Spatial and Temporal Distribution of the *Vibrionaceae* in Coastal Waters of Hawaii, Australia, and France

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Abstract

Relatively little is known about large-scale spatial and temporal fluctuations in bacterioplankton, especially within the bacterial families. In general, however, a number of abiotic factors (namely, nutrients and temperature) appear to influence distribution. Community dynamics within the Vibrionaceae are of particular interest to biologists because this family contains a number of important pathogenic, commensal, and mutualist species. Of special interest to this study is the mutualism between sepiolid squids and *Vibrio fischeri* and *Vibrio logei*, where host squids seed surrounding waters daily with their bacterial partners. This study seeks to examine the spatial and temporal distribution of the Vibrionaceae with respect to *V. fischeri* and *V. logei* in Hawaii, southeastern Australia, and southern France sampling sites. In particular, we examine how the presence of sepiolid squid hosts influences community population structure within the Vibrionaceae. We found that abiotic (temperature) and biotic (host distribution) factors both influence population dynamics. In Hawaii, three sites within squid host habitat contained communities of *Vibrionaceae* with higher proportions of *V. fischeri*. In Australia, *V. fischeri* numbers at host collection sites were greater than other populations; however, there were no spatial or temporal patterns seen at other sample sites. In France, host presence did not appear to influence *Vibrio* communities, although sampled populations were significantly greater in the winter than summer sampling periods. Results of this study demonstrate the importance of understanding how both abiotic and biotic factors interact to influence bacterial community structure within the Vibrionaceae.

Introduction

Recent work in marine microbial ecology has uncovered the importance of bacterioplankton in many ecological processes [4, 15, 24, 27, 31]. Given the ecological significance of these communities, there is a need to understand the dynamic forces shaping their structure. Changes in abiotic factors, such as temperature, salinity, and nutrients, have been shown to be important causes of community changes. More recently biotic factors, including bacterivory, bacteriophage parasitism, and mutualistic relationships, have been shown to affect bacterioplankton [22, 34]. One mutualism that appears to influence bacterial community structure is the relationship between sepiolid squids (Cephalopoda: Sepiolidae) and their *Vibrio* symbionts.

In the association between sepiolid squids and *Vibrio fischeri* and/or *Vibrio logei*, the squid host benefits from *Vibrio*-produced light that is used in counterillumination (an antipredation mechanism), whereas vibrios benefit from a nutrient-rich habitat in which near-maximum growth rates can be achieved [5, 17]. In this environmentally transmitted symbiosis, juvenile squids acquire their bacterial symbionts from the surrounding seawater upon hatching. After colonization, the host exhibits a daily venting behavior in which 90–95% of the symbionts are released into the surrounding seawater [19, 32]. Because bacterial populations in mature squids can reach levels of approximately 10^8–10^{11} cells within the light organ [33], the daily flux of *Vibrio* into the environment can potentially influence local bacterioplankton community structure.

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On the island of O'ahu, Hawaii, studies using *lux* gene probes [20, 21] have demonstrated that the presence of the squid host *Euprymna scolopes* does appear to influence community structure within the *Vibrionaceae*. However, Hawaiian waters represent a relatively simple system to study bacterioplankton community dynamics because of the relatively constant water temperature and lack of a predominant thermocline within host habitats. It appears that in different locations where this symbiosis occurs, other factors may influence community dynamics within the *Vibrionaceae*. For example, at sites in Banyuls-sur-mer, France, studies using plate counts of *V. fischeri*, a warm-adapted symbiont, and *V. logei*, a cold-adapted symbiont, have demonstrated that the presence of a thermocline presents a barrier to mixing between these two species [28]. The profound impact on the spatial distribution of *Vibrio* species at this location suggests that there may be additional factors capable of influencing local bacterioplankton community structure. Further adding to this complexity are a number of different strains of *V. fischeri* in nature, many of which cannot colonize at all (nonsymbiotic) or can only partially colonize the light organ of sepiolid hosts [21].

The goal of this study was to examine the distribution of *V. fischeri* and *V. logei* in reference to host location using fluorescence *in situ* hybridization (FISH) with a nested set of probes (i.e., γ-Proteobacteria > *Vibrionaceae* > *V. fischeri* and *V. logei*) in an attempt to understand the complex spatiotemporal patterns of members of the *Vibrionaceae*. Using a modification of traditional FISH, which incorporates direct counts (DC) on filters [23], we have accumulated an extensive dataset examining the *Vibrionaceae*, *V. fischeri* and *V. logei*, at field sites in southern France and along the east coast of Australia to obtain a more definitive picture of how various factors influence population structure within the *Vibrionaceae*.

