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Dynamics of *Vibrio* with virulence genes detected in Pacific harbor seals (*Phoca vitulina richardii*) off California: implications for marine mammal health

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DYNAMICS OF *VIBRIO* WITH VIRULENCE GENES DETECTED IN PACIFIC HARBOR SEALS (*PHOCA VITULINA RICHARDII*) OFF CALIFORNIA: IMPLICATIONS FOR MARINE MAMMAL HEALTH

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Master of Science

by

Stephanie N. Hughes

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December 2012

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ABSTRACT

DYNAMICS OF *VIBRIO* WITH VIRULENCE GENES DETECTED IN PACIFIC HARBOR SEALS (*PHOCA VITULINA RICHARDII*) OFF CALIFORNIA: IMPLICATIONS FOR MARINE MAMMAL HEALTH

by Stephanie N. Hughes

Given their coastal site fidelity and opportunistic foraging behavior, harbor seals (*Phoca vitulina*) may serve as sentinels for coastal ecosystem health. Seals using urbanized coastal habitat can acquire enteric bacteria, including *Vibrio*, that may affect their health. To understand *Vibrio* dynamics in seals, demographic and environmental factors were tested for predicting potentially virulent *Vibrio* in free-ranging and stranded Pacific harbor seals (*P. v. richardii*) off the coast of California. *Vibrio* prevalence did not vary with season and was greater in free-ranging seals (29%, $n = 319$) compared with stranded seals (17%, $n = 189$). Of the factors tested, location, turbidity, and/or salinity best predicted *Vibrio* prevalence in free-ranging seals. The relationship of environmental factors with *Vibrio* prevalence differed by location and may be related to oceanographic or terrestrial contributions to water quality. *Vibrio parahaemolyticus*, *V. alginolyticus*, and *V. cholerae* were observed in seals with *V. cholerae* found almost exclusively in stranded pups and yearlings. Additionally, virulence genes (*trh* and *tdh*) were detected in *V. parahaemolyticus* isolates. *Vibrio cholerae* isolates lacked targeted virulence genes, but were hemolytic. Three out of four stranded pups with *V. parahaemolyticus* (*trh*+, and/or *tdh*+) died in rehabilitation, but the role of *Vibrio* in causing mortality is unclear, and *Vibrio* expression of virulence genes should be investigated. Considering that

humans share the environment and food resources with seals, potentially virulent *Vibrio* observed in seals also may be of concern to human health.

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Introduction

Marine mammals are sensitive to changing environmental conditions and can serve as sentinels for ecosystem health [11, 13, 35, 60]. Long-term monitoring of marine mammal health includes measuring contaminants, trace elements, biotoxins from harmful algal blooms, baseline blood values, and prevalence of infectious disease so that deviations from normal values may be detected [10, 12, 32, 33, 35-38]. Cumulative effects of one or more of these factors may have contributed to the deterioration of marine mammal health [10, 11, 13, 35]. Because infectious marine diseases appear to be increasing and are of immediate concern to marine mammal health, the dynamics and virulence potential of aquatic microbes should be investigated [4, 10, 28, 38, 48, 75].

Baseline epidemiological data on marine pathogens can be obtained from a representative marine mammal to aid in disease mitigation in marine mammals [10, 36, 48, 65, 83]. Harbor seals (*Phoca vitulina*) are an excellent indicator species because they are long-lived and upper-level trophic consumers that inhabit coastal areas throughout the northern hemisphere. Harbor seals often have strong site fidelity to areas near dense human populations (e.g., San Francisco Bay), and forage opportunistically on available benthic and pelagic prey [9, 23, 62, 69, 84, 91]. Terrestrial inputs from urbanized coastal communities may alter the quality of habitat or food resources exploited by harbor seals [32, 53, 58, 70, 72, 82]. These inputs also may contribute to increases in marine pathogens [39, 42], thereby leading to an increasing incidence of disease in marine animals including harbor seals.

