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ORIGINAL ARTICLE

Aquaculture production of hatchling Hawaiian Bobtail Squid (*Euprymna scolopes*) is negatively impacted by decreasing environmental microbiome diversity

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Abstract

Aims: The Hawaiian Bobtail Squid (*Euprymna scolopes*) is a model organism for investigating host–symbiont relationships. The current scientific focus is on the microbiome within *E. scolopes*, while very little is known about the microbiome of the tanks housing *E. scolopes*. We examined the hypothesis that bacterial communities and geochemistry within the squid tank environment correlate with the production of viable paralarval squid.

Methods and Results: Total DNA was extracted from sediment and filtered water samples from 'productive' squid cohorts with high embryonic survival and paralarval hatching, 'unproductive' cohorts with low embryonic survival and paralarval hatching. As a control total DNA was extracted from environmental marine locations where *E. scolopes* is indigenous. Comparative analysis of the bacterial communities by the 16S rRNA gene was performed using next generation sequencing. Thirty-eight differentially abundant genera were identified in the adult tank waters. The majority of the sequences represented unclassified, candidate or novel genera. The characterized genera included *Aquicella*, *Woeseia* and *Ferruginibacter*, with *Hyphomicrobium* and *Rhizohapis* were found to be more abundant in productive adult tank water. In addition, nitrate and pH covaried with productive cohorts, explaining 67% of the bacterial populations. The lower abundance of nitrate-reducing bacteria in unproductive adult tank water could explain detected elevated nitrate levels.

Conclusions: We conclude that microbiome composition and water geochemistry can negatively affect *E. scolopes* reproductive physiology in closed tank systems, ultimately impacting host-microbe research using these animals.

Significance and Impact of study: These results identify the tight relationship between the microbiome and geochemistry to *E. scolopes*. From this study, it may be possible to design probiotic counter-measures to improve aquaculture conditions for *E. scolopes*.

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KEYWORDS

aquaculture, biodiversity, Bobtail squid, environmental microbiology, *Euprymna scolopes*, next generation sequencing

INTRODUCTION

Many of the most utilized eukaryotic model systems in Biology are aquatic vertebrates or invertebrates, from both marine and freshwater habitats. These models are typically housed in a research institute in a variety of different tank systems. Aquatic animals are sensitive to environmental conditions and incompatible conditions can lead to unsuccessful aquaculture (Ahmed et al., 2019). Therefore, maintaining aquatic animals in artificial environments requires extensive upkeep and monitoring of animal health and environmental parameters including, but not limited to temperature, salinity, pH and nitrogenous compounds (Fiorito et al., 2015; Hamlin et al., 2008).

The microbiome can most simply be defined as the micro-organisms in a particular environment. Artificial aquatic environments, as with natural aquatic environments, contain a community of microbes collectively termed the microbiome that directly or indirectly interact with the eukaryotic occupants and, like the eukaryotic occupants, can react to changing environmental conditions. Fluctuating environmental conditions can arise from changes in biotic and abiotic factors, and can impact environmental microbial diversity. For example, limiting the colloidal organic material in a recirculating aquaculture system housing Atlantic salmon parr (*salmo salar)* through filtration resulted in decreased water microbial diversity (Fossmark et al., 2020). The relationship between biotic and abiotic factors in an environment form a self-perpetuating cycle where micro-organisms reinforce a change in abiotic factors potentially leading to loss of productivity for aquaculture. For example, nutrient loading can directly impact animal health, by indirectly enriching micro-organisms that produce toxins, as demonstrated by harmful algal blooms (Anderson et al., 2002). The ubiquitous nature of microbes and their influence on health makes the microbiome an attractive target for monitoring and influencing animal health and disease as evidenced by commercial products designed to promote or discourage microbial growth, or add specific microbes as probiotics (Infante-Villamil et al., 2021; Peixoto et al., 2020; Ringø & Vadstein, 1998). However, the relationship between biotic and abiotic factors, microbial communities and animal health is surprisingly understudied given the importance of aquaculture to food and commercial products as well as habitat and animal conservation (Froehlich et al., 2017). An improved understanding of micro-organisms in aquaculture could advance sustainable aquaculture given the diverse roles micro-organisms play in environment and animal health.

The Hawaiian Bobtail Squid (*Euprymna scolopes*) is a member of the Cephalopoda class and serves as a model organism for developmental biology and host–microbe interactions (Callaerts et al., 2002; McAnulty & Nyholm, 2017; Tischler et al., 2019). As the majority of labs working on *E. scolopes* are not located near an ocean, maintaining *E. scolopes* outside of its natural marine environment for study requires an artificial housing system that mimics oceanic environments. Standard protocols indicate that *E. scolopes* is best reared in a continually recirculating aquaculture systems (RAS) (Lee et al., 2009; van Kessel et al., 2010). RAS offers sustainable aquaculture approaches by reducing the amount of water needed for upkeep with the option for varying scales from small internal tanks to full outdoor ponds (Badiola et al., 2018; Zhang et al., 2011). In marine waters, microbes play crucial roles in geochemical cycles in marine ecology, and this may take on heightened importance in a closed tank system where microbial and geochemical input is limited (Finney et al., 2015; Giovannoni & Vergin, 2012).

Research utilizing *E. scolopes* aquaculture has primarily focused on the horizontally acquired beneficial bacterial symbioses present as a bacterial consortium in the accessory nidamental gland (ANG) (Collins et al., 2012) and as a bacterial monoculture of *Vibrio fischeri* in the light organ (Claes & Dunlap, 2000), with the majority of studies conducted on juvenile animals. Female animals will lay multiple egg clutches containing tens to several hundred eggs that take 18–25 days to hatch. While environmental conditions in the adult and egg tanks are monitored to ensure the health of the animals and development and hatching of eggs, the microbial populations in the tanks and the role they might play in animal health and development is poorly understood.

Research conducted on the symbiosis between *E. scolopes* and *V. fischeri* heavily relies on the use of hatchling animals to investigate the process of symbiont acquisition by the host and colonization of host tissues. Therefore, for the purpose of this study, a 'productive' cohort of adult animals is one where females lay multiple egg clutches and the eggs develop normally and hatch numerous paralarval squids. In 2017, we experienced an unproductive cohort of adult animals wherein eggs laid by the unproductive females failed to fully develop and did not hatch. Surprisingly, the adult animals (female and male) that comprised the animal cohorts monitored in this study did not display behaviour that is consistent

with poor health. All animals displayed proper hunting and feeding behaviour, were responsive to light, either during the night or at dawn, by quickly burying in the substrate, were responsive to physical stimuli by inking and swimming away from stimuli, did not display difficulty in respiration, displayed typical morphology, and did not have lesions or signs of infection. Female animals laid multiple, typically sized clutches containing 50–200 eggs during their tenure in the tank system. In addition, the tank/water conditions were daily monitored and maintained at optimal levels (see Section 2). Given that the unproductive females showed no obvious difference in health and behaviour in comparison to females from productive cohorts, we hypothesized that the microbiome of aquaculture systems plays a role in *E. scolopes* reproductive pathophysiology (Hou et al., 2017; Ramírez & Romero, 2017; Sykes et al., 2017; Zoqratt et al., 2018). However, to our knowledge, no studies have focused on the membership of microbial communities within the aquaculture tanks housing the squid, and whether those communities differ between successive cohorts. Therefore, in this study we aim to identify whether differences in bacterial communities and water geochemistry are correlated with successful hatching of paralarval squid.