Methods

Probe Construction and Optimization.   Probes used in this study are listed in Table 1. To design the Vib749 probe (*Vibrionaceae*), 16S rRNA sequences of representative members of the *Vibrionaceae* (obtained from GenBank; http://www.ncbi.nlm.nih.gov/) and *Escherichia coli* K-12 (as the outgroup; GenBank accession number U00096) were aligned in MacVector™ 6.5 using the ClustalW alignment algorithm. Conserved sequences of 18–23 base pairs in length within the family *Vibrionaceae* that contained at least one base difference from *E. coli* K-12 were screened visually. Sequence specificity of each candidate probe sequence was determined using the Probe Match function in the Ribosomal Database Project II [6], and the probe that represented the most species within the *Vibrionaceae* was found at *E. coli* K-12 base position 749. The analogous procedure was implemented for designing Vfsh84a and Vlog251 probes. In these cases, a number of GenBank 16S rRNA sequences from *V. fischeri* strains [29] (accession numbers AY292941, AY292938, AY292922, AY292921, AY292920, and AY292919) were aligned against two *V. logei* strains (accession numbers X74708 and AY292934), and probe sequences were determined as above.

Stringency conditions for *in situ* hybridization of each probe were empirically optimized to allow the probe to bind only to target sequences containing zero mismatches (Table 1). To do so, probes were hybridized against bacterial strains with zero, one, and two mismatches in hybridization solutions containing various formamide concentrations between 5–50%. The Vib749 probe was tested against *V. fischeri* ES114 (zero mismatches), *Photobacterium damselae* (one mismatch; accession number X78105), and *E. coli* K-12 (two mismatches). For Vfsh84a, test strains were *V. fischeri* ES114 (0 mismatches), *P. damselae* (1 mismatch), and *Vibrio parahaemolyticus* strain 113 (two mismatches; accession number AY527396). Vlog251 was tested against *V. logei* (zero mismatches; accession number X74708) and *V. fischeri* ES114 (1 mismatch). All *Vibrio* and *Photobacterium* strains were grown overnight at 28°C in Luria–Bertani high salt solution (1% tryptone, 0.5% yeast extract, 2% NaCl, 0.3% glycerol, and 50 mM Tris–HCl at

Table 1. Probes used in this study

Probe	Target group	Test strain	Sequence	Percent formamide
Gam42a[a]	γ-Proteobacteria	N/A	5′GCCTTCCCACATCGTTT	35
Vib749	*Vibrionaceae*	*V. fischeri* ES114	5′TCGCATCTGAGTGTCAGT	35
		P. damselae	3′AGCGUAGACUCGCAGUCA	
		E. coli K12	3′AGCGUGGACUCGCAGUCA	
Vfsh84a	*V. fischeri*	*V. fischeri* ES114	5′ACGCCCTTAACGTTCCCCG	50
		P. damselae	3′GGCGGGAAUUGCAAGGGGC	
		V. parahaemolyticus	3′UGCGGCAAUAGCAAGGGGC	
Vlog251	*V. logei*	*V. logei*	5′CCTTGGTGAGCTCTTACCCC	40
		V. fischeri ES114	3′GGAACCACUCGAGAAUGGAG	

The optimal concentration of formamide was empirically derived for Vib749, Vfsh84a, and Vlog251. Each probe was hybridized at varying concentrations of formamide ranging from 0–50% against test strains containing zero, one, or two mismatches against the probe. The target ribosomal sequences were listed from 3′ to 5′ for test strains containing one or two mismatches to clearly identify the location of each underlined mismatch.
[a][2]

pH 7.4). *Escherichia coli* was grown overnight at 37°C in Luria broth (Invitrogen, Carlsbad, CA, USA).

For probe optimization hybridizations, overnight cultures were fixed in 3% paraformaldehyde and 5 µL spots of individual test strains were allowed to dry in each of 10 wells (5 mm diameter) on a Teflon-treated slide (Cel-Line Associates, Inc., Newfield, NJ, USA). Wells were treated with 20 µL of hybridization solution (using Cy-3-labeled probes; see below for recipe) containing increasing concentrations of formamide at 5% intervals from 5–50%. Wash steps and viewing are described below. To determine the optimal formamide concentration for each probe, each test strain was examined at every formamide concentration. The optimal concentration was that at which the one and two mismatch strains showed no visible fluorescence, but the zero mismatch strain was strongly fluorescent.

Field Sampling.   Water samples were collected from field sites in O'ahu, Hawaii, on the SE coast of Australia, and southern France for sampling. In Hawaii, eight sites were sampled around O'ahu in March 2003 (Fig. 1). At these sites, samples were collected during the morning low tides from waist-deep water. In Australia, samples were collected predawn and postsunset from waist-deep water at ten sites along the east coast between Sydney and Melbourne in March and September 2004 (Fig. 2). For logistical reasons, not every site was sampled during both months. In France, samples were collected at various depths at two research sites (Bay of Banyuls and Bay of Elmes in Southern France; Fig. 3) in November 2003 and August 2004. Access to conductivity–temperature–depth recorder (CTD) and research vessels at these sites enabled a more complete examination of the bacterioplankton. At each site, samples were taken in triplicate.

