Microbiomes of pathogenic *Vibrio* species reveal environmental and planktonic associations

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Abstract

Background Many species of coastal Vibrio spp. bacteria can infect humans, representing an emerging health threat linked to increasing seawater temperatures. Vibrio interactions with the planktonic community impact coastal ecology and human infection potential. In particular, interactions with eukaryotic and photosynthetic organism may provide attachment substrate and critical nutrients (e.g. chitin, phytoplankton exudates) that facilitate the persistence, diversification, and spread of pathogenic Vibrio spp. Vibrio interactions with these organisms in an environmental context are, however, poorly understood.

Results We quantified pathogenic Vibrio species, including V. cholerae, V. parahaemolyticus, and V. vulnificus, and two virulence-associated genes for one year at five coastal sites in Southern California and used metabarcoding to profile associated prokaryotic and eukaryotic communities, including vibrio-specific communities. These Vibrio spp. reached high abundances, particularly during Summer months, and inhabited distinct species-specific environmental niches driven by temperature and salinity. Associated bacterial and eukaryotic taxa identified at fine-scale taxonomic resolution revealed genus and species-level relationships. For example, common Thalassiosira genera diatoms capable of exuding chitin were positively associated with V. cholerae and V. vulnificus in a species-specific manner, while the most abundant eukaryotic genus, the diatom Chaetoceros, was positively associated with V. parahaemolyticus. Associations were often linked to shared environmental preferences, and several copepod genera were linked to low-salinity environmental conditions and abundant V. cholerae and V. vulnificus.

Conclusions This study clarifies ecological relationships between pathogenic Vibrio spp. and the planktonic community, elucidating new functionally relevant associations, establishing a workflow for examining environmental pathogen microbiomes, and
highlighting prospective model systems for future mechanistic studies.

Background

Coastal bacterial Vibrio species can cause severe human infections, which are an emerging international health concern linked to rising global temperatures. V. cholerae, the causative agent of the disease cholera, infects millions of people each year, killing thousands, and is typically spread through ingesting contaminated drinking water [1]. Two other species of major concern are V. parahaemolyticus and V. vulnificus, which can cause severe wound infections, septicemia, and gastroenteritis from ingesting Vibrio-colonized seafood [2]. Several particularly dangerous pandemic strains have been identified, and non-virulent strains may become virulent via horizontal gene transfer as many infection-related genes are mobile [3]. Additionally, at least a dozen other species can infect humans or animals, extending the threat to aquaculture operations. Climate change may exacerbate the extent of these infections. Increasing air and water temperatures can facilitate increased metabolic growth capacity and temporal and geographic range expansion of Vibrio spp. pathogens [4–6]. Furthermore, V. cholerae epidemics have been linked to global temperature rise on decadal scales- a representative case study for understanding the link between the environment and human disease [7, 8]. Vibrio spp. interactions with the planktonic community have implications for both coastal ecology and human health. Coastal communities are highly productive environments; diverse and abundant populations of microbes and multicellular organisms are supported by primary productivity driven by ample nutrient availability. Vibrio spp. attach to and form biofilms on particles and eukaryotic organisms, living and dead, [9–12], presumably to better acquire carbon and nutrients and avoid environmental stress. These “close quarters” incite competition and enable cooperation and horizontal gene transfer with co-
occurring bacterial and eukaryotic species. An important facet of these interactions involves chitin, an abundant polymer produced by many marine eukaryotes. In addition to providing nutrients and an attachment substrate, chitin facilitates bacterial competition and horizontal gene transfer in Vibrio spp. [13, 14], which may spread virulence and antibiotic resistance genes among populations. Attachment also enables environmental persistence and dispersal; for example, Vibrio spp. attach to copepod exoskeletons by the thousands with high copepod abundances linked to cholerae epidemics. Vibrio spp. can also attach to chitin-producing diatoms [15, 16], though these dynamics and the ecological consequences are poorly understood.

Genus, species, and even strain-level distinctions likely modulate the functional characteristics of Vibrio-community interactions, but these are challenging to address because quantifying and characterizing environmental microbes is time and labor intensive and their identification often lacks clear criteria. Total quantities of Vibrio spp. are frequently used to infer ecological associations and pathogenicity potential, however, pathogenic species often occupy distinct environmental niches driven by temperature, salinity, and other biotic and abiotic factors (reviewed in Takemura et al., 2014). Virulence mechanisms, while poorly understood, are also species dependent. Likewise, eukaryotes are often grouped into broad categories such as total copepods or in the case of algae, by bulk chlorophyll a concentration, total pigment concentration, or at a broad taxonomic level (e.g. diatoms, dinoflagellates). But physiological differences at lower taxonomical ranks may have functional consequences for interactions; for example, some diatom genera exude chitin while others may not [18, 19], and algae are known to host distinct bacterial communities [20]. Thus, broad taxonomic groupings may be responsible for apparent conflicts and lack of consensus among prior studies.

Next generation sequencing based metabarcoding now enables large-scale community
analyses with improved taxonomic resolution that can be used to infer potential functional
significance from observed associations and co-occurrence patterns. In our study, we
sampled monthly for one year at 5 sites in Southern California (Fig. 1A-D), pairing
environmental data and pathogenic Vibrio species and virulence-associated gene
abundance obtained via digital droplet PCR with high resolution community composition
data derived from three different DNA amplicons. We used 16S and 18S rRNA gene
sequences derived from total RNA and cDNA to characterize the active communities of
bacteria, archaea, and eukaryotes, including both phytoplankton and multicellular
organisms. We further sequenced HSP60 gene sequences to characterize the vibrio-
specific community in both whole-community and vibrio isolate samples. Our findings
establish a workflow for examining environmental pathogen microbiomes, offer insights
into conflicting results from previous studies, elucidate new functionally relevant
associations, and facilitate future environmentally realistic model laboratory studies.