Current knowledge of the diversity and ecology of marine pathogens in harbor seals is limited to clinical cases, serological surveys, zoonotic cases (marine mammal caretakers), and epidemics associated with animal-stranding events [10, 37, 38, 73, 78, 83, 92]. The most common cause of live harbor seal strandings in central California during the last ten years was malnutrition (52%) [16]. Malnourished individuals may be more susceptible to enteric pathogens [44]. To better understand impacts of enteric pathogens on the health of harbor seals, enumerating these pathogens in healthy and stranded seals is warranted [10, 16, 32, 48, 51].

Marine pathogens of the genus *Vibrio* are of concern to marine mammal and ecosystem health. Vibrios are facultative anaerobes that can be found in aquatic environments throughout the world [18, 24]. Pathogenic strains may proliferate following environmental perturbation, and nonpathogenic strains may become competent pathogens via inter-microbial gene transfer [4, 7, 18, 24, 34, 39, 57, 63, 79]. The diversity and versatility of this group of bacteria allows their persistence in a variety of ecological niches and hosts [17, 20, 23, 33, 47, 93]. Infectious species and serotypes of *Vibrio* can deleteriously affect a broad range of marine taxa causing mass mortality events [18]. *Vibrio* may persist in the water column, although greater concentrations of some pathogenic species of *Vibrio* have been observed in sediment, zooplankton, mussels, and fish [19, 21, 43, 45, 55, 78]. In humans, enteric *Vibrio* infections are acquired by ingesting water or raw seafood that is contaminated with virulent or pathogenic strains of *Vibrio.* Ingesting pathogenic *Vibrio* may lead to gastroenteritis, dehydration, septicemia and in some cases death in human and experimental hosts [7, 18, 78]. Given their

mammalian physiology, marine mammals may be similarly affected following a *Vibrio* infection.

Vibrio species have been detected in marine mammals suffering from enteritis and septicemia [58, 83]. Species of *Vibrio* also were detected in harbor seals, although the virulence of these strains was unknown [32]. Measuring the abundance, distribution, and virulence potential of *Vibrio* from seals and corresponding environmental conditions may aid in identifying processes that promote pathogen proliferation and thus may impact seal health [58, 83]. The goal of this study was to investigate the dynamics and virulence potential of *Vibrio* among free-ranging and stranded Pacific harbor seals (*P. v. richardii*) off the coast of California to identify risks associated with the presence of *Vibrio*. We determined the temporal and spatial prevalence of *Vibrio* spp. in seals and examined demographic risk factors (age, sex, and body condition) and environmental conditions (precipitation, nutrients, temperature, pH, salinity, and turbidity) associated with *Vibrio* detection. *Vibrio* prevalence and species distribution in free-ranging seals were compared with those of stranded seals to better understand the role that *Vibrio* may play in the health of harbor seals. Lastly, the virulence potential of *V. parahaemolyticus* and *V. cholerae* isolates was determined by screening for virulence genes, and clinical signs associated with potentially virulent *Vibrio* were examined.

Materials and Methods

Isolation and characterization of Vibrio *spp. from free-ranging and stranded seals*

Free-ranging harbor seals (n = 220, Fig. 1) were captured on mud flats, sand spits, or rocky outcrops in San Francisco Bay (SFB), Elkhorn Slough (ES), Tomales Bay (TB) and Humboldt Bay (HB) using beach seine, drift net, or hand-held salmon nets [32]. Seals in SFB and TB were sampled during the dry (May to October) and wet (November to April) seasons from May 2010 to June 2011. Seals in ES were sampled from August to December 2010, and those in HB were sampled in June of 2011. Free-ranging seals were not sampled during the pupping season (March to April) to avoid disturbance of mother and pup pairs. Seals were weighed (± 1 kg) and restrained physically and chemically with 5mg/ml of diazepam (Hospira, Inc., Lake Forest, Illinois USA) at a dose of 0.25mg/kg. Standard length (± 1 cm) and axillary girth (± 1 cm) also were measured. Sex and age class were determined using external characteristics, mass, standard length criteria, and time of year [9, 32].