Upon collection of 100 mL of seawater, the samples were immediately fixed with paraformaldehyde (3% final concentration) at 4°C for 4–12 h. After incubation, various volumes of the sample (5 mL for the γ-Proteobacteria, 10 mL for the *Vibrionaceae*, and 20 mL for *V. fischeri* and *V. logei*) were filtered onto 0.2-µm pore size nucleopore filters (Whatman, Clifton, NJ, USA) coated with 0.1% poly-L-lysine, and backed with 8-µm pore size filters. Filtration took place using a vacuum below 40 kPa. Dried filters were stored at −20°C until hybridization.

In Situ Hybridization.   Fluorescence *in situ* hybridization incorporating DC was performed on each filter according to previous methods with minor modifications [23]. Each filter was aseptically cut into a square, which fit in a 15 × 15-mm Frame-Seal Incubation Chamber (MJ Research, Waltham, MA, USA) and placed on a microscope slide. The sample was then covered with 40 µL of hybridization solution (0.9 M of NaCl, 5 mM of EDTA, 0.5% of sodium dodecyl sulfate [SDS], 50 mM of sodium phosphate buffer [pH 7], 10× Denhardt's solution (Sigma), 1 µg/µL of poly(A), and 1 ng/µL of Cy3 or Cy5-labeled oligonucleotide) supplemented with the appropriate formamide concentration depending on the probe used (Table 1). The *V. logei* probe was labeled with Cy-5, whereas the *Vibrionaceae* and *V. fischeri* probes contained Cy-3. Each chamber was sealed and placed in a DNA Engine Dyad® Peltier Thermal Cycler equipped with a slide chamber Alpha Unit (MJ Research) at 42°C for 4 h.

After 4 h of hybridization, each filter was washed (0.9 M of NaCl, 0.1% of SDS, and 50 mM of sodium phosphate buffer at pH 7) for 30 min at 46°C. Filters were rinsed in 0.2 µm of filtered distilled water and air-
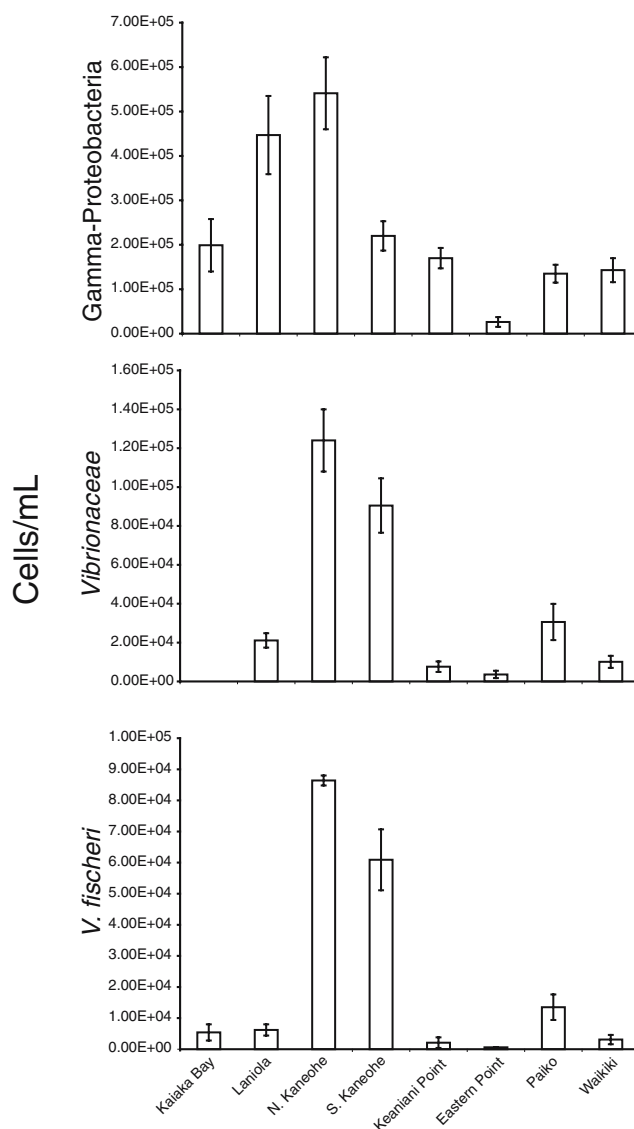