Results

Environmental niche of pathogenic vibrio species and virulence-associated
gene prevalence

We quantified three pathogenic vibrio species with high human health relevance: Vibrio
parahaemolyticus, V. vulnificus, and V. cholerae. Digital droplet PCR (ddPCR) was
performed to absolutely quantify single copy number genes specific to each species in
known filtration volumes using primers previously designed for qPCR (Additional File 1)
[21–25]. A wide range of temperatures (13.2–33 °C), and salinities (2.6–42.4 ppt) were
sampled (Fig. 1E,F), with highly variable chlorophyll a concentrations (Fig. 1G). Both
salinity and chlorophyll a were positively associated with temperature (Fig. 1H). Each
species was detected at all sites, often simultaneously, and exhibited species-specific
environmental niches (Fig. 2A-C). Target species were predominantly found above 20 °C. 

*V. parahaemolyticus* was detected in 80% of samples (Additional File 2) and was most abundant at warm temperatures and high salinities (Fig. 1A), though only the association with temperature was significant (p < 0.05)(Fig. 2D). We observed *V. parahaemolyticus* at extremely high salinities (> 40 ppt, Fig. 1A), while *V. vulnificus* and *V. cholerae* were both significantly associated with low salinity but not temperature (Fig. 2D). *V. vulnificus* was most abundant at moderate and *V. cholerae* at low salinity sites (Fig. 2B,C). High numbers of *V. cholerae* (> 280,000 copies/100 mL) were detected at Los Peñasquitos Lagoon (LPL) from March through May, corresponding with low salinity caused by lagoon closure and subsequent urban freshwater accumulation (Fig. 1F, Fig. 2C). All three species peaked between March and July at the LPL and San Diego River (SDR) sites. At the Tijuana River Estuary (TJ) sites, *V. parahaemolyticus* was predominantly detected, occurring between February and October and peaking at > 33,000 copies/100 mL in September (Fig. 2A).

We further screened for the presence of virulence-associated genes and quantified two genes associated with *V. vulnificus*: pilF and vcgC. We measured pilF and vcgC only in *V. vulnificus*-positive samples (Figs. 2E,F). Both were most abundant at the SDR sites, with pilF reaching > 7,000 copies/100 mL (Figs. 2F). When *V. vulnificus* was detected, 50% of samples also tested positive for one or both of the virulence-associated genes (Fig. 2G).

The ratio of these targets to *V. vulnificus* copies (potentially reflective of number of virulence-associated gene copies per *V. vulnificus* cell) was often below 1, though sometimes closer to 2 as in the LPL April sample (Fig. 2H).

**Vibrio community abundance and composition**

We assessed relative abundance of *Vibrio* spp. in the context of the entire bacterial community using HSP60 DNA amplicon sequences, amplified using DNA as a template. We characterized *Vibrio*-specific community composition using sequences generated from
culture-dependent Vibrio communities isolated on CHROMagar Vibrio plates (CHROMagar) (Fig. 3A, Additional File 3) and also culture-independent whole community filtered samples (Fig. 3B), which we then compared to Vibrio community composition determined via the 16S community data. Based on whole community sequencing, Vibrio spp. represented 0.03–4.9% of the 16S bacterial community (Mean = 0.44%, Additional Files 4 and 5) and 0–4.5% of the HSP60 community (Mean = 0.4%, Additional File 4). As in previous studies (e.g. Jesser and Noble, 2018), the composition of 16S Vibrio spp. sequences were poorly resolved at the species levels, with the majority identified only as “Vibrio sp.” (Additional File 5B). Of the pathogenic species detected via ddPCR, only V. cholerae was detected by 16S, and both V. cholerae and V. parahaemolyticus were detected using HSP60. Nearly 4 times as many 16S sequences were obtained across whole-community-samples than the HSP60 sequences (Additional File 4), with a higher percentage of assigned reads, resulting in the apparent absence of Vibrio spp. in some HSP60 samples though they are known to be present at these sites based on 16S, ddPCR, and isolate community sequencing (Fig. 3B). Additionally, in whole community samples, the HSP60 amplicon identified different but not substantially more Vibrio species (Fig. 3B).

Culture-based sequencing of the HSP60 amplicon from isolates revealed diverse Vibrio communities with higher taxonomic resolution than both whole 16S and HSP60 culture-independent sequencing approaches. HSP60 isolate community sequencing, which produced substantially more reads overall and a higher percentage of assigned reads (Additional File 4), identified more than 100 unique ASVs and identified all three quantified species and additional potential Vibrio spp. pathogens (Fig. 3A). Alpha and Shannon diversity were elevated during the summer months sampled, May and July, which coincided with moderate temperatures (Fig. 3C-E). Assuming not all Vibrio spp. in the samples were cultured/culturable, this may be an underestimate of species diversity.
16S and 18S Community Composition and Diversity

For 16S rRNA gene sequences, which were sequenced using RNA as a template, ~29K exact sequence variants (ASVs) were identified after removing eukaryotic, mitochondrial, and chloroplast sequences. A few major bacterial classes dominated community composition, including Gammaproteobacteria (encompassing Vibrio spp.), Bacteroidia, and Alphaproteobacteria (Fig. 4A). LPL and SDR sites had sizeable populations of Oxyphotobacteria (i.e. cyanobacteria), while other prominent classes included Campylobacteria and Verrucomicrobia. In contrast, 18S communities comprising ~17K ASVs were more diverse, with many rare species. For example, >50% of classes at some sites were <5% of relative abundance (Fig. 4B). Diatoms were the most common eukaryotes, comprising ~28% of 18S reads (Fig. 4A), and while common at the LPL and SDR sites, they were particularly abundant at the TJ sites, frequently representing >75% of 18S reads. Other abundant groups included unicellular Spirotrichea ciliates, photosynthetic Cryptophyceae, and chitin-producing Arthropoda organisms.

