island, Australia	Subtidal	[-23.4431, 151.9118]	0.1083 g	Linckia laevigata	Epidermis	33469
Heron Island, Australia	Subtidal	[-23.4431, 151.9118]	0.1801 g	Linckia laevigata	Gonads	8127
Heron Island, Australia	Subtidal	[-23.4431, 151.9118]	0.1053 g	Linckia guildingii	Pyloric Caeca	6860
Heron Island, Australia	Subtidal	[-23.4431, 151.9118]	0.1205 g	Linckia guildingii	Gonads	1215
Heron Island, Australia	Subtidal	[-23.4431, 151.9118]	0.1225 g	Linckia guildingii	Pyloric Caeca	33538
Washington State USA	24 m	[47.3934, -122.2626]	2 mL	Hippasteria spinosa	Coelomic Fluid	24785
Washington State USA	24 m	[47.3934, -122.2626]	0.1242 g	Hippasteria spinosa	Pyloric Caeca	19848
Washington State USA	24 m	[47.3934, -122.2626]	0.1121 g	Hippasteria spinosa	Gonads	14130
Washington State USA	24 m	[47.3934, -122.2626]	0.1131 g	Hippasteria spinosa	Epidermis	20150
Washington State USA	24 m	[47.3934, -122.2626]	2 mL	Hippasteria spinosa	Coelomic Fluid	28628
Washington State USA	24 m	[47.3934, -122.2626]	2 mL	Hippasteria spinosa	Coelomic Fluid	22589

Washington State USA	30 m	[47.5810, -122.3339]	2 mL	Evasterias trochelli	Coelomic Fluid	22736
Washington State USA	30 m	[48.2000, -122.5181]	2 mL	Evasterias trochelli	Coelomic Fluid	2726
Washington State USA	30 m	[48.2000, -122.5181]	0.0891 g	Evasterias trochelli	Gonads	2683
Washington State USA	30 m	[48.2000, -122.5181]	0.0952 g	Evasterias trochelli	Pyloric Caeca	14402
Washington State USA	30 m	[48.2000, -122.5181]	0.0998 g	Evasterias trochelli	Epidermis	19155
Aquarium Tank	-	-	2 mL	Evasterias trochelli	Coelomic Fluid	17335
Aquarium Tank	-	-	0.0356 g	Evasterias trochelli	Epidermis	20357
Aquarium Tank	-	-	0.0959 g	Evasterias trochelli	Pyloric Caeca	36655
Aquarium Tank	-	-	2 mL	Evasterias trochelli	Coelomic Fluid	26273
Aquarium Tank	-	-	0.0343 g	Evasterias trochelli	Epidermis	53410
Aquarium Tank	-	-	0.0911 g	Evasterias trochelli	Pyloric Caeca	22569
Aquarium Tank	-	-	2 mL	Evasterias trochelli	Coelomic Fluid	18860
Aquarium Tank	-	-	0.0345 g	Evasterias trochelli	Epidermis	44363
Aquarium Tank	-	-	0.1087 g	Evasterias trochelli	Pyloric Caeca	23483
Washington State USA	35 m	[48.2934,-122.4296]	0.1026 g	Crossaster papposus	Gonads	15961
Washington State USA	35 m	[48.2934,-122.4296]	0.1027 g	Crossaster papposus	Pyloric Caeca	31905
Washington State USA	35 m	[48.2934,-122.4296]	0.1231 g	Crossaster papposus	Epidermis	23960
Washington State USA	37 m	[48.1133, -123.0648]	0.0916 g	Crossaster papposus	Pyloric Caeca	23425
Washington State USA	37 m	[48.1133, -123.0648]	0.0977 g	Crossaster papposus	Gonads	10616
Washington State USA	37 m	[48.1133, -123.0648]	0.0880 g	Crossaster papposus	Epidermis	17564
Washington State USA	50 m	[48.4228, -122.5716]	0.1085 g	Crossaster papposus	Pyloric Caeca	21774
Washington State USA	50 m	[48.4228, -122.5716]	0.0997 g	Crossaster papposus	Gonads	34069
Washington State USA	30 m	[48.3529, -122.5476]	0.1053 g	Pteraster tesselatus	Gonads	5897
Washington State USA	30 m	[48.3529, -122.5476]	0.0992 g	Pteraster tesselatus	Pyloric Caeca	33023