In this study, we compare bacterial communities between an 'unproductive' cohort of adult *E. scolopes*, and 'productive' cohorts, with productivity being defined as the total number of viable offspring produced by that cohort. The goal of this study was to assay for differences in diversity and abundance of environmental bacteria that may correlate to unproductive aquaculture systems. Creating a better understanding of the microbial communities provides opportunities for targeted interventions in future instances and is an initial step toward actively managing the microbiome of aquaculture tanks.

MATERIALS AND METHODS

Squid collection and aquaculture

The symbiosis between *V. fischeri* and *E. scolopes* is a model system used to study many aspects of beneficial bacterial–host relationships. The association is uniquely specific and facilitates research on the host and bacterial factors that promote the horizontal transmission of *V. fischeri*, and colonization of paralarval *E. scolopes* by *V. fischeri*. Adult *E. scolopes* were collected in shallow water off the shores of Oahu Hawaii (Figure 1a) in 2017, 2018 and 2019. Squid were individually placed in 3 ml thick fish bags (JEHM Co, Inc.) with 2 litres of ocean water and oxygen, and were transported to the Southern Illinois University in Carbondale (SIUC) by air cargo. After approximately 15–20 h, travel (including transport to and from the airport, and to the lab), animals were acclimatized to the tank environment (water and temperature) slowly and incrementally over approximately 4–6 h. After acclimatization, squid were maintained in two 760 L recirculating tanks containing Instant Ocean (IO) (Instant Ocean) with UV sterilization (Pentair Aquatic Eco-Systems Inc.). Tanks consisted of eight individual compartments (condos) all receiving water-flow, and squid were placed one or two to a condo. IO was made by dissolving Reverse Osmosis (RO) water (Culligan) and IO crystals in clean 130 L garbage cans to a salinity of 32–36 ppt. Adult animals were kept on a 14-h/10-h day/night cycle and fed 3–6 ghost shrimp per animal per night. Tanks were inspected

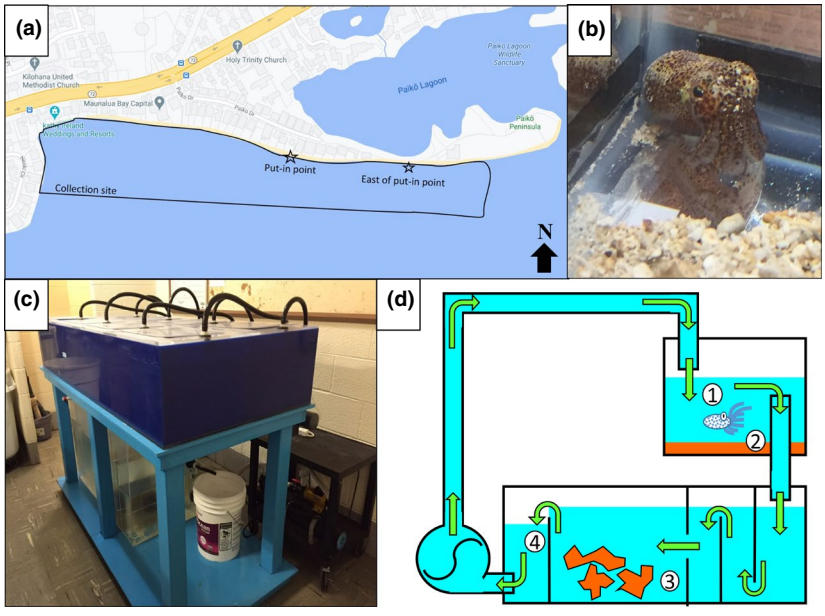

FIGURE 1 Hawaiian Bobtail squid and aquaculture design. (a) Map of *Euprymna scolopes* collection site in Oahu, Hawaii, (b) Sexually mature adult Hawaiian Bobtail squid (*E. scolopes*), (c) aquaculture squid 'condos'; and (d) pumped recirculating water tank diagram with sampling locations: (1) condo water, (2) condos sediment, (3) sump rock and (4) post sump rock

each morning for newly laid egg clutches, and partial or whole shrimp carcasses, which were removed to prevent fouling. Tank water was maintained at ambient room temperatures (17–26°C) and water changes consisting of the replacement of ~242 L of IO with freshly made IO occurred either once a week or as needed based on water chemistry. Nitrate concentration, nitrite concentration, and ammonia were tested daily using a kit designed for colorimetric assessment (API Saltwater Master Test Kit, Petco) and kept within the following parameters: Nitrate 0–10 ppm; Nitrite 0–0.25 ppm; ammonia 0–0.25 ppm. Salinity was measured by refractometer (Petco) and maintained between 32 and 36 ppt.

Physical parameters and aquaculture sample collection

Four samples were taken from the adult squid tanks in 2017, 2018 and 2019 for this study: a water and a sediment sample from the condo or squid living space; and a water and sediment sample from the sump tank. Microbial samples for DNA extraction from planktonic cells were collected by pumping 2–5 L of water by peristaltic pump through a 0.2 μm Sterivex filter (Millipore). Water was pumped through the filters until plugged or >90% of flow of water was restricted, after which all remaining water was pushed through filter until dry, then stored in sterile 50 ml conical centrifuge tube at −80°C. Sediment samples were collected using a sterile spatula, removing ~5 g of sediment and stored in 50 ml conical tubes at −80°C.

Two water samples and two sediment samples were collected between two different days at the environmental ocean locations where the animals were caught. The site of entry into the collection site Put-in-Point (PIP) and a site east of the Entry Put-in-Point (EPIP) on 11 February and 14 February 2019 (Figure 6a). These samples were further classified as unproductive (2017), productive (2018–2019), and ocean (2019, Table 1), and a full outline of sampling collection scheme can be found in Figure S1.

DNA extraction

Sediment and filter samples were stored at −80°C until DNA extractions were performed. Extractions were performed following manufacturers protocols using the MoBio Power Soil DNA isolation kit (Qiagen). For sediment samples, 0.25 g of pulverized material was used for extraction. For filter samples, the entirety of the 0.2 μm filter was aseptically removed from the filter casing, cut into on average 5 mm × 5 mm pieces and placed into the provided DNA extraction kit bead tubes. DNA purity and concentrations were determined using Nanodrop (ThermoFisher).

Extracted total environmental DNA samples with concentrations ranging from 1.3 to 17.3 ng/μl (Table 1), were sent for Illumina Miseq paired end sequencing (2 × 300 bp) using universal bacterial primers (515F, 806R) targeting the V4 region of the 16S rRNA gene. Amplification, library preparation and Illumina sequencing were conducted by sequencing facilities offered by the University of Illinois at Chicago Research Resources Center Genome Research Core.