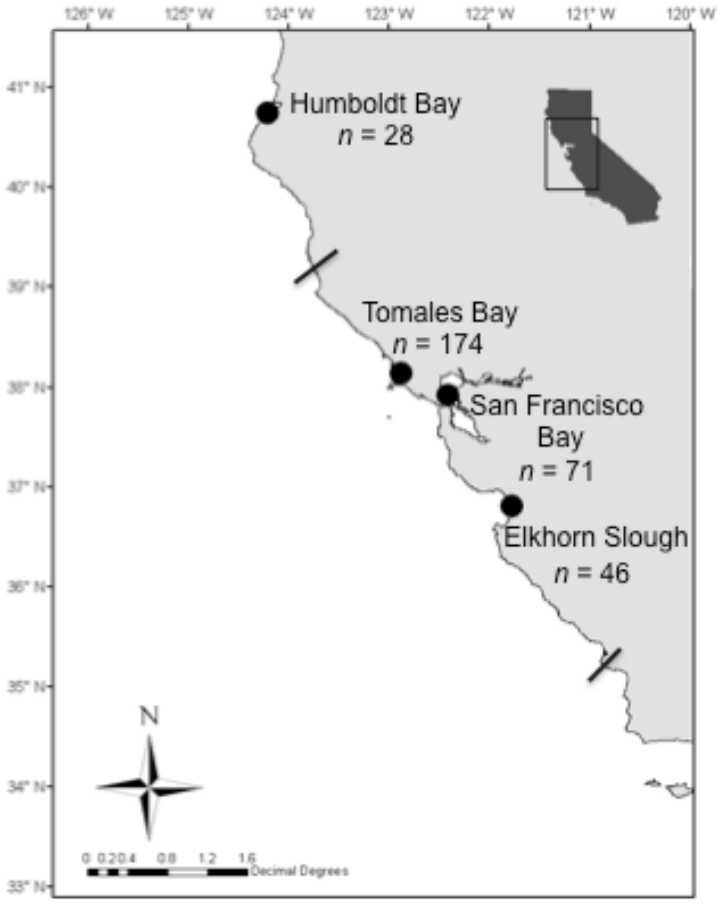


Figure 1. Sampling sites and sample sizes for free-ranging harbor seals (*n* = 319, black dots) and area between San Luis Obispo and Mendocino (black bars) where stranded animals were sampled (*n* = 189) during admission to The Marine Mammal Center in 2007 through 2011.

Harbor seals that stranded between San Luis Obispo and Mendocino were opportunistically sampled during admission for rehabilitation from May 2010 to July 2011 (Fig. 1). These individuals were primarily pups and yearlings that beached alive (*n* = 53) and appeared ill, emaciated, or were recently deceased (*n* = 4, died in transit to the rehabilitation facility, or sampled less than six hours post-mortem). Mass (± 1 kg), standard length (± 1 cm), axillary girth (± 1 cm), sex, and age class were noted as described above. Locations of stranded seals were assigned to the nearest capture location, Monterey Bay (MB, -121.95ºW / 36.61ºN to -122.11ºW / 36.95ºN), SFB (-

122.45ºW / 37.49ºN to -122.80ºW / 37.99ºN), and TB (-122.98ºW / 38.08ºN to -123.09ºW / 38.39ºN), to compare with free-ranging seals.

Fecal samples were collected from each seal using sterile double-tip cotton swabs in Amies transport media (HealthLink, Inc. USA) inserted into the rectum of each seal. Swab samples were placed on ice, and transported to the University of California, Davis Veterinary Medical Teaching Hospital Microbiology for sample processing. One swab from each seal was placed in alkaline peptone water (Hardy Diagnostics USA) incubated at 35ºC overnight in an oxygenated incubator. Plates of thiosulfate citrate bile salts agar (TCBS, Hardy Diagnostics USA), selective for *Vibrio* growth, were inoculated with the enriched swab and incubated at 35ºC overnight. Individual green or yellow colonies on TCBS agar were subjected to biochemical testing to confirm genus using triple sugar iron agar slants, Christensen's urease agar slants, spot indole test, and cytochrome oxidase test. Species identification was determined using API 20E test strips (API 20E Test Kit, bioMereux, Inc., Hazelwood, MO USA). Isolates from 2010 to 2011 were cryogenically preserved (MicroBank vials, Copan Diagnostics USA) and stored at -80ºC. Isolates that were not identified to the species level using biochemical testing were genetically characterized by targeting the species-specific *ToxR* gene region using the polymerase chain reaction (PCR) amplification and amplicon visualization methodology of Bauer and Rorvik (2007) [6]. If species could not be determined, or more than one species of *Vibrio* was isolated from an individual seal, the sample was categorized as *Vibrio* mixed culture for data analysis purposes. *Vibrio* prevalence and species distribution data in free-ranging seals were compared with stranded seals among locations for all years sampled.