Figure 1. Numbers of γ-Proteobacteria, *Vibrionaceae*, and *V. fischeri* at the eight sampling sites in O'ahu, Hawaii (see Table 3). Error bars represent one standard deviation. There are no data available for the *Vibrionaceae* estimate in Kaiaka Bay.
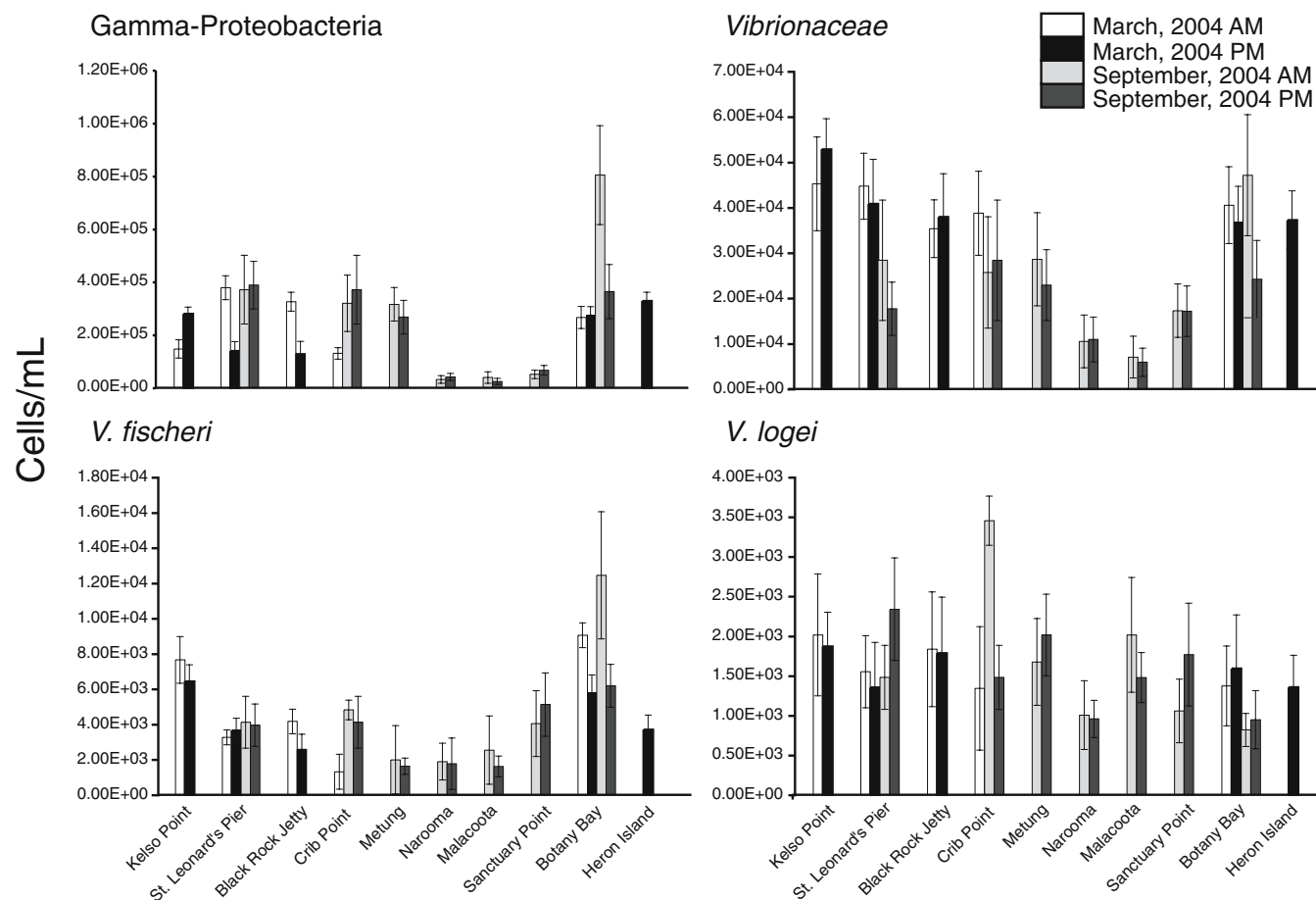
Figure 2. Numbers of γ-Proteobacteria, *Vibrionaceae*, *V. fischeri*, and *V. logei* at ten different sites during March and September 2004 in Australia. The *Y*-axis represents numbers of bacteria at each site. Samples were taken in the morning at sunrise and in the evening after sunset. Only the St. Leonard's Pier and Botany Bay sites were sampled in the morning and evening both years. Error bars represent one standard deviation.

dried. Immediately before viewing, filters were mounted with ProLong Antifade (Molecular Probes, Eugene, OR, USA). Samples were viewed with a Nikon E800 epifluorescence microscope equipped with excitation/emission filters of 546/565 for Cy3 and 620/700 for Cy5. Samples were counted manually and a total of 30 random fields and at least 400 cells were counted for each probe in every sample. When Cy5 was used, samples were enumerated by counting from pictures taken with a CCD camera.

Results

Probe Development and Optimization. The *Vibrionaceae*, *V. fischeri*, and *V. logei* probes were found to have optimal formamide concentrations of 35, 50, and 45%, respectively. Database searches and sequences from our own laboratory strains found the *V. fischeri* probe to be specific for all known symbiotic *V. fischeri* strains. Database searches also found that the *V. fischeri* probe also hybridizes to *Vibrio orientalis*, *Vibrio hepatarius*,

and *Vibrio agarivorans*. The *V. logei* probe also hybridizes to an uncultured marine γ-Proteobacterium, *Aquicella lusitana*, and *Oscillatoria limnetica*. Given the lack of 100% specificity to only *V. fischeri* and *V. logei* by these probes, the numbers presented in this study may be slight overestimates of actual populations.