Both sampling site and month influenced community diversity, and samples collected at nearby sites or close in time were more similar to each other than to other communities. Based on Bray-Curtis dissimilarity, locations with two sampling sites clustered together for both 16S (Fig. 4C) and 18S (Fig. 4D). LPL samples more similar to the SDR samples for 16S communities and displayed tighter intra-site clustering for 18S samples. PERMANOVA tests revealed significant differences (p < 0.001) in community dissimilarity based on site and month for both 16S (Site: F-value: 2.6776, Month: F-value: 2.589) and 18S communities (Site: F-value: 2.1763, Month: F-value: 1.7862). Alpha diversity based on observed ASVs was also similar between sites, with LPL more similar to the SDR sites than the TJ sites. While 18S alpha diversity was significantly different based on both site (p = 0.004, Kruskal-Wallis statistic: 15.273) and month (p = 0.02, Kruskal-Wallis statistic: 22.618), for
16S only site (p-value: 0.005, Kruskal-Wallis statistic: 14.666) varied significantly across all samples, and not month (p = 0.22086, Kruskal-Wallis statistic: 14.224).

Relationships between pathogenic Vibrio spp. and planktonic community taxa

To investigate associations between pathogenic Vibrio spp. and planktonic community members we used spearman rank correlations to compare quantities of target species with abundant and functionally relevant planktonic classes, genera, and ASVs, and conducted linear discriminant analysis effect size (LEfSe) analyses to identify additional taxa associated with high concentrations of pathogenic Vibrio spp. For 16S communities, V. parahaemolyticus and V. vulnificus were positively associated with Verrucomicrobiae, a class containing mostly bacteria isolated from freshwater, soil, and human feces (Fig. 5A). V. vulnificus, V. cholerae, and the V. vulnificus virulence-associated gene pilF were positively associated with cyanobacteria and negatively associated with Camphylobacteria, a pattern mirroring the negative association between these three marker genes and salinity. Additionally, V. cholerae was negatively associated with Bacteroidia and Kirimatiellae (Fig. 5A). Individual ASVs, however, often exhibited associations masked at class level. For example, the Gammaproteobacteria and Alphaproteobacteria classes had no significant associations with any species or virulence genes (Fig. 5A), but in observing relationships with the top 20 most abundant ASVs (Fig. 5B), individual gammaproteobacterial taxa were negatively associated with V. parahaemolyticus (e.g. members of the genera Glaciecola and Marinobacterium unidentified at the species level) while alphaproteobacterial ASVs including a member of the SAR11 group, a Salinihabitans sp., and a Litorimicrobium sp. were positively associated; the former two were also positively associated with temperature and chlorophyll a. Bacterial taxa from both of these classes were also significantly associated with the other Vibrio spp. and genes quantified, indicating ASV-level associations and
revealing shared environmental preferences.

Eukaryotic taxa also exhibited ASV-level associations unapparent at higher taxonomic ranks. The most abundant class, Bacillariophyta (containing diatoms), was positively associated with *V. parahaemolyticus* and temperature, salinity, and chlorophyll a (Fig. 5C), but individual diatom taxa exhibited species-level associations. For example, two Chaetoceros pumilum ASVs, representing the most abundant diatom taxa (Fig. 5D, Additional File 6A), and a Thalassiosira weissflogii ASV followed this class-level pattern of positive *V. parahaemolyticus* association, but an abundant Skeletonema ASV was negatively associated. In the case of Thalassiosira, at the genus level (Additional File 6B) the only significant associations are with low temperature and salinity and high *V. vulnificus* concentrations. However, an abundant ASV annotated as *T. pseudonana* was positively associated with all three quantified species while a *T. weissflogii* was associated with high salinity and low *V. cholerae*. Other abundant community members included the photosynthetic Cryptophyceae and chitin-producing arthropods, which as a class were positively correlated with the *V. vulnificus* virulence-associated gene pilF and negatively correlated with salinity (Fig. 5D). Rather than being driven by the most abundant copepod genus (Additional File 6C), Pseudodiaptomus, which shows no correlations with target species or environmental variables at the genus (Additional File 6D) or ASV level (Fig. 5D), this association instead appears to be related to several less abundant copepod genera found in low salinity samples, including the genera Canuella, Tigriopus, Sinocalanus, and Cyclops, which are also linked to the pathogenic *Vibrio* spp. commonly found in lower salinity samples (Additional File 6D).

Target pathogenic *Vibrio* spp. were often co-abundant with particular organisms despite the absence of significant correlations across all samples. Pseudodiaptamus was not significantly associated with any target *Vibrio* species but was common and a dominant
part of the 18S arthropod community at high V. vulnificus and V. cholerae sites, particularly LPL during May (V. vulnificus and V. cholerae) and March (V. cholerae) (Additional File 6). For diatoms, Cyclotella spp. were abundant at both SDR sites during April, and May for SDR2, and Chaetoceros was abundant when V. cholerae concentrations were highest, at LPL March through May. While Chaetoceros diatoms were positively associated with V. parahaemolyticus across all samples, Thalassiosira and Cyclotella diatoms, which were not, were some of the most abundant eukaryotes in the high-V. parahaemolyticus samples, for example, at TJ2 in September, TJ1 in April, and SDR2 May (Fig. 4C). This suggests that despite a lack of correlation across samples, certain diatom genera or ASVs, including Thalassiosira and Chaetoceros diatoms, are an important component of high-Vibrio communities.