Banked isolates from 2010 and 2011 characterized as *V. parahaemolyticus* and *V. cholerae* were screened for genes encoding virulence factors. Multiplex PCR amplification procedures previously outlined were followed with minimal modification [6, 8, 77]. Crude deoxyribonucleic acid (DNA) was extracted from each isolate using the boiling method [56], and a 1:10 dilution was used as a template for multiplex PCR amplification. Primer dilutions (Invitrogen, Inc USA), reaction buffer concentrations (dNTP Mix, GeneAmp UK; Hot Start Taq Kit, Qiagen USA), and PCR (Thermocycler, Eppendorf, USA) amplification conditions follow those of previous studies listed above. Thermostable direct hemolysin (*tdh;* 269 bp), related thermostable direct hemolysin (*trh;* 500 bp), and thermolabile hemolysin (*tl;* 450 bp) gene regions were targeted for *V. parahaemolyticus* isolates, whereas cholera toxin (*ctx;* 617 bp) and toxin co-regulated pilus (*tcp;* 385 bp) gene regions were targeted for *V. cholerae* isolates. Amplicons were separated via gel electrophoresis and visualized using 7 µl of GelStar nucleic acid stain (Gel Star, Lonza, ME USA) per 100mL of 1.5% agarose (USB Corporation, OH USA) in tri-acetate-EDTA (TAE) buffer (Bio-Rad, Inc., USA). A subset (n = 7) of positive samples and sample controls were sequenced (ElimBio, CA USA) to confirm amplification of target regions (Geneious 5.5, NZ). Virulence gene profiles from isolates collected from free-ranging seals were compared with stranded seals among locations. Lastly, clinical signs in seals carrying potentially virulent *Vibrio* were also documented.

Environmental data collection

Water quality and weather data collected at a resolution of 15-minute intervals were downloaded from NOAA's National Estuarine Research Reserve System (NERRS) database from SFB (SCQC1, 38.21° N / -122.03°W; SFX1, 38.22°N / -122.03°W) and ES (ELQC1 and ELXC1, 36.82°N / -121.74°W). Daily averaged salinity (ppt), temperature (ºC), turbidity (NTU), pH (standard units), nutrient data (NO_3- uM, available only for ES), and daily cumulative precipitation (mm) were computed from raw, quality assured, and quality controlled NERRS data files (Matlab R2011a, Mathworks, Inc., USA) for both sampling locations. Missing or flagged data from NERRS quality control and assurance checks were excluded from analyses [94].

Data analysis

Previous *Vibrio* prevalence data collected from free-ranging seals (n = 99) in SFB and TB (May, June, and December of 2007, and May and June of 2008) using similar methodology were included to test the effects of environmental and demographic predictors among locations and years [32]. Chi-squared (χ^2) tests were performed to determine if the presence or absence of *Vibrio* was dependent on year, season (wet or dry), or location (SFB or TB) in free-ranging seals. A separate χ^2 test was used to compare presence or absence of *Vibrio* in free-ranging seals from ES to that of other locations sampled in 2010 to 2011. If no significant differences were detected, data were combined for subsequent analyses. Factors that were significant were either separated by factor level, or used as covariates in a logistic regression assessing risk factors. The

magnitude of effect of categorical predictor variables used in logistic regression was determined by comparing odds ratios (OR = proportion of cases/ 1- proportion of cases for reference category). Age classes were collapsed into two main categories; pups were grouped with yearlings, and subadults with adults. Residuals from the linear regression of length versus mass were used as an indicator of body condition (BCI). A forced entry, exploratory logistic regression analysis was used to determine whether age, sex, and body condition predicted the presence or absence of *Vibrio* in free-ranging harbor seals.