The *Vibrionaceae* probe outperformed two previously constructed probes by Amann et al. [2] and Nishimura et al. [30] for estimating total number of species hybridized (Table 2). Whereas the probe of Amann et al. recognized amore species of *Enterovibrio* and *Photobacterium*, the probe developed in this study recognized more species of *Vibrio*, *Listonella*, and unclassified species of the *Vibrionaceae*.

Field Test. In Hawaii, total numbers of *Vibrionaceae* ranged from 3.6×10^3 to 1.2×10^5 cells/mL, and the percentage of *Vibrionaceae* that was *V. fischeri* ranged from 17 to 31% at sites not known to harbor large numbers of *E. scolopes* (Kaiaka Bay, Laniola, Keaniani
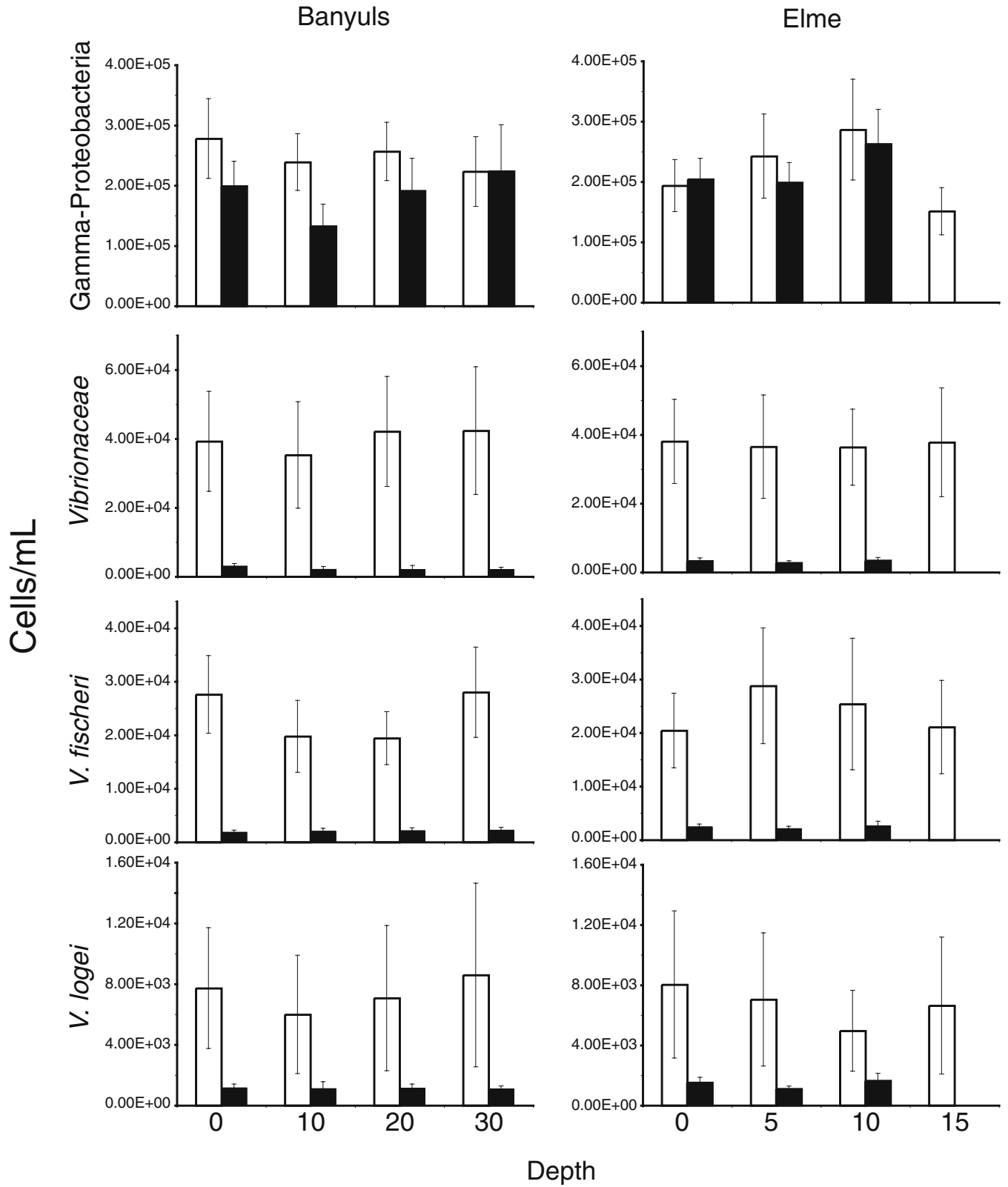
Figure 3. Community numbers at Banyuls and Elmes field sites in France. White colored bars are from the field season in November 2003, whereas black bars are from August 2004. There was no sampling at 15 m at the Elmes field site in August 2004. Error bars represent one standard deviation.