Vibrio fischeri Polymerase Chain Reaction (PCR) assay

Primer pairs for detection of *Vibrio fischeri* were designed to amplify *luxR* (Forward: 5′-TAATGAGTCCCGATTCC-3′ and Reverse: 5′-ACGGCTTAATTCGACTA-3′), VF1206 (Forward: 5′-TCACCACTACGCTTTAC-3′ AND Reverse: 5′-CCTTGGAAGTTAATTGGG-3′) and VF1209 (Forward 5′-TGTGGGTTGAGACGGTATTG-3′ and Reverse: 5′-TCTAAATGCCTCAAGTGCTGA-3′) ORFs. Reactions were performed using the GoTaq® Rapid Master Mix (Promega, WI) with Rapid 2-Step protocols for 20 μl reactions. Thermocycler (Applied Biosystems) program started with a one-minute 95°C activation step followed by 30 cycles of 95°C denaturation for 2 s, and 65ºC in a combined annealing/extension step for 6 s and a final 15 s elongation step at 72°C.

NGS sequencing processing

The quality of resulting raw FASTQ files was evaluated using the software Fastqc (0.11.8) (Andrews, 2010). Sequencing primers were removed with Cutadapt (V 2.8) (Martin, 2011). Raw FASTQ files were then processed using Mothur (V1.43.0) following the Mothur MiSeq SOP (https://mothur.org/wiki/miseq_sop/) (Kozich et al., 2013; Schloss et al., 2009). Processing included, paired reads merged into contigs and removal of contigs longer than 275 nucleotides or containing one or more ambiguous bases. Filtered contigs were aligned to the Silva database and chimeric reads were removed using VSearch (Rognes et al., 2016). Sequences were clustered into Operational Taxonomic Unites (OTUs) at 97% similarity using Opticlust algorithm and any non-bacterial lineages were removed (Westcott & Schloss, 2017). The final shared, taxonomy, and metadata files were converted to BIOM format for downstream analysis. Using R Studio (V 1.2.0), the OTU table was filtered to remove any OTUs that were less than 0.005% abundance in the

TABLE 1 Hawaiian Bobtail Squid study sample identification and metadata

Sample name	Sample ID	Sample Type	Category	Extracted DNA concentration (ng/μL)
Sump Pump Rock Water 2017	SPR17	Water	Unproductive	3.4
Egg Chamber Water 2017	EC17	Water	Unproductive	2.5
Squid Condo Water 2017	C17	Water	Unproductive	3.2
Sump Pump Rock Water 2018	SPR18	Water	Productive	4.7
Egg Chamber Water 2018	EC18	Water	Productive	9.3
Squid Condo Water 2018	C18	Water	Productive	3.9
Sump Pump Rock Water 2019	SPR19	Water	Productive	3
Egg Chamber Water 2019	EC19	Water	Productive	1.7
Squid Condo Water 2019	C19	Water	Productive	2.2
East of Put in Point Water Feb 11th	EPIP8	Water	Ocean	2
Put in Point water Feb 14th	PIP11	Water	Ocean	4.8
East of Put in Point water Feb 14th	EPIP11	Water	Ocean	7.5
Squid Condo Sediment 2017	SC17	Sediment	Unproductive	3
Sump Pump Rock Sediment 2017	SSR17	Sediment	Unproductive	4.9
Squid Condo Sediment 2018	SC18	Sediment	Productive	3.6
Sump Pump Rock Sediment 2018	SSR18	Sediment	Productive	2.9
Squid Condo Sediment 2019	SC19	Sediment	Productive	1.3
Sump Pump Rock Sediment 2019	SSR19	Sediment	Productive	7.1
Put in Point Sediment Feb 11th	SPIP11	Sediment	Ocean	12.4
East of Put in Point Sediment Feb 11th	SEPIP11	Sediment	Ocean	17.3
Put in Point Sediment Feb 14th	SPIP14	Sediment	Ocean	6.2
East of Put in Point Sediment Feb 14th	SEPIP14	Sediment	Ocean	5.1

dataset, as no mock community was sequenced alongside these samples. This threshold is recommended to account for sequencing errors in the absence of a mock community during sequencing (Bokulich et al., 2013). NCBI BioSample Accession SAMN18718163 to SAMN18718184.

Statistical analysis

Hypothesis testing and visualizations of significant differences among communities conducted using were conducted in R studio using vegan and phyloseq packages (McMurdie & Holmes, 2013; Oksanen et al., 2013). As needed, raw counts were transformed using variance stabilizing transformation (VST) for non-parametric data in the DESeq2 package (Love et al., 2014; McMurdie & Holmes, 2014). Alpha diversity metrics were summarized by observed OTUs and Shannon diversity index. Differences among groups, control, productive cohorts, unproductive cohort and ocean water, were detected using Kruskal–Wallis rank-sum test with post-hoc testing on significant results done with Dunn's test. Bray–Curtis and Jaccard matrices were analysed using Analysis of Similarities (ANOSIM) and Permutational Analysis of Variance (PERMANOVA) using the Anosim and Adonis functions in Vegan. Tests for homogenous dispersion were performed with betadisper prior to PERMANOVA and ANOSIM testing. No additional transforms were used prior to PERMANOVA or ANOSIM testing. Benjamini-Hochberg correction was used on any statistical test requiring correction for multiple hypothesis testing. P-values <0.05 were considered significant unless otherwise stated. Distance matrices were visualized with Principal Coordinate analysis (PCoA) and hierarchical clustering. Hierarchical clustering was carried out using Bray–Curtis distance with hclust function in the vegan package. Differentially abundant OTUs from the untransformed OTU table between productive and unproductive tanks were discovered using DESeq2, with $p < 0.001$ as the significance threshold. To account for spurious OTUs, they were combined at the genus level before differential abundance analysis Inference testing was conducted to evaluate the influence of water chemistry on bacterial communities. The explanatory variables used in this analysis included pH, temperature, dissolved oxygen and nitrate. Other aspects of tank conditions including nitrites, ammonium and salinity levels

were not included because their values were the same across all three years and would not serve as distinguishing driving factors for community differences. Inference was conducted comparing Bray–Curtis matrices to tank conditions using PRIMER-E software (V 6.1.12) (Clarke & Gorley, 2006). Distance-based redundancy analysis (dbRDA) was used to visualize the water chemistry with vectors representing influences of measured chemistries on microbial communities. Distance-based linear modeling (DistLM) was used to test for overall significance of a particular chemistry measurement. For model selection in DistLM, we used Akaike Information Criterion (AICc; adjusted for small sample size) to select the model and included variables with p-values less than <0.10 as a conservative measure to avoid overfitting on the data. Given the stochastic nature of ecological studies, we deemed it logical to raise acceptance criteria from the traditional $\alpha = 0.05$ threshold.