Stepwise lagged correlation analysis (Matlab, Student Version R2011a, Mathworks, Inc., USA) was used to determine if *Vibrio* prevalence in free-ranging seals sampled in SFB and ES was related to rainfall events for up to twenty-two days before sampling date. Environmental predictor variables measured for SFB and ES were tested for multicollinearity and normality. Daily average nutrients (ES only), temperature, salinity, pH, and turbidity values for each sample day were assigned into categories using the bin criteria determined by the mean and/or median for SFB (Appendix A) and ES (Appendix B). *Vibrio* prevalence corresponding to each category was tested for goodness of fit (Pearson's χ^2, or Cochran's for $df = 1$). Variables with the greatest χ^2 value and correlation coefficients less than 0.70 were retained for further analysis. A backwards stepwise logistic regression was used to assess the effect of environmental predictors on the presence or absence of *Vibrio* in free-ranging seals. Odds ratios were calculated to determine the magnitude of effect.

Similarly, previous *Vibrio* prevalence data from live ($n = 102$) and recently deceased stranded seals ($n = 30$) seals in January 2007 to September 2008 were included

to test the effects of predictors on the presence or absence of *Vibrio* [32]. Chi-squared tests were performed on categorical (year, season, location, sex) predictor variables, and t-tests were performed on continuous (admission mass, BCI) predictor variables. Only significant predictors were included in a backwards stepwise logistic regression of *Vibrio* prevalence. The overall model fit was determined using the likelihood (LR) ratio test and/or the Homer and Lemeshow (HL) test statistic [68]. Statistical analyses were performed using PSAW Statistics (19.0, IMB, USA), and statistical significance was assumed for an alpha level less than 0.05 for all analyses.

Results

Vibrio *prevalence in free-ranging harbor seals*

From 2007 to 2011, the overall prevalence of *Vibrio* in free-ranging seals was 29% (n = 319). Neither year ($\chi^2_{0.05,3} = 5.512$, $P = 0.138$ for years sampled in SFB, and $\chi^2_{0.05,3} = 4.643$, $P = 0.200$ for TB) nor season (Cochran's $\chi^2_{0.05,1} = 0.276$, $P = 0.599$) had an effect on presence of *Vibrio* in free ranging seals, therefore, these data were pooled within locations for further analyses. Significant differences in *Vibrio* presence were detected among locations ($\chi^2_{0.05,2} = 56.237$, $P < 0.001$; Fig. 2). Elkhorn Slough had the greatest proportion of seals confirmed with *Vibrio* (58.7%, n = 46), followed by SFB (45.0%, n = 71), and TB (11.5%, n = 174). Seals sampled in ES were ten times more likely to carry *Vibrio* (OR = 10.94), whereas seals in SFB were six times more likely (OR = 6.32) than seals in TB ($P < 0.001$). The overall model was significant (LR, $P < 0.001$; HL, $P <$

1.000), and was 75% accurate in classifying presence or absence of *Vibrio* (Table 1). Because location significantly predicted *Vibrio* in seals, data were separated by location for further analyses.