Table 2. Comparison of published *Vibrionaceae* family specific probes

	Vibrio	*Photobacterium*	*Listonella*	*Enterovibrio*	*Salinovibrio*	Unclassified
VIB749	**53.5**	4.4	**51.0**	0.0	0.0	**22.3**
GV[a]	30.8	**26.6**	44.5	**1.6**	0.0	21.2
Vir1[b]	48.2	4.4	30.3	0.0	0.0	15.7

Each cell lists the percentage of sequences in the five genera that are 100% complimentary to the three probes. Sequences were gathered from the Ribosomal Database Project II, release 9.34. Numbers in bold indicate the highest percentage within each genus.
[a][2].
[b][30].

Point, Eastern Point, and Waikiki; Fig. 1 and Table 3). Sites containing stable populations of *E. scolopes* (N. Kaneohe, S. Kaneohe, and Paiko) had approximately 44 to 70% of the *Vibrionaceae* that were identified as *V. fischeri*. The total number of *V. fischeri* ranged from 0.6×10^3 to 8.6×10^4 cells/mL among the different sites sampled in Hawaii (Fig. 1). *Vibrio logei* was not detected in Hawaiian waters.

In Australia, bacterial populations were relatively constant between sites and between morning and evening sampling times (Fig. 2), with two notable exceptions being the September 2004 AM samples for Botany Bay and Crib Point. Within the γ-Proteobacteria, populations were similar to France sites, with the exception of Narooma, Malacoota, and Sanctuary Point, which had very low numbers. Comparatively low numbers of *Vibrionaceae* were also observed at these sites. *Vibrio fischeri* distribution exhibited pronounced variation in cell density, with the highest numbers enumerated from Botany Bay. *Vibrio logei* numbers were

lower than *V. fischeri*, and exhibited no apparent trends between seasons (Fig. 2).

Conductivity–temperature–depth recorder sampling in France enabled depth profiling of communities at different temperatures and salinities (Figs. 3 and 4). The summer thermocline generally resulted in a decrease from about 21 to 14°C. One-way analysis of variance (ANOVA) failed to reject the null hypothesis of no difference in cell numbers at different depths for each nested group of bacteria at both France sites (p values ranging from 0.29 to 0.64), which meant there was no apparent reduction or increase in community numbers across the thermocline. Numbers did, however, differ significantly between the summer and winter sampling times, as the null hypothesis of no difference in cell numbers between seasons was rejected for *Vibrionaceae*, *V. fischeri*, and *V. logei* using ANOVA ($p < 0.001$ for each sample, except for the γ-Proteobacteria where $p = 0.007$). The most notable difference in cell numbers was within the *Vibrionaceae*, *V. fischeri*, and *V. logei*. There was

Table 3. Sites sampled in this study

	Site name	Latitude	Longitude
Hawaii	Kaiaka Bay	N 21°34′56″	W 158°07′46″
	Laniola	N 21°38′31″	W 157°55′02″
	Keaniani Point	N 21°33′50″	W 157°52′23″
	N. Kaneohe	N 21°29′31″	W 157°50′47″
	S. Kaneohe	N 21°25′45″	W 157°47′32″
	Eastern Point	N 21°19′01″	W 157°39′51″
	Paiko	N 21°16′49″	W 157°43′49″
	Waikiki	N 21°16′10″	W 157°49′23″
Australia	Kelso Point	S 41°3′24″	E 146°47′52″
	St. Leonard's Pier	S 38°10′14″	E 144°43′8″
	Black Rock Jetty	S 38°13′37″	E 145°01′29″
	Crib Point	S 38°21′01″	E 145°13′11″
	Metung	S 37°53′02″	E 147°58′35″
	Narooma	S 33°29′05″	E 150°06′54″
	Malacoota	S 37°33′59″	E 149°45′59″
	Sanctuary Point	S 35°06′	E 150°39′
	Botany Bay	S 34°00′	E 151°12′
	Heron Island	S 23°26′53″	E 151°55′49″
France	Banyuls	N 42°35′29″	E 3°02′57″
	Elmes	N 42°37′35″	E 3°02′22″