Ethics statement

Research conducted on cephalopods at Southern Illinois University does not require approval by an animal research ethics committee as cephalopods are not included in federal laws governing the use and welfare of animals. All field collection of research animals was done in accordance with state and federal regulations. Current guidelines were followed in the care of *Euprymna scolopes* as outlined in (Fiorito et al., 2015).

RESULTS

Cohort productivity

During this study, three cohorts of adult *E. scolopes* were housed in the Rader laboratory facility. Females from all three cohorts of animals produced clutches with females from Spring 2017 laying fewer clutches on average then Spring 2018 and Spring 2019. However, the eggs from Spring 2017 females arrested late in development, leading to no hatchling animals from that cohort (Table S1).

Sampling and sequencing summary

Total DNA was isolated from 19 samples collected from adult squid tanks and squid collection sites (Figure S1 and Table 1), and the V4 region of the 16S rRNA gene was sequenced. Processing of NGS data yielded 1,712,800 total sequences grouped into 45,207 OTUs at 97% sequence similarity across the dataset. Filtering out sequences with less than 0.5% abundance resulted in 11,476 OTUs remaining.

Water bacterial community composition

Productive adult water samples contained 1,927 unique OTUs while ocean samples had 757 unique OTUs; however, 4,772 of the total (combined) 8,616 observed OTUs were strictly shared between these two groupings. Unproductive water samples had 1,112 unique OTUs and shared 168 additional OTUs with the other groupings. In total, 121 OTUs were present in all water samples (Figure 2a).

The predominant phyla within the nine water samples were *Proteobacteria* (33–53%), *Acidobacteria* (3–11%), *Bacterioidetes* (4–15%), *Actinobacteria* (4–12%), *Patescibacteria* (1–13%), *Planctomyetes* (3–21%) and *Verrucomicrobia* (1–6%) (Figure 2b). The *Proteobacteria* phylum was primarily composed of *Alphaproteobacteria* and *Gammaproteobacteria* with unproductive tank water showing more *Alphaproteobacteria* and less *Gammaproteobacteria* relative abundance values compared to productive tank water and ocean water. In the *Planctomycetes* phylum, both orders within the phylum, *Planctomycetacia* and *Physcisphaerae*, were present. The unproductive adult water samples contained less *Planctomycetacia* and more *Physcisphaerae* compared to productive water and ocean specimens. No OTU's classified as *Vibrio* were discovered in any water sample.

The top 50 most abundant OTUs were analysed for abundance distributions across the dataset. Of the most abundant OTUs, few were found in the unproductive water samples, these samples did not share abundance patterns observed with ocean, and productive water samples (Figure 3). For unproductive systems, within the phylum Proteobacteria at the class level tank water had 10% fewer Gammaproteobacteria and 5% more Alphaproteobacteria OTUs than productive adult water. The second most abundant phyla among water samples were Planctomycetes. Within unproductive adult tank water, at the class level, there was a 20% decrease in Planctomycetacia and a 20% increase in Phycisphaerae compared to productive adult water.

Sediment bacterial community composition

After processing the NGS data, a total of 2,851 OTUs were recovered from sediment samples. Only 57 OTUs were recovered from unproductive sediments, compared 1,928 and 2,355 for productive and ocean sediments respectively.

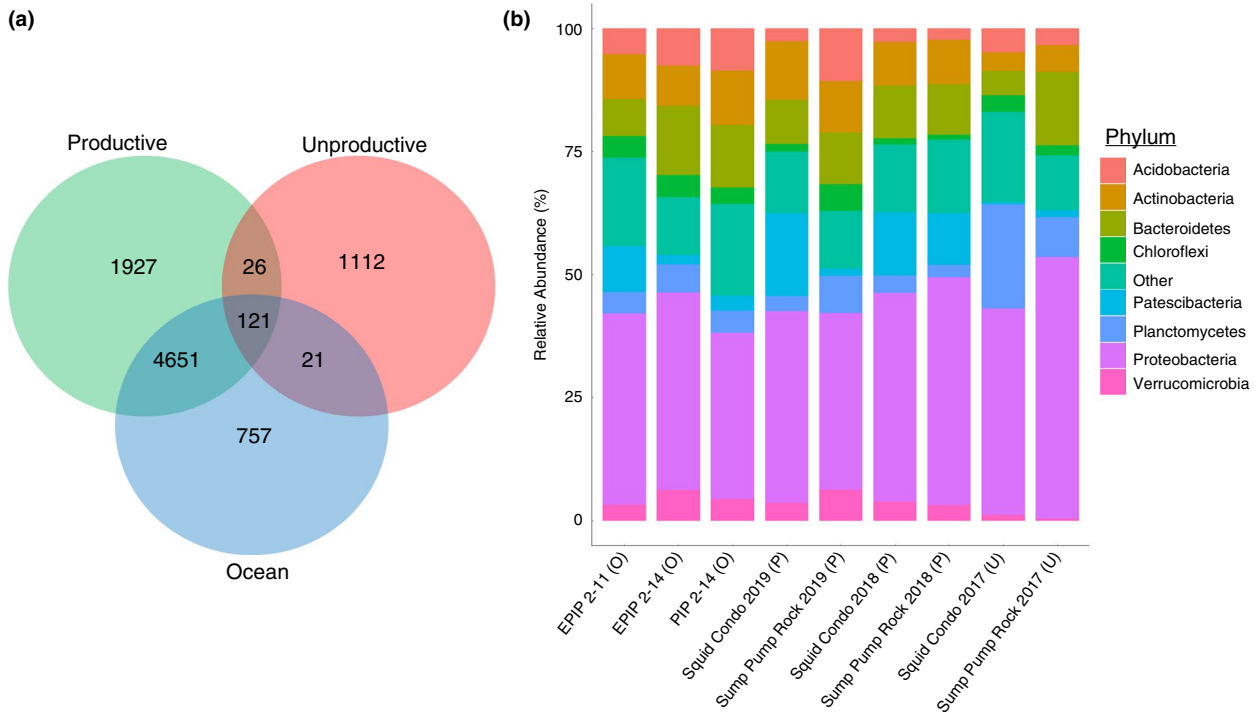

FIGURE 2 Overview of OTU distribution and phylum level relative abundance. (a) Venn diagram of OTU distribution based on sampling source groupings. (b) Overview of phyla distribution within water samples depicted as percentage of overall composition within the sample. 'Other' category contains all phyla which were <5% of composition within any sample. Sampling source denoted as (O) for ocean, (P) for productive adult tank and (U) for unproductive adult tank

Productive adult tank sediment samples had 475 unique OTUs, ocean sediments had 893 unique OTUs, and 20 unique OTUs were found in unproductive adult tank sediments. Ocean and productive sediments shared 1,452 OTUs (Figure S2a).