Lastly, we performed a linear discriminant analysis effect size (LEfSe) analysis to identify particular genera and ASVs, regardless of abundance, associated with different quantities of pathogenic Vibrio spp. Bacterial genera including Dovosia and Mycobacteria were more abundant in samples with high or very high levels of the lower salinity species V. cholerae and V. vulnificus (Additional File 7A,B) while different genera were associated with higher V. parahaemolyticus samples (Additional File 7C). Several Cyanobacteria (class: Oxyphotobacteria) genera and ASVs were associated with the pathogenic species; Anabaena was associated with low levels of V. cholerae, Prochlorococcus with low levels of V. parahaemolyticus, and two genera, Prochlorothrix and Pseudoanabaena were linked to very high levels of V. vulnificus. Additionally, two Picochlorum ASVs were associated with medium-high levels of V. cholerae. For eukaryotic ASVs, additional non-diatom algae that were less common (i.e. not among the most abundant 20 ASVs) were associated with high levels of pathogenic targets, including poorly characterized Prymnesiophytes (f_Prymnesiaceae) and Chrysophytes (Chrysophyceae clade F and D) associated with high
V. vulnificus and V. cholerae. Additional diatom species were found to be differentially abundant with high and very high levels of V. vulnificus (Cyclotella striata, C. scaldensis, Cylindrotheca Closterium, and Skeletonema subsalsum), while a poorly characterized Thalassiosira sp. ASV was associated with low levels of V. parahaemolyticus.

Discussion

Environmental niche and diversity of pathogenic Vibrio species

We observed distinct environmental niches among V. cholerae, V. vulnificus, and V. parahaemolyticus related to salinity and temperature (Fig. 2A-C). While these environmental factors are known to drive Vibrio distribution (reviewed in Takemura et al., 2014) many studies have focused on individual species or on the Vibrio genus as a whole, potentially overlooking species shifts in response to surrounding environmental community changes. By screening all three species, we capture some of these dynamics. We also present the first quantification and ecological analysis of pathogenic Vibrio spp. in the Southern California coastal region, and area of emerging risk due to warm coastal seawater temperatures, high residential and tourism recreational water use, and seafood cultivation. Vibrio spp. infections in Southern California have increased in recent years[30], particularly in San Diego County; the most recent year assessed, 2018, showed the highest number of infections ever reported and an infection rate substantially higher than both the California and US infection rates [31].

All three species were detected at all sites, occasionally simultaneously. This suggests either a continuous presence at all times, sometimes below detectable concentrations, or a temporal residence in the sediments or a viable but non-culturable (VBNC) [32] state until conditions become ideal for proliferation in the water column [33]. Peak abundance among the three species varied noticeably with salinity (Fig. 1A-C), with V. cholerae highest at the lowest salinity sites (~ 2–5 ppt), V. vulnificus highest at moderate salinities
(~ 17–25 ppt), and V. parahaemolyticus highest at high salinity sites (> 30 ppt). The distribution patterns of these three species were also linked to site, with V. cholerae most abundant at LPL, V. vulnificus most common at the SDR sites, and V. parahaemolyticus most abundant at the TJ sites. It is unclear whether those sites happened to present an ideal ecological niche at a given time, or if other factors such as biotic interactions limit concentrations of species that would otherwise be abundant.

V. cholerae and V. vulnificus were significantly associated with low salinity and were most abundant in warm temperatures though there was no significant temperature association across all samples (Fig. 1B,C). While V. cholerae has been reported in high salinity conditions, it is most common in low salinities, hence it’s tendency to contaminate drinking water. Likewise, V. vulnificus grows poorly at salinities higher than 25 ppt, preferring the range of 10-18 ppt [34, 35]. Both species peaked during warm summer months, typically a month or two before the peak temperature, and high abundances (i.e. > 1000 copies/ mL) were only found from March through July (Additional File ). As temperature was associated with high salinity, intermediate conditions where temperatures are warm, but salinity is low or moderate may be ideal. Both species were abundant only above 20 °C, a temperature above which human Vibrio infections are a serious concern [35–37].

V. parahaemolyticus abundance was significantly associated with high temperatures, but not salinity, suggesting V. parahaemolyticus may be a more halotolerant species. This is supported by a meta-analysis finding that in contrast to V. cholerae, V. parahaemolyticus was distributed across a broader salinity range of 3-35 ppt, with a warmer, more narrow temperature range [17]. The abundant V. parahaemolyticus populations we observed at extremely high salinities (> 40 ppt) (Fig. 2A) were out of the reported range in the meta-analysis and for other prior studies we examined, perhaps suggesting unique high-salinity
adaptations. However, the fundamental ecological niche of many Vibrio species, particularly in terms of salinity, is often larger than realistic environmental conditions [38]. Comparative analyses examining specific physiological properties of these populations, other high-salinity V. parahaemolyticus strains if identified, and those with “typical” salinity ranges may shed light on whether the strains we observed were atypical. As the high-salinity populations were found at moderate temperatures, salinity tolerance may allow V. parahaemolyticus to take advantage of fortuitous warm temperatures, though other site-specific factors are likely to be involved. In general, our study supports the well-established temperature and salinity preferences previously observed in these species in a different geographic region.

Some of our findings suggest local Vibrio populations may be of regional concern, perhaps necessitating a monitoring and surveillance plan. The potentially pathogenic V. vulnificus targets we examined appeared to contain a high percentage of virulence-associated genes as measured by ddPCR (Fig. 2G,H). Half of the samples that tested positive for V. vulnificus also tested positive for one or both of the virulence-associated genes tested. For example, the vcgC gene, which is a marker more common in clinical V. vulnificus strains than environmental counterparts, was detected in 20% of these samples. Along the North Carolina Coast Williams et al. found that 5.3% of the V. vulnificus examined possessed the vcgC gene [39]. The pilF gene, which based on human serum sensitivity is highly correlated with pathogenicity potential [40], was also detected in 45% of these samples. Additionally, we detected high concentrations of V. cholerae (> 2800 cells/ mL) at the LPL site. While it is unclear whether these strains possess virulence-associated genetic markers such as the ctxA toxin-associated gene, V. cholerae can infect immunocompromised humans without these virulence genes and Vibrio communities lacking these virulence genes can acquire them rapidly via viral infection [41] or other
horizontal gene transfer events.