In Hawaii and Australia samples were taken from surface waters approximately 20 m offshore. In France samples were taken at various depths from boat using a CTD.
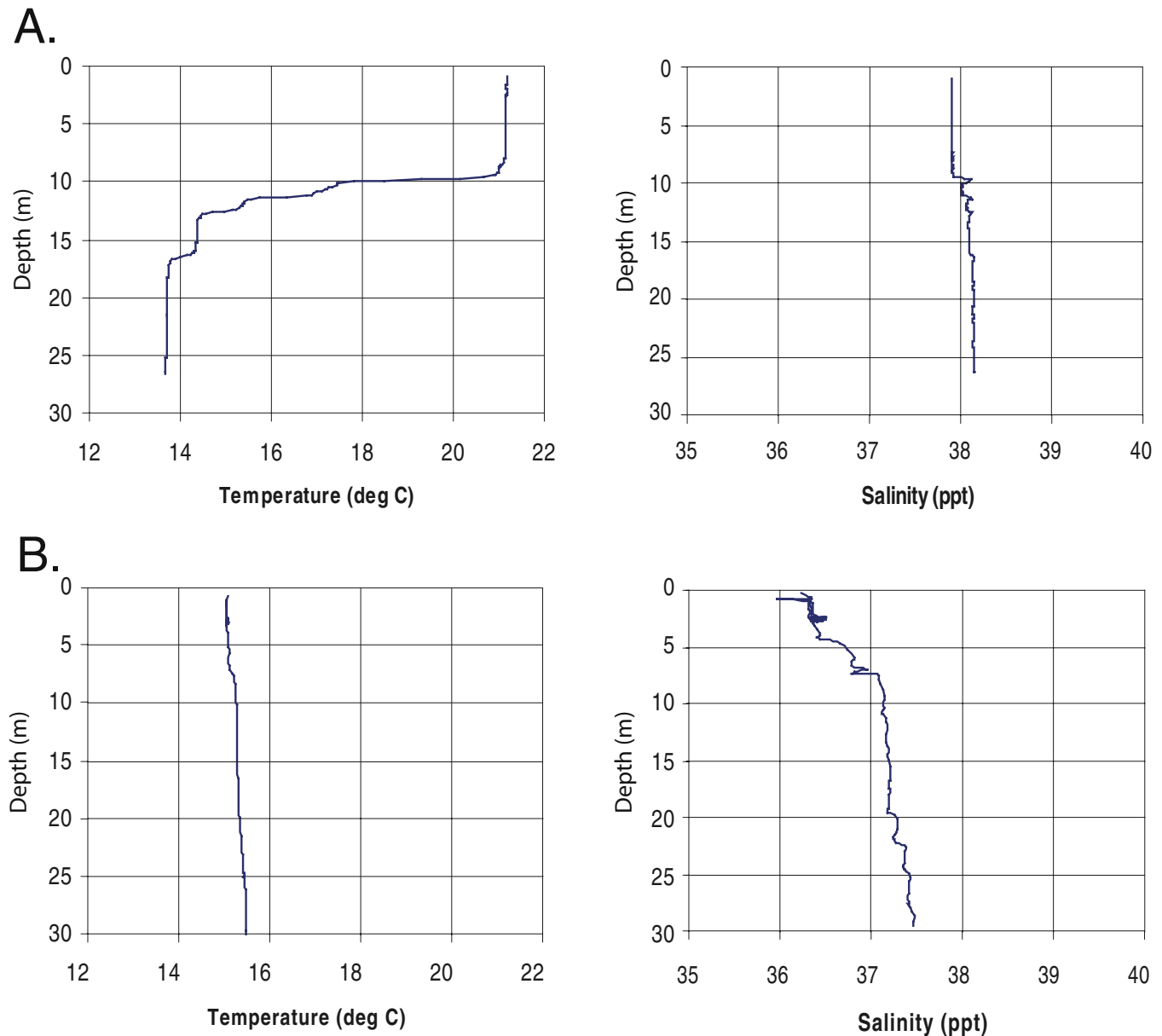
A.

B.

Figure 4. Representative salinity and temperature profiles from France study sites during the summer sample period (A) and the winter sample period (B). Note the lack of the thermocline during the winter.

approximately a tenfold difference in total counts of these groups of bacteria at both sites between seasons.

Discussion

Fluorescence *in situ* hybridization, a technique commonly used to examine microbial communities [1, 2, 7–9, 23], has provided a wealth of information to researchers through "snapshots" of microbial populations. Fluorescence *in situ* hybridization has been successfully applied to oceanographic studies examining bacterioplankton, and has provided tremendous insight as to how these

communities are structured [10–14]. Although past studies have mainly focused on the α-Proteobacteria, it is interesting to note that members of the *Vibrionaceae* still constitute a large proportion of the bacterioplankton community in the areas we were able to sample, with or without the presence of squid hosts. This strengthens the fact that multiple factors (abiotic and biotic) are probably responsible for trends in the distribution of any division of bacteria, particularly vibrios.

Results from the Hawaiian dataset confirm previous reports [20, 21], which exhibited greater numbers of *V. fischeri* in the water column within the Kaneohe Bay study sites (Fig. 1). This bay is known to harbor large

numbers of *E. scolopes*, and daily expulsion of *V. fischeri* may explain the elevated levels. In addition, this location also contains a large number nutrient inputs from streams entering the Bay, which may allow certain species of bacteria to flourish in these waters [16]. The Paiko site also had statistically significant numbers of *V. fischeri*. This site is also a common *E. scolopes* collection location, but because Paiko is not a bay it is more open to ocean currents than Kaneohe. These currents may flush out excess vibrios, preventing the increased accumulation observed in North and South Kaneohe Bay.

The results presented in this study also demonstrate a marked increase in numbers of *V. fischeri* detected in Hawaiian waters compared to previous studies [20, 21]. Specifically, Lee and Ruby [21] found the abundance of *V. fischeri* to be between 276–400 cells/mL based on *luxA* quantitative DNA–DNA hybridization and between 130–1680 cells/mL based on most-probable-number analysis using *luxA*-specific primers for polymerase chain reaction from cell lysates. Indeed, the lowest estimate of *V. fischeri* abundance at Eastern Point (600 ± 100 cells/mL) falls into the high end of the estimates of Lee and Ruby [21]. This could be because of binding of the probe to complementary sequences from bacterial species other than *V. fischeri* that were not catalogued in the Ribosomal Database Project.