The top five bacterial phyla in the sediment samples *Firmicutes* (1–98%), *Proteobacteria* (2–67%), *Bacteroidetes* (0–17%), *Actinobacteria* (1–30%) and *Planctomycetes* (0–16%). Unproductive samples contained more *Firmicutes* compared to productive and ocean samples (Figure S2b). At the class level within *Firmicutes* phylum, over 90% of these OTU's were placed within the *Clostridiales* class. The rarer classes of *Ersyipelotrichales* and *Negativicutes* were notably absent in the unproductive sediment samples. The second most abundant phylum in sediment samples was *Proteobacteria*. Productive tanks and ocean sediments had comparable levels of *Alpha-*, *Delta-* and *Gammaproteobacteria*. Unproductive tank sediments had a greater variation in their *Alpha-* and *Gammmaproteobacteria* abundances and did not contain any OTU's classified as *Deltaproteobacteria*.

Although unproductive sediments contained fewer OTUs, 37 of them were shared with the ocean or productive sediments. The heatmap of OTU abundance shows unproductive sediments lacking many the OTUs that were most abundant across other sediment samples (Figure S3).

Detection of *Vibrio fischeri*

Given that the *E. scolopes* light organ symbiosis undergoes a diel rhythm in which ~95% of the bacterial contents, comprised specifically of *V. fishceri*, are vented out into the surrounding environment at dawn, we argued that *vibrio* species would be detectable in our tank water and sediment samples. NGS analysis found that *Vibrio* and *Aliivibrio* genera were in low abundance (<1% relative abundance) for all water and sediment samples. An unclassified genus within the Vibrionaceae family was present in productive sediments (1–20% relative abundance) and in unproductive waters (0–3% relative abundance). To conduct a more sensitive analysis, detection of *Vibrio* was performed on all DNA samples using end-point PCR with primers specific to *luxR*, the transcriptional regulator of the lux operon for bioluminescence, and uncharacterized *Vibrio* specific ORFs VF1206 and VF1209 (Table 2). PCR detected the presence of *Vibrio* DNA in a small number of tank samples, and only one sample, Sump Water 2019, was positive for all three primer sets.

Unproductive bacterial diversity

To determine if the tanks from the unproductive year differed in alpha diversity from productive years or ocean

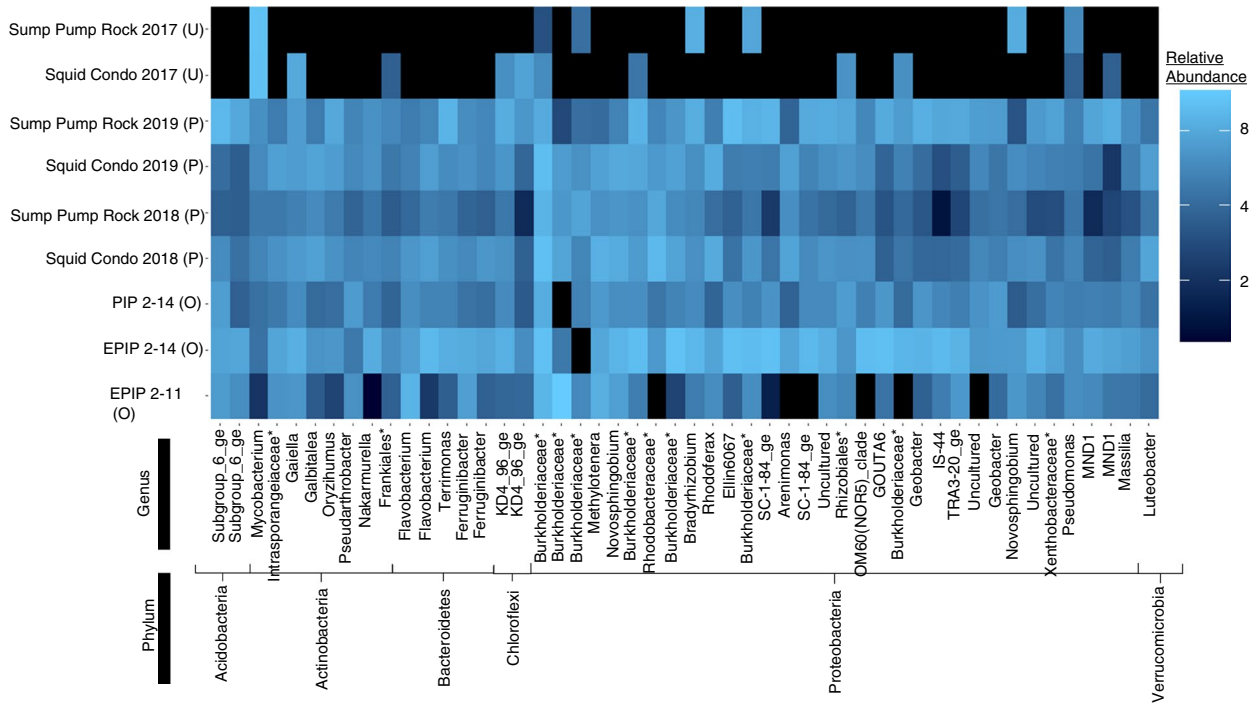

FIGURE 3 Heatmap of the top 50 most abundant OTUs across all water samples. OTUs are labelled under their genus names with brackets labelled by respective phylum taxonomic classification. *Indicates an unclassified member of the respective genus. Marine samples, Put-In-Point (PIP) and East of Put-In-Point (EPIP) were all collected in 2019 on either February 11th or 14th as designated. Sampling source denoted as (O) for ocean, (P) for productive adult tank, and (U) for unproductive adult tank

samples, water and sediment samples were grouped together based on productivity and ocean samples were grouped together for analysis. Alpha Diversity estimates were extrapolated from filtered OTU counts using the observed number of OTUs and Shannon Diversity index (Figure 4a,b and Figure S4a,b). Raw values for water and sediments show that samples from unproductive adult tanks contain fewer total number of OTUs, however, this observation was not statistically significant.

Kruskal–Wallis tests indicated that bacterial diversity differed overall among water samples ($p = 0.038$). Pairwise testing with Dunn's test revealed significant differences in diversity between productive and unproductive adult tank water ($p = 0.018$). Sediment samples were not significantly different. No statistical significance was found in the location of sampling within tank, sump or condo, for observed OTUs or Shannon Diversity.

Differences between bacterial communities

Hierarchical clustering on both water and sediment samples revealed that unproductive adult tank samples clustered separately from the productive adult tank and ocean samples (Figure S5a,b). Principal Coordinates Analysis (PCoA) based on Bray–Curtis and Jaccard dissimilarity matrices support the clustering pattern observed in hierarchical clustering analysis (Figure 5). PERMANOVA indicated that water from productive adult tanks, unproductive adult tanks, and Ocean samples were statistically different ($p = 0.008$ Bray–Curtis, $p = 0.006$ Jaccard). These findings were also supported by ANOSIM ($R = 0.5692$, $p = 0.006$). PERMANOVA on sediments was not statistically significant ($p = 0.169$), however, ANOSIM found a difference in bacterial community similarity within this grouping ($R = 0.3942$, $p = 0.035$) (Figure S6).

Since two sites within the adult tanks were sampled, sump and condo, we wanted to test if there were differences between microbiomes within the sampling location. No statistical significance was found to support a difference between sampling location within the tanks (Condo or Sump) in either water or sediment samples using PERMANOVA ($p = 0.700$ Water, $p = 0.500$ Sediments) between productive and unproductive years.