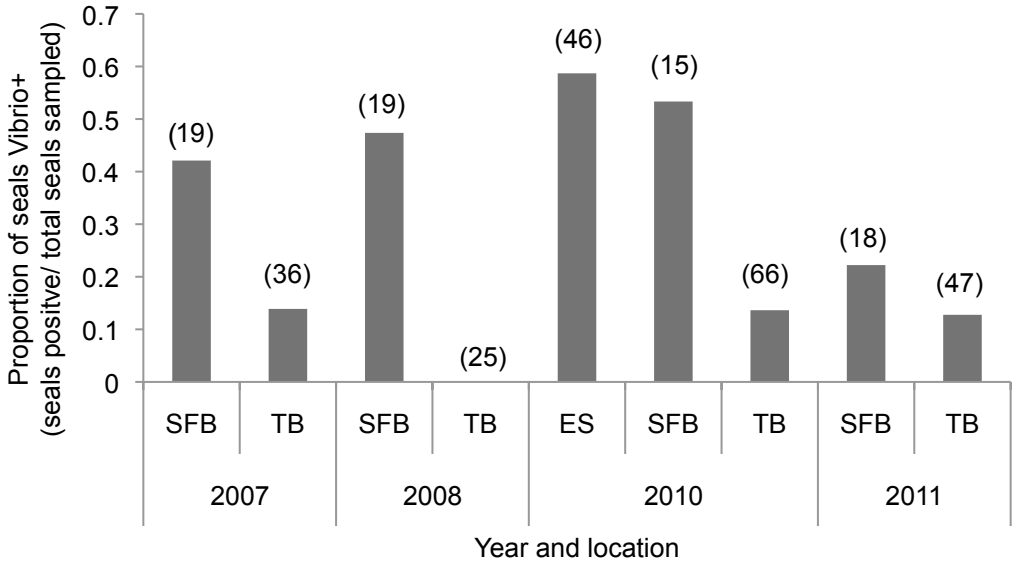


Figure 2. Proportion of free-ranging harbor seals with *Vibrio* (seals confirmed with *Vibrio*/ total seals sampled) by year and location (SFB = San Francisco Bay, TB = Tomales Bay, ES = Elkhorn Slough). Seals were sampled from ES only in 2010. Sample sizes are indicated in parentheses.

Table 1. Results from logistic regression testing location as a predictor for *Vibrio* presence and absence in seals sampled in 2007 to 2011.

Location	*n*	n_1[a]	Prevalence (%)	*P* value	Odds Ratio (OR)	CI for OR
Location 2007-2011	291	79	30.00			
Elkhorn Slough	46	27	58.69	**<0.001***	**10.94**	5.17- 23.15
San Francisco Bay	71	32	45.07	**<0.001***	**6.32**	3.27-3.22
Tomales Bay[b]	174	20	11.49	**<0.001***	-	-
Constant				<0.001	0.17	-

[a]Vibrio+, [b]Reference Category

Free-ranging pups and yearlings from SFB were more likely to carry *Vibrio* (52%) compared with adults and subadults (36%), although age class was not a significant predictor of *Vibrio* when tested using logistic regression ($P = 0.26$). Body condition ($P = 0.93$) and sex ($P = 0.41$) also were not significant predictors of *Vibrio* in free-ranging seals from SFB. Demographic risk factor analysis was not performed on data from seals sampled at other locations because pups and yearlings were poorly represented (ES) or presence of *Vibrio* was rare (TB).

No relationship was detected between *Vibrio* prevalence of free-ranging seals and daily average precipitation in SFB and ES, and no lag in *Vibrio* prevalence with rainfall was detected using lagged correlation analysis. The occurrence of *Vibrio* in seals was related to pH (Cochran's $\chi^2_{0.05,1} = 3.82$, $P = 0.051$ for SFB, and $\chi^2_{0.05,1} = 5.81$, $P = 0.016$ for ES), and turbidity (Cochran's $\chi^2_{0.05,1} = 8.02$, $P = 0.005$ for SFB, and $\chi^2_{0.05,1} = 7.94$, $P = 0.005$ for ES). Salinity was not significantly related, however this parameter met the criteria to be included in logistic regression (greatest chi-squared; $r < 0.70$). Temperature was significantly correlated with nutrients ($r = 0.988$, $P < 0.01$), and salinity ($r = 0.999$, $P < 0.01$), but neither temperature nor nutrients were related to *Vibrio* presence

(Appendices A & B). Salinity, pH, and turbidity ($r < 0.70$) in SFB and ES were then selected for logistic regression analysis. The most parsimonious model (LR, $P = 0.005$; HL, $P < 1.000$) for predicting *Vibrio* in free-ranging seals from SFB included only turbidity ($P = 0.008$). *Vibrio* was