Australian total numbers of all bacterial samples probed compared similarly to Hawaiian estimates of γ-Proteobacteria, *Vibrionaceae*, and *V. fischeri* presented in this study (Fig. 2). Within the *Vibrionaceae*, however, the percentage of the *Vibrionaceae* that was *V. fischeri* was much lower than Hawaiian estimates. Reasons for this discrepancy are unclear, but because Australian and Hawaiian habitats are geographically separated and ecologically distinct, there is no reason to expect that the bacterial communities in these areas should be structured in a similar manner, nor follow patterns that link *Vibrio* concentration to host squid number.

Numbers of *V. fischeri* in Botany Bay are of particular interest because of the aims of this study. Although specific population size of the sepiolid host is unknown in this area, Botany Bay is a common collection site because of the abundance of *Euprymna tasmanica*. Much like Kaneohe Bay in Hawaii (but on a much greater scale), Botany Bay is a large enclosed body of water in which numbers of vented *V. fischeri* could accumulate. In this population, numbers of *V. fischeri* are greater than every other site, with the exception of Kelso Point (another site where *E. tasmanica* is commonly collected) in Tasmania. In addition, the morning *V. fischeri* numbers are greater than those collected in the evenings at these sites. At these locations, the presence of *E. tasmanica* may be the reason for this phenomenon, although no causal relationship can be inferred with the present data.

In France, there were greater numbers of all groups of bacteria detected in the November sampling season (Fig. 3). In general, the greatest discrepancies between years were observed when comparing *Vibrionaceae*, *V. fischeri*, and *V. logei*, whose November populations were much higher than the summer populations. Greater availability of nutrients during the winter when the thermocline is absent and less competition from other bacteria during this time may account for this general increase in *Vibrio* numbers.

Population structure of the symbiotic bacteria *V. fischeri* and *V. logei* was not affected by depth at Banyuls-sur-mer or Bay of Elmes study sites during the two seasons samples were collected. This is counterintuitive for a number of reasons. First, it is known that light organs of sepiolid squids from greater depths tend to have a higher proportion of the cold-adapted *V. logei* than the more warm-adapted *V. fischeri* [28]. Second, there is a predominant thermocline in summer months, which prevents mixing of deep, cold waters with warm surface waters at both study sites (Fig. 4). In late autumn, the thermocline disappears, and waters become a homogeneous mixture. Given this information, it was hypothesized that *V. logei* would be more abundant than *V. fischeri* in deep waters below the thermocline in summer months because of increased expulsion of *V. logei* by deep water sepiolid squids and increased growth rates over *V. fischeri* in these colder waters. The analogous hypothesis was made for *V. fischeri* distribution in warmer waters above the thermocline. Given the data, the hypotheses were clearly rejected, as no depth-related differences were observed.

There are several factors that may explain why these differences were observed. First, during the summer months in Southern France, periods of periodic winds known as the mistral can produce heavy gusts that can potentially disrupt the thermocline for short periods of time (1–4 days). If the thermocline is continually disrupted throughout the sampling period, bacterial temperature-related distribution patterns would disappear or at least be transient. The second possibility is that there is no temperature-dependent distribution of *V. fischeri* and *V. logei* associated with the thermocline. Given that sepiolid squid light organs are environmentally colonized upon hatching, this may infer that initial colonization might be equally parsimonious for either *V. fischeri* or *V. logei*, and subsequent dominance in the organ could be because of *V. logei* outcompeting *V. fischeri* at colder temperatures in a high nutrient environment where the squid resides. This was demonstrated *in vitro* with two species of Mediterranean sepiolids, *Sepiola affinis* and *Sepiola ligulata* with both *V. fischeri* and *V. logei* [28]. Thus, the symbiosis is probably more specific once infection has occurred by one of the two symbiont species available in the Mediterranean.

In conclusion, there appears to be multiple factors that structure bacterioplankton community composition

in natural environments. Whereas overall bacterial numbers were similar between Australia, France, and Hawaii, fine-scale processes appear to influence bacterial community structure in both space and time. Our hypothesis that sepiolid squid presence influences bacterial community structure was supported to an extent, but future sampling efforts will need to take into account as many abiotic and biotic factors (including temperature, salinity, dissolved organic matter, blooms of other species, bacterivory, etc.) for an increased understanding of what determines microbial community structure. Other studies with molluscan and vertebrate hosts have demonstrated that salinity and temperature influence a number of factors [3, 18, 25, 26], which not only influence colonization, but also the physiological state of *Vibrio* species in the water column. Thus, deciphering the mechanisms of how the ecology of squid hosts and their *Vibrio* symbionts affect their surrounding habitat and whether they are influential players in the overall microbial community structure are important aspects that still need to be addressed in future studies.

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