Culture dependent and independent sequencing methods highlight Vibrio diversity and additional pathogenic species of interest

We gained insight into Vibrio community diversity by sequencing the HSP60 gene in both whole community samples (culture independent) and Vibrio isolate communities (culture dependent) (Fig. 3). The HSP60 amplicon is better at phylogenetically resolving certain bacterial taxa, including Vibrio spp. [29, 42, 43], compared to the more commonly used 16S amplicon. Consistent with these prior findings, in our study, for both culture dependent and independent HSP60 sequencing methods, more Vibrio ASVs were identified at the species level than the 16S rRNA gene sequencing which identified the large majority of Vibrio spp. only at the genus level only (Fig. 3B, Additional File 5). In our HSP60 whole community analysis we did not observe as many species as Jesser and Noble 2018, which introduced this method for profiling Vibrio communities; this could be the result of differences in sequencing depth and/or regional differences in Vibrio spp. abundance as that study was conducted in the Neuse River Estuary of North Carolina.

However Sequencing Vibrio isolate communities from 24 sites allowed us to identify nearly twice as many species as the 16S or HSP60 amplicons, including all 3 pathogenic species detected using ddPCR and additional human pathogenic species including V. alginolyticus, V. fluvialis, V. furnissi (also identified by HSP60), and V. metschnikovi [44]. With a large number of sequence reads obtained, the majority of which belonged to the Vibrio genus (Additional File 4) the diversity of these communities was better captured than with either the HSP60 or 16S rRNA gene amplicon - particularly regarding intraspecific diversity (Fig. 3A). This method does present some limitations; sequences are limited to cultivated organisms, and this method precludes an analysis of relative abundance within the
community as some species likely outgrow others. Nonetheless, our results reveal an impressive, and likely underestimated, picture of Vibrio diversity.

Combining these methods, we substantially expanded our understanding of what other Vibrio bacteria exist with abundant pathogenic species populations. For example, an interesting species, V. antiquarius (formerly known as Vibrio sp. Ex25), was a highly abundant member of the Vibrio community in both culture-dependent and independent analyses. This species is closely related to V. parahaemolyticus and V. alginolyticus and was originally isolated from deep-sea hydrothermal vents [45]. Based on genome sequencing, its predicted to possess both the functional potential to survive in extreme conditions and factors potentially involved in human disease caused by coastal Vibrio spp. More recent studies have identified isolates in heat-shocked oysters, confirming that it inhabits diverse environments [46], though given its similarity to other Vibrio species its taxonomic designation is still being explored [47]. In our study, this species co-occurred with highly abundant pathogenic Vibrio spp., and while the ecological role and pathogenicity potential of V. antiquarius is unknown, its close phylogenetic relationship to and copresence with the pathogenic species at these sites suggests they may be interacting and potentially even horizontally sharing genes.

Pathogenic Vibrio spp. are commonly associated with prokaryotic and eukaryotic community members, including chitin producers. Our study elucidates links between three pathogenic Vibrio species, the environment, and the planktonic community. In conducting these analyses, it is clear that taxonomic resolution plays an important role in defining potential functionally relevant relationships, and in establishing an ecologically relevant context for prior and future studies. Using Vibrio spp. abundance to assess community interactions, ecological niche, and even health risk is a common practice. However, these results can be conflicting or misleading,
unsurprisingly since Vibrio spp. occupy distinct ecological niches and possess unique physiological capabilities, including virulence mechanisms and modes of infection. This applies to community associations as well; demonstrating the potential importance of species-level associations, Turner et al. [16, 48] found that while total Vibrio spp. bacteria were negatively correlated with copepods in a particular size fraction (63–200 µm), the pathogenic species V. parahaemolyticus and V. vulnificus were actually positively associated with copepods.

Bacterial species interacting with Vibrio spp. may impact virulence and environmental persistence through horizontal gene transfer, population dynamics via viral infection, and growth through competition or cooperation. We observed that common bacterial classes were similarly present and abundant across most sites and months, however, particular genera exhibited species and virulence gene-specific correlations. Individual ASVs of the most abundant classes were either positively or negatively associated with pathogenic Vibrio spp. despite no clear correlations at the class level (e.g. Gammaproteobacteria and Alphaproteobacteria) (Fig. 4A, Fig. 5A). Notably, we observed associations between members of the class Oxyphotobacteria (Cyanobacteria), which was negatively associated with V. vulnificus, V. cholerae, and pilF (Fig. 5A). Jesser and Noble 2018 also found a negative association between Cyanobacteria and V. vulnificus using a comparative relative abundance approach. In our dataset only one ASV, a Prochlorothrix sp., was among the 20 most abundant and appeared to drive this pattern. Multiple less abundant ASVs had more variable associations, as revealed by LEfSe, which may guide future studies. A prior laboratory study investigated the response of Synechococcus sp. WH8102 to co-culture with V. parahaemolyticus, finding significant transcriptional changes including evidence of possible phosphate stress and utilization of specific nitrogen sources [49]. While we didn’t observe this organismal pairing in our dataset, future transcriptomic studies may identify
Similarly important Vibrio-cyanobacterial interactions based on our dataset. A primary focus of our study was assessing pathogenic Vibrio spp. interactions with eukaryotes in the community, including those that produce chitin and organisms crucial to ecosystem function such as primary producers and grazers which are frequently overlooked in environmental microbiome studies. Of particular interest are diatoms (class Bacillariophyta) and copepods (class Arthropoda) as both of these groups are capable of chitin production and have been shown to interact with pathogenic Vibrio spp. in laboratory studies. For most known Vibrio spp., chitin serves as a nutrient source and a substrate for biofilm formation and subsequent protection from environmental stressors and predation [12, 50]. It also induces a well-studied suite of cellular interactions initiating bacterial competition via the Type VI secretion system (T6SS) and natural competence, which may be the mechanism for how non-virulent populations become virulent (Meibom, 2005; Borgeaud et al., 2013; Lutz et al., 2013; Sun et al., 2013; Antonova and Hammer, 2015; Erken et al., 2015).