Differential abundance

There were 39 differentially abundant genera including unclassified and candidatus genera in both productive and

TABLE 2 Results of PCR detection of *V. fischeri*

Sample name	LuxR	VF1206	VF1209
Sump Pump Rock Water 2017			
Egg Chamber Water 2017	+		
Squid Condo Water 2017			
Sump Pump Rock Water 2018		+	
Egg Chamber Water 2018	+		+
Squid Condo Water 2018			
Sump Pump Rock Water 2019	+	+	+
Egg Chamber Water 2019			
Squid Condo Water 2019			
East of Put-in-Point Water Feb 11th			
Put in Point water Feb 14th			
East of Put-in-Point water Feb 14th			
Squid Condo Sediment 2017			
Sump Pump Rock Sediment 2017			
Squid Condo Sediment 2018	+		
Sump Pump Rock Sediment 2018	+		
Squid Condo Sediment 2019			
Sump Pump Rock Sediment 2019			
Put-in-Point Sediment Feb 11th			
East of Put-in-Point Sediment Feb 11th			+
Put-in-Point Sediment Feb 14th			+
East of Put-in-Point Sediment Feb 14th			+

unproductive water. Unproductive water samples were found to be enriched with members of *Pseudohongiella* (7.1), *Blastopirellula* (6.8) and *Woeseia* (6.0) genera. Productive water contained more differentially abundant genera than unproductive water, which may reflect differences seen in diversity. The three genera with the greatest log-fold change in the productive water were *Flavobacterium* (8.5), *Geobacter* (8.3) and *Ferruginibacter* (8.3) (Figure 6).

Nitrate concentrations and pH levels

Results of the Distance-Based redundancy analysis (dbRDA), which was conducted only on samples that came from the water samples, supported the same clustering patterns observed in the PCoA (Figure 7). Of the explanatory variables used in this analysis (pH, temperature, dissolved oxygen and nitrate), pH ($p = 0.055$) and nitrate ($p = 0.066$) were the only significant variables (Table S2). Nitrate concentrations and pH explained 67% of the variance ($R^2 = 0.67$) among the bacterial communities.

DISCUSSION

We reasoned that this lack of productivity could have been, at least in part, caused by a dysbiosis in the adult squid tank microbiome, effecting female reproductive physiology without compromising overall health through environment-tank microbiome-squid interactions. The following observations were used in our reasoning: (a) all females in all cohorts appeared healthy and produced egg clutches and therefore lack of hatchlings was not specific to any one animal, (b) the environmental conditions in the 9.5 L tanks housing the eggs was similar between all 3 cohorts (Table S2), (c) *E. scolopes* eggs received from a colleague at the University of Florida and placed in the same environment developed fully and hatched, and (d) no changes to the RO system used to produce IO took place between cohorts.

This study is one of the first to analyse the microbiome of artificial marine habitats for *Euprymna scolopes* in aquaculture. Given the increasing body of research detailing the influences of microbial communities on marine animals, it is reasonable to expect that environmental microbiomes influence the well-being of *E. scolopes* (Bentzon-Tilia et al., 2016; Rajeev et al., 2021). To begin effectively defining a healthy microbial community for captive *E. scolopes*, we sought to provide critical insights into the distinctions in the bacterial assemblages of productive and unproductive squid cohorts. NGS is an excellent tool to assay bacterial populations to understand if they have a role in aquaculture health and to ascertain which microorganisms are present. Although there are limitations with the 250 bp at assigning species level identification. Despite this caveat, broad descriptive taxonomic overviews can still be performed.

At the phylum level, bacterial populations between all samples did not have major differences in abundance and Proteobacteria were the most populous phylum across all samples. Unproductive tank water samples contained an increased percentage of Planctomycetes along with decreased Patescibacteria compared to productive and ocean water samples. Nearly all of the 50 most abundant OTUs from the water dataset were absent or had lower relative abundance in the unproductive water compared to productive water and ocean water. This result is suggestive that the taxonomic shifts are occurring at the lower taxonomic rankings, and that while the phylum compositions are similar, their constituents at the genus level and below differ between sample sources.

Few OTUs were recovered from unproductive sediments, however unproductive tank sediment samples had a greater concentration (30% of total community) of *Firmicutes* than productive sediments. The unproductive sump rock sediment had the second highest raw

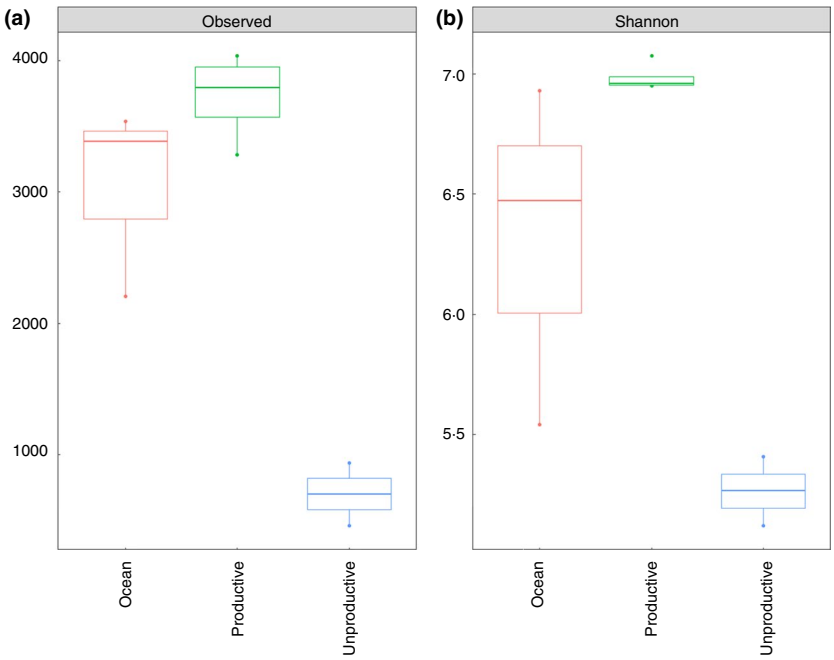

FIGURE 4 Diversity estimates within sampling sites. (a) Number of observed OTUs present in each grouping. (b) Shannon Diversity index. Sample groupings are Ocean samples (Red), Productive (Green), Unproductive (Blue)