Washington State USA	30 m	[48.3529, -122.5476]	0.1192 g	Pteraster tesselatus	Epidermis	59682
Washington State USA	37 m	[48.1133, -123.0648]	2 mL	Pteraster tesselatus	Coelomic Fluid	30003
Washington State USA	37 m	[48.1133, -123.0648]	0.1509 g	Pteraster tesselatus	Pyloric Caeca	160056
Washington State USA	37 m	[48.1133, -123.0648]	0.1233 g	Pteraster tesselatus	Gonads	31433
Washington State USA	37 m	[48.1133, -123.0648]	0.1276 g	Pteraster tesselatus	Epidermis	33854
Washington State USA	30 m	[48.2000, -122.5181]	2 mL	Pycnopodia helianthodies	Coelomic Fluid	2250
Washington State USA	30 m	[48.2000, -122.5181]	0.1195 g	Pycnopodia helianthodies	Pyloric Caeca	38053
Washington State USA	30 m	[48.2000, -122.5181]	0.1052 g	Pycnopodia helianthodies	Gonads	12212
Washington State USA	30 m	[48.2000, -122.5181]	0.0996 g	Pycnopodia helianthodies	Epidermis	13231
Washington State USA	37 m	[48.1133, -123.0648]	0.1032 g	Orthasterias koehleri	Pyloric Caeca	38329
Washington State USA	37 m	[48.1133, -123.0648]	0.1257 g	Orthasterias koehleri	Gonads	19589
Washington State USA	37 m	[48.1133, -123.0648]	0.1221 g	Orthasterias koehleri	Epidermis	39991
Washington State USA	28 m	[47.5589, -122.3553]	2 mL	Dermasterias imbricata	Coelomic Fluid	11606
Washington State USA	28 m	[47.5589, -122.3553]	0.1152 g	Dermasterias imbricata	Pyloric Caeca	27980
Washington State USA	28 m	[47.5589, -122.3553]	0.1245 g	Dermasterias imbricata	Epidermis	22635

Washington State USA	Surface	[47.5810, -122.3339]	23L	Bacterioplankton	Sea Water	98365
Washington State USA	Surface	[48.1412, -122.4779]	21L	Bacterioplankton	Sea Water	18243
Washington State USA	Surface	[48.2934,-122.4296]	21L	Bacterioplankton	SeaWater	12251
Washington State USA	Surface	[48.3529, -122.5476]	21L	Bacterioplankton	Sea Water	3309
Washington State USA	Surface	[48.4228, -122.5716]	21L	Bacterioplankton	Sea Water	21224
Washington State USA	Surface	[48.2000, -122.5181]	21L	Bacterioplankton	Sea Water	28569
Washington State USA	Surface	[48.1133, -123.0648]	21 L	Bacterioplankton	Sea Water	16520
Washington State USA	30 m	[48.5810, -122.3339]	0.1144 g	Henricia spp.	Epidermis	3710
Washington State USA	25 m	[47.3934, -122.2626]	0-1 cm	Sediment	Sediment	9721
Washington State USA	40 m	[48.1412, -122.4779]	0-1 cm	Sediment	Sediment	73201
Washington State USA	58 m	[48.4269, -122.5211]	0-1 cm	Sediment	Sediment	7561
Washington State USA	30 m	[48.2000, -122.5181]	0-1 cm	Sediment	Sediment	8582
Washington State USA	41 m	[48.1054, -123.0589]	0-1 cm	Sediment	Sediment	50155
Moreton Bay, Australia	Surface	[-27.4802, 153.4034]	1.2L	Bacterioplankton	Sea Water	46581
Moreton Bay, Australia	Surface	[-27.4802, 153.4034]	1.2L	Bacterioplankton	Sea Water	20447
Heron Island, Australia	Surface	[-27.4802, 153.4034]	6L	Bacterioplankton	Sea Water	12928
Heron Island, Australia	Surface	[-27.4802, 153.4034]	6L	Bacterioplankton	Sea Water	2638
Heron Island, Australia	Subtidal	[-23.4431, 151.9118]	200 mg	Sediment	Sediment	17482
Heron Island, Australia	Subtidal	[-23.4431, 151.9118]	200 mg	Sediment	Sediment	30443