The most abundant eukaryotic organisms found in our samples (> 28% of 18S sequences) were diatoms, which in prior studies were frequently associated with high Vibrio spp. concentrations [16, 55, 56] but are often analyzed as a single group or under the “bulk” algae category even though individual species host distinct microbial communities [20], release unique dissolved organic matter substrates [57–59], have variable susceptibility to viral or bacterial infection [60–63], or exude chitin. In our study the class Bacillariophyta was positively associated with V. parahaemolyticus, temperature, salinity, and chlorophyll a (Fig. 5C). However, looking at the more resolved genus level, this affect appears to be driven by the most abundant diatom genus Chaetoceros (Additional File 6B). In particular, two ASVs most closely related to Chaetoceros pumilus comprised the majority of Chaetoceros diatoms (Fig. 5D).
Though many diatom genera contain chitin synthesis genes or full pathways, and may potentially produce chitin as a component of the cell wall [64, 65] (though to the best of our knowledge this hasn’t yet been reported), only two have been shown to date to actually exude chitin: Thalassiosira and Cyclotella [18]. These chitin producing diatoms were highly abundant in our samples, with 3 ASVs among the most abundant 20 eukaryotic taxa (Fig. 5D). Notably, they exhibited ASV-specific relationships with the pathogenic Vibrio spp.; T. pseudonana was positively linked to all of the target species while T. weissflogii was negatively correlated with V. cholerae. While the genus Cyclotella had no significant correlations with any of the targets overall, individual ASVs closely related to C. scaldensis and C. striata (one of the 20 most abundant ASVs) did exhibit significant associations, particularly with V. vulnificus. T. pseudonana, which has been observed to have algicidal interactions with chitinase-producing bacterium in laboratory studies [66] is a well-characterized and genetically tractable model organism. Our findings support a prior laboratory study where Frischkorn et al. 2013 observed V. parahaemolyticus attaching to the chitin-producing diatom T. weissflogii, suggesting an unexplored mechanism of environmental persistence [15]. In addition to revealing new environmental associations, our findings establish an ecologically relevant foundation for studying interactions between chitin-producing diatoms, pathogenic Vibrio spp., and the environment.

The interaction between pathogenic Vibrio spp. and planktonic copepods is an important, well-studied coastal phenomenon with demonstrated human health implications. Huq et al. 1983 found that V. cholerae 01 and non-01 serovars attached to living but not dead Acartia tonsa, Eurytemora affinis, and Scottolana spp. copepods from natural samples [9]. Another laboratory study investigating these same living copepod species found that V. cholerae preferentially attached to Acartia tonsa copepods over Eurytemora affinis, and
that individual V. cholerae strains exhibited different attachment efficiencies [67]. In contrast, an O1 V. cholerae serovar (strain N16961) and two non-O1/O139 V. cholerae isolates, were found to preferentially attach to dead, rather than living, Tigriopus californicus copepods, as well as dinoflagellates [11]. It is unclear whether this difference is due to experimental methodology, the copepod species, or the Vibrio strains.

Environmental studies accounting for copepod taxonomy are rare and inconclusive: one found no association between V. cholerae and co-occurring Diaptomus and Cyclops genera copepods [68], and while others have reported qualitative associations in field samples [69, 70] relationships based on quantitative data and copepod specificity are rare and consequently poorly defined.

We observed positive correlations between pathogenic Vibrio spp., particularly those found in lower salinities, and several copepod genera (Additional File 6D). The most abundant copepod was identified as Pseudodiaptamus inopinus, an invasive species originating in Asia [71, 72], which was not significantly associated with any Vibrio species across all samples but was highly abundant during the months where the highest levels of V. cholerae and V. vulnificus were detected at LPL and the SDR sites (Fig. 4B, Fig. 5D).

Other abundant copepods were the Harpacticoid genera Canuella and Tigriopus, both positively associated with V. vulnificus and the virulence-associated gene pilF (Fig. 5C, D). In laboratory studies, the type IV pilus (containing the pilF subunit) has been shown to be involved in chitin attachment to Vibrio spp. [12]. Tigriopus was also positively associated with V. cholerae. Though we could not obtain species-specific taxonomic resolution for Tigriopus, it is a well-established laboratory model genus with gene-silencing capabilities and full or partially assembled genomes for several species [73–75]. Thus, Tigriopus and Canuella spp. may be good candidate genera for future laboratory studies involving ecologically relevant Vibrio-plankton interactions.
Conclusions

Our study quantifies for the first time potentially pathogenic Vibrio spp. in Southern California while using a metabarcoding approach to identify community diversity and potential interactions. In addition to observing abundant populations of V. parahaemolyticus, V. vulnificus, and V. cholerae that conform to previously observed temperature and salinity niches, culture-dependent and culture-independent sequencing approaches revealed diverse Vibrio communities including additional potentially pathogenic species. High abundances in previously unstudied areas with high potential for human exposure, along with the detection of multiple genes associated with human infection, suggest that future sampling and risk modelling for these areas may be appropriate.

In characterizing the microbial and eukaryotic communities co-occurring with these individual Vibrio species, we identified ASV-specific relationships with potential functional implications including interactions between target Vibrio spp. and chitin producing diatoms and copepods. Ultimately, our characterization of Vibrio communities, other community members, and their shared environmental preferences can be used to develop and test new hypotheses about the role of the environment and biotic interactions in Vibrio persistence, proliferation, and disease risk.

Methods

Environmental sampling and Vibrio isolate culturing

Monthly sampling was conducted from December 2015 to November 2016 at 3 locations in San Diego County: Los Peñasquitos Lagoon (LPL), the San Diego River (SDR), and the Tijuana River Estuary (TJ) (Fig. 1A-D). For intra-site comparisons, two different sites at SDR (SDR1 and SDR2) and TJ (TJ1 and TJ2) were sampled, totaling 5 sampling sites.
Temperature and salinity were measured between 12 pm and 1 pm using a YSI Pro 30 field instrument (YSI Inc.). Unfiltered water samples were collected in 4 L opaque bottles and processed in lab beginning no more than 2 hours after collection. These samples were kept in a cool area at roughly room temperature rather than at 4 °C to prevent a viable but non-culturable (VBNC) state in Vibrio bacteria [76].

Water samples were gently filtered and flash-frozen in the lab for downstream processing. For chlorophyll a quantification, 10-100 mL samples were collected on GF/F filters (Whatman) and stored at -20 °C. Samples were later extracted in 90% acetone overnight and measured on a 10AU fluorometer (Turner), followed by addition of HCL and re-measurement to account for the chlorophyll a degradation product pheophytin [77]. For downstream nucleic acid extractions 50-400 mL samples were filtered onto 0.4 µm polycarbonate filters (Whatman) and stored at 80 °C until processing.

Live Vibrio isolate communities were collected at 24 sampling points by filtering 10-100 µL of whole seawater onto 0.45 µm polycarbonate filters, which were transferred to CHROMagar Vibrio (CHROMagar Microbiology) plates and incubated overnight at 37 °C. These communities (examples in Additional File 3) were resuspended in 1 mL of either LB broth (Amresco) or Zobell Marine Broth 2216 (HiMedia), depending on sampling salinity and frozen as 15% glycerol stocks at -80 °C. Half of each glycerol stock was pelleted and used for downstream DNA extraction.

**DNA and RNA extraction and cDNA synthesis**

Nucleic acids were extracted from filter samples using the NucleoMag Plant kit (Macherey-Nagel) for genomic DNA (gDNA) and the NucleoMag RNA kit (Macherey-Nagel) for RNA. Initial sample lysis buffer resuspension and vortexing was completed manually, the remainder using an epMotion liquid handling system (Eppendorf). RNA was reverse-transcribed into cDNA using the SuperScript III First-strand cDNA Synthesis System
gDNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) and RNA using the Quant-iT RiboGreen RNA Assay Kit. Nucleic acid integrity was confirmed using an Agilent 2200 TapeStation (Agilent). Duplicate filters were extracted for all RNA and DNA samples with the exception of SDR1 December and April, for which two RNA but only one DNA sample was extracted. Genomic DNA was extracted from Vibrio isolate pellets using a DNeasy Blood and Tissue Kit (Qiagen), with subsequent quantification and quality control as described above.

Vibrio digital droplet and end-point PCR

Select pathogenic Vibrio species and virulence genes were quantified using the QX200 digital droplet PCR (ddPCR) System (BioRad), following the manufacturer’s protocols and recommended reagents. Previously published assays based on qPCR were optimized for ddPCR, including running temperature gradients for each target to establish optimum reaction temperature. Results from technical replicates were merged for analysis, and more than 19,000 droplets were measured per sample. Target-specific gBlocks (Integrated DNA Technologies) were used as positive controls for all ddPCR and end-point PCR targets. Single copy-number gene targets for the species V. parahaemolyticus, V. vulnificus, and V. cholerae were quantified and used to approximate cell number per 100 mL of sample (Additional File 1). We targeted toxR for V. parahaemolyticus [23], vvhA for V. vulnificus [24], and ompW for V. cholerae [25]. We also quantified the virulence-associated V. vulnificus genes vcgC [21] and pilF [22].

Amplicon library construction and sequencing

Amplicon libraries were constructed and sequenced using cDNA or DNA template for whole community and Vibrio “isolate community” samples to characterize composition of multiple co-occurring communities. RNA template was used to characterize biologically active community members using 16S and 18S amplicons, while DNA was used to
characterize vibrio-specific diversity using the HSP60 amplicon. The 16S rRNA gene small subunit (SSU-rRNA) V4-5 region was targeted to characterize the prokaryotic bacterial and archaeal community using primers 515F-926R [78]. The V9 region of the 18S rRNA gene was targeted for eukaryotic community composition using primers 1389F and 1510R [79]. The universal regions of heat shock protein 60 (HSP60), also known as chaperonin 60 (cpn60), sequence was amplified and sequenced as described in Jesser and Noble 2018 using primers identified in previous studies [42, 80].

Replicate samples from each site and date were sequenced for the 16S and 18S rRNA gene sequences, and a single replicate was sequenced for HSP60 amplicons (both whole community and isolate community samples). For the HSP60 amplicon, 24 select samples (Additional File 4) were sequenced for Vibrio isolate communities, and whole community samples were not sequenced for SDR1 April and December. No template controls and mock communities were sequenced for 16S and 18S amplicon sequencing runs (one replicate run for each). ZymoBIOMICS™ Microbial Community DNA Standard mock communities (Zymo Research) were sequenced with the 16S run and the custom eukaryotic communities “even” and “stag” were obtained from the Fuhrman lab and sequenced with the 18S run [81].