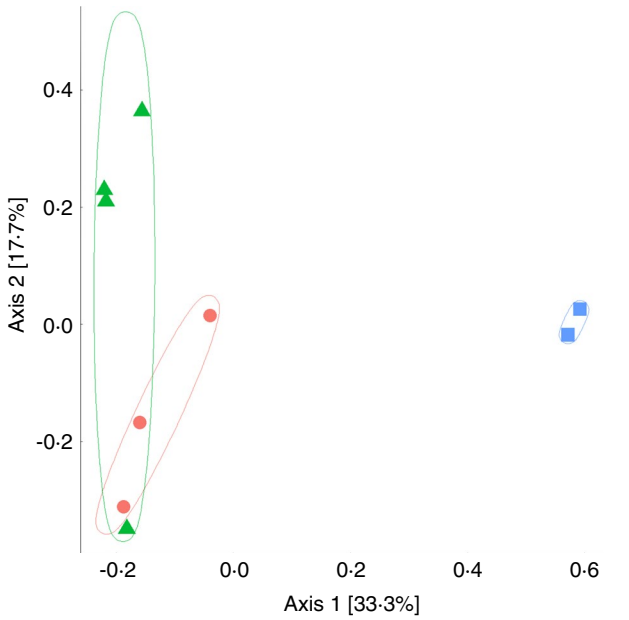

FIGURE 5 Differences between bacterial communities. Principle Coordinates Analysis (PCoA) on Bray-Curtis distance of bacterial communities with OTUs with 97% sequence similarity. Groupings include Ocean samples (Red Circles), Productive (Green Triangles), Unproductive (Blue Squares)

number of reads compared to the other sediment samples. These reads were primarily found distributed across three OTUs of unclassified Clostridia and were 97% of the recovered sequences from the unproductive sump rock sediment. However, these proportions are exaggerated graphically as only 57 OTUs were recovered from the unproductive sediment samples. The dominance of the Clostridia appears to coincide with overall decreased diversity. Recovering high quality DNA from sediments can be problematic which has effects on diversity estimates and comparisons (Pearman et al., 2020). While this is a caveat to consider, the proportion of Clostridia to other microbes in the unproductive sediments could indicate that sediment bacterial communities were perturbed in this instance.

Diversity estimates, OTU counts and Shannon Diversity, showed lower values from unproductive adult tank water and sediment samples compared to productive adult tanks and ocean samples. In both water and sediment sampling cases, productive adult tanks and ocean samples shared similar numbers of OTUs and diversity values. This result indicates that productive adult tanks had greater diversity whereas unproductive adult tanks had lower diversity suggesting their communities were predominantly made up of a few OTUs. The similarity between productive adult tanks and the ocean suggest that microbial diversity is crucial for optimal *E. scolopes* husbandry in artificial marine environments.

Comparisons between the pooled microbial communities of ocean, productive adult tank and unproductive adult tank water samples indicated statistically significant differences between sample sources. However, no significant differences were found to exist within the productive tank and unproductive tank water samples between the squid condo and sump portion of the tanks. This suggests overlap in bacterial compositions exists throughout the adult tank apparatus. Hierarchical clustering and PCoA show distinct groupings with unproductive samples clustering separately for both water and sediment. Differences in abundance levels and presence/absence of bacteria

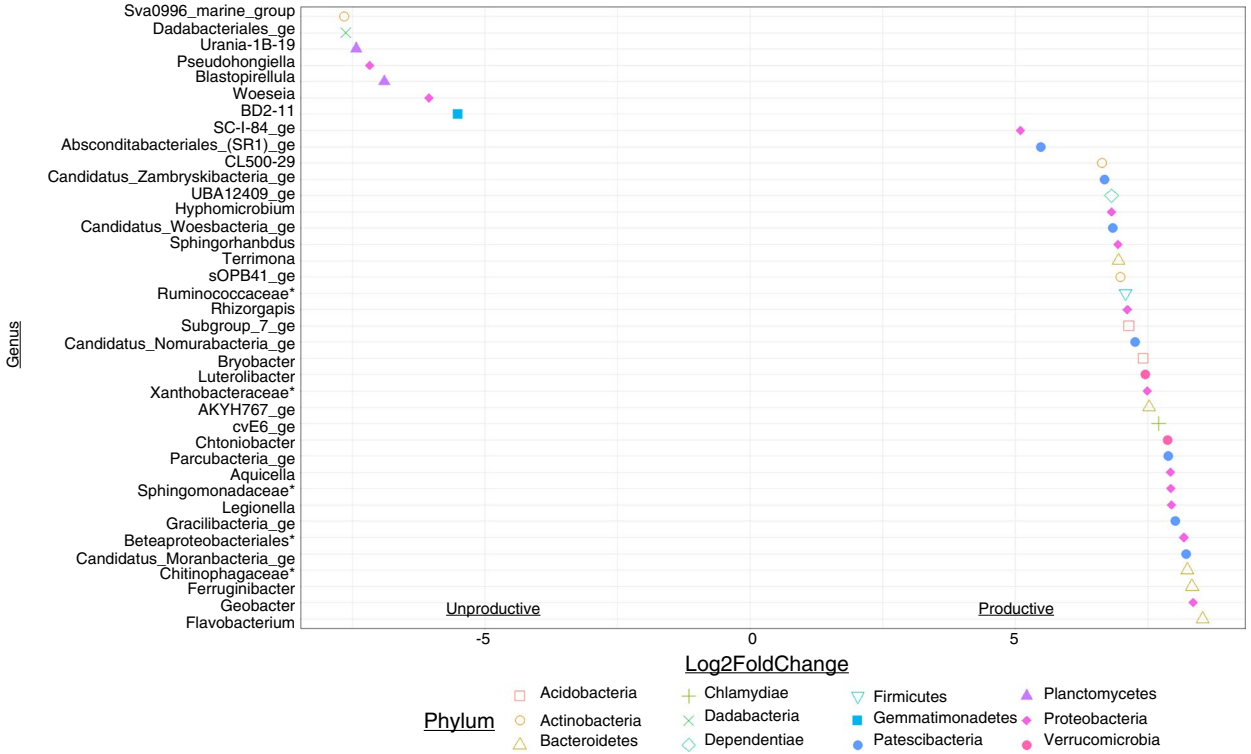

FIGURE 6 Potential drivers of community divergence. Differentially abundant genera between productive and unproductive cohorts. *Represents unclassified member of respective genus

support that distinct bacterial communities are associated with *E. scolopes* productivity.

Given that *E. scolopes* vents up to 95% of its dense *V. fischeri* light organ symbionts into the environment daily at dawn, we assumed that *V. fischeri*, or *Aliivibrio* OTUs, would be present in all samples in some abundance (Visick & McFall-Ngai, 2000). This association of *V. fischeri* with *E. scolopes* makes it a potential biomarker for health based on the likelihood of its presence, particularly if there is a presence/absence relationship with productive and unproductive cohorts. NGS analysis unexpectedly indicated low relative abundances of *Vibrio* and *Aliivibrio* genera across all samples. PCR amplification of genes conserved genes to the *Vibrio* genus included the uncharacterized ORFs VF1206 and VF1209 and the *luxR* gene responsible for regulating the lux operon. VF1209 is more widely distributed in the *Vibrio* genus than is VF1206. Other samples were positive for one or two of the gene targets, this was true primarily in the sediments. These results indicate the presence of *Vibrio*, but not necessarily *V. fishceri*. NGS data found unclassified members of the Vibrionaceae family along with *Vibrio* and *Alioivibrio* genera were present in low abundance in productive and ocean sediments. No pattern of detection between productivity of the tanks was observed. Potential explanations for this could be sampling errors such as insufficient numbers of samples, or incorrect timing of sampling, for example sampling when squid are not venting. It is also possible that filtering with the sump rocks (Figure 1C) helps to remove *V. fischeri* from the water. In either case, the results here suggest that detection of *V. fischeri* is not indicative of adult tank productivity.