Sequencing was conducted using an Illumina MiSeq platform at either the Institute for Genomic Medicine (IGM, University of California, San Diego) or at the UC Davis Genome Center (https://dnatech.genomecenter.ucdavis.edu/), with 300-bp paired end sequencing for the 16S and HSP60 amplicons and 150 bp paired end sequencing for the 18S amplicon. Demultiplexed sequences were analyzed using the Qiime2 [82] pipeline and additional analyses and visualizations were conducted using the R package phyloseq [83] and the web-based tool MicrobiomeAnalyst [84]. Sequences were quality filtered, chimeric sequences were removed, and exact amplicon sequence variants (ASVs)[85] were defined
using dada2 [86] with a maximum expected error cutoff rate of 2 for 16S and 18S rRNA
gene amplicons and 5 for the HSP60 amplicon. For 16S and 18S amplicons, replicate
samples were merged using the “qiime feature-table group” function. Taxonomy was
assigned using Silva [87] version 132 for bacterial and archaeal 16S sequences, and PR2
[88] for 18S sequences, and the cpn60 database [89] with taxonomic designations derived
from NCBI for HSP60 sequences as described in Jesser and Noble 2018 [29]. Chloroplast,
mitochondrial, and eukaryotic sequences were removed from 16S datasets prior to
downstream analyses. Alpha and beta diversity metrics for community composition were
calculated using Phyloseq and MicrobiomeAnalyst, respectively. Singleton ASVs were
retained for alpha diversity analyses. Principle coordinate analyses (PCoA) were conducted
using Bray-Curtis dissimilarity to examine beta diversity after filtering out ASVs with fewer
than 4 reads in at least 10% of samples.
Spearman’s rank correlation coefficients were calculated to explore relationships between
environmental variables, Vibrio quantification data, and relative abundance of groups of
interest in the amplicon sequencing. Correlations were visualized as correlograms using
the corrplot package in R [90], or as network graphs using the R package iGraph [91].

Declarations

Ethics approval and consent to participate: Not Applicable

Consent for publication: Not Applicable

Availability of data and material: The sequencing datasets generated and/or analyzed
during the current study are available in the NCBI repository, (BioProject accession no.
PRJNA593265; BioSample accession nos. SAMN13474661-SAMN13474785, SAMN13475110-
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Location of the sampling sites mapped in the context of the San Diego region using Google Earth. Site abbreviations are as follows: (A) LPL = Los Peñasquitos Lagoon, (B) SDR = San Diego River (site 1 and 2), and (C) TJ = Tijuana River Estuary (site 1 and 2), with (D) depicting the regional context. Environmental conditions, including (E) temperature, (F) salinity, and (G) chlorophyll a, a proxy for photosynthetic organism abundance, were measured monthly at each site (LPL = red, SDR1 = mustard, SDR2 = green, TJ1 = blue, and TJ2 = purple) for one year from December 2015-November 2016. Spearman rank correlations identified relationships between environmental variables, where values closer to 1 (dark blue) are positive correlations and values closer to -1 (dark red) are negative correlations, and * represents significant correlations (p > 0.05).
Figure 2

Number of single-genome copy genes (a proxy for cell numbers) per 100 mL detected by digital droplet PCR. (A) the *Vibrio parahaemolyticus* (Vp) species-specific gene target *toxR* (B) the *Vibrio vulnificus* (Vv) species-specific target *vvhA*, and (C) and the *Vibrio cholerae* species-specific target *ompW*, with marker size corresponding to copy number and color corresponding to site, plotted against temperature and salinity. (D) Spearman’s rank correlation coefficients of
associations between environmental the variables temperature, salinity, and chlorophyll a, and Vibrio species and virulence gene targets. Blue represents a strong positive correlation, while red represents a strong negative correlation, significant correlations (p<0.05) are denoted with *. Number of copies detected per 100 mL by digital droplet PCR for the Vibrio vulnificus virulence-associated genes (E) vcgC and (F) pilF, plotted against temperature and salinity. (G) The percent of V. vulnificus samples where no virulence gene was detected, either vcgC or pilF were detected, or both were detected. (H) the ratio of the number of pilF and vcgC copies detected to total V. vulnificus determined by vvhA copy number.
Figure 3

Relative abundance of Vibrio ASVs determined by HSP60 amplicon sequencing and alpha diversity metrics. (A) Heatmap of Vibrio spp. and ASVs identified using isolate community sequencing for 24 samples, with metadata including temperature, salinity, chlorophyll a, and relative Vibrio target quantities below. (B) Heatmap of Vibrio spp. and ASVs identified by whole community sequencing. Diversity metrics including (C) number of observed ASVs and (D) Shannon diversity, along with temperature and salinity (E), are also presented for the isolate communities.
Figure 4

Taxonomic composition and principle coordinate analyses of prokaryotic and eukaryotic communities. Taxonomic composition of 16S (A) and 18S (B) communities by class, and principle coordinate analyses (PCoA) based on Bray-Curtis dissimilarity for 16S (C) and 18S (D) communities grouped by site and labelled by month.
Figure 5

Class and ASV-level associations between abundant prokaryotic and eukaryotic community members, Vibrio species and virulence gene targets, and environmental variables. (A) Spearman’s Rank Correlations between the top 10 most abundant bacterial classes, Vibrio target species (toxR = V. parahaemolyticus, vvhA = V. vulnificus, ompW = V. cholerae) and virulence-associated genes (pilF and vcgC), and temperature, salinity, and Chlorophyll a. Blue correlation coefficients indicate positive associations, red indicates negative associations, and statistically significant associations (p < 0.05) are denoted with an *. (B) Statistically significant Spearman’s Rank Correlations between the 20
most abundant prokaryotic ASVs (P1-20) organized by class, with relative strength corresponding to correlation coefficient, blue lines indicating a positive association, and red lines indicating a negative association. (C) Correlations as described in (A) for the top 10 most abundant eukaryotic classes, and (D) Associations as described in (B) for the 20 most abundant eukaryotic ASVs (E1-20) organized by class.

Supplementary Files

This is a list of supplementary files associated with the primary manuscript. Click to download.

AdditionalFile3_CHROMagarVibrioPlates.pdf
AdditionalFile7_LEfSE.pdf
AdditionalFile5_16SVibrioCommunity.pdf
AdditionalFile6_DiatomCopepodGenera.pdf
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AdditionalFile2_ddPCR.pdf
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