There is growing evidence suggesting that 'bacterial dysbiosis' is associated with unproductivity of the aquaculture system (Infante-Villamil et al., 2021; Wang et al., 2020). Dysbiosis is a broad category distinguished by losses of microbial diversity and lower abundance of the dominant genera present in the 'normal', or in our case, productive condition (Petersen & Round, 2014). There were 38 differentially abundant genera were identified in the adult tank waters. Of the 38 genera, a majority were under or poorly studied candidate genera and five were novel, unclassified genera. The remaining characterized genera are commonly associated with marine or aquatic environments including *Aquicella*, *Woeseia* and *Ferruginibacter*. Two other genera, *Hyphomicrobium* and *Rhizohapis*, were found to be more abundant in productive adult tank water and have members associated with the nitrate reduction (Francis et al., 2014; Martineau et al., 2015). The lower abundance of nitrate-reducing bacteria in unproductive adult tank water could explain the elevated nitrate levels.

Multiple factors such as feeding and human handling could also alter the tank water conditions with the ecological repercussion of promoting bacterial dysbiosis (Mente et al., 2006; Sykes et al., 2017). Of the conditions evaluated

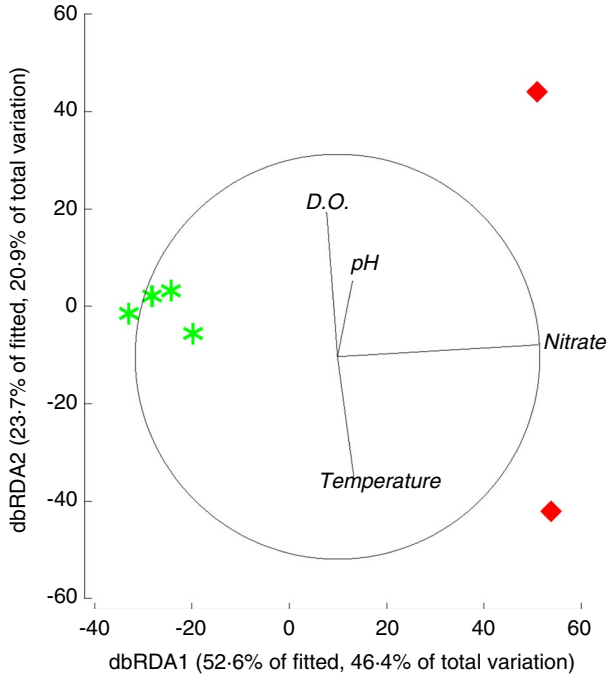

FIGURE 7 Impact of water chemistry on bacterial communities. Distance-Based Redundancy Analysis (dbRDA) of measured water chemistries explanatory potential of bacterial communities based on Bray–Curtis dissimilarity. Vectors show direction and magnitude of explanatory variables. Sample groupings are by Unproductive (Red Diamonds) and Productive (Green Asterisks)

in this study, pH and nitrate are important determinants for bacterial communities. Although pH levels were within the normal limits for water in the tank system the nitrate levels were well above for the unproductive adult tank water (Table S2). Collectively, the geochemistry of the tank water system could explain 66% of the community variation.

Bacterial dysbiosis associated with unproductivity of *E. scolopes* aquaculture and provides guidance to future detailed experiments where no information existed before. This dysbiosis is distinguished by loss of microbial diversity and abundance of dominant communities relative to productive years. In each analysis productive samples measured closely with those from the natural ocean habitat of *E. scolopes*. This result is not surprising as the productive adult tanks reflect a tolerable microbiome for *E. scolopes*, which is analogous to the microbiome found in its natural oceanic habitat. Clearly distinguishing taxonomic and functional profiles for an unproductive microbiome will be key for future studies to support the findings in this study. By doing so, early detection of the microbial drivers towards this phenotype could be used for preventative or mitigative strategies. The data suggest that bacterial community's composition and abundance affect the productivity of the tank. Due to the lack of a timescale in this study, it is not possible to determine whether bacterial perturbations precede or are result of an unhealthy tank system. The association of nitrate and pH levels correlating with bacterial compositions coupled with the increased abundance of nitrate-reducing bacteria presents an interesting dynamic. Nitrate and nitrogenous compounds have been shown to be toxic in aquaculture (Robles-Porchas et al., 2020). Lower abundance of nitrate-reducing bacteria in unproductive adult tank water could explain the elevated nitrate levels. A disproportionate nitrogen cycle would be very disruptive to aquatic lifeforms and thus lead to unproductivity (Infante-Villamil et al., 2021; Xie et al., 2020). We were also unable to attribute functionality to the bacterial communities characterized in this study due to the absence of metagenomic data, and the limited number of samples, both microbial and environmental, taken. Increasing sampling and conducting metagenomic sequencing in future studies will provide greater insight into nitrogen cycling and its role in squid aquaculture.

Distinguishing an unproductive microbiome may be key for future studies as early detection of the microbial drivers would be useful for preventative or mitigative strategies. Determining the core and healthy bacterial communities in the aquaculture environment for *E. scolopes* is of importance for sustainability in aquaculture. An imperative question to answer is how environmental bacteria impact the health of *E. scolopes*. A similar study on Tilapia suggested that the bacteria in the environment may not directly colonize the fish but shifts in environmental bacterial communities correlate with shifts on host microbiota (Giatsis et al., 2015). Establishing a mechanism of how biotic or abiotic factors drive colonization of *E. scolopes* and what implications that has on health would be important for building upon this work.

This study was the first to capture and compare the bacterial compositions for healthy and unhealthy cohorts of *E. scolopes*. In doing so, we described a broad fundamental picture of a decrease in bacterial diversity in both water and sediment being attributed with unproductive cohorts of *E. scolopes*. Though already of known importance for overall health, these results show the tight relationship of geochemical and microbial communities can have on fastidious organisms such as *E. scolopes*. It may be possible from this study to design and explore possible probiotic counter measures with the goal of improving aquaculture conditions for squid.

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CONFLICT OF INTEREST

No conflict of interest declared.

AUTHOR CONTRIBUTIONS

Trevor R. Murphy contributed to data curation, formal analysis, funding acquisition and writing—original draft. Rui Xiao contributed to conceptualization, methodology and writing—original draft. Scott D. Hamilton-Brehm contributed to conceptualization, methodology, formal analysis, supervision, project administration, funding acquisition, resources and writing-review and editing. Marjorie L. Brooks contributed to methodology, formal analysis, writing-review and editing and supervision. Bethany A. Rader contributed to conceptualization, formal analysis, investigation, resources, writing-review and editing, supervision and project administration.

DATA AVAILABILITY STATEMENT

NCBI BioSample Accession SAMN18718163 to SAMN18718184, Tax ID: 1169740, and BioProject PRJNA721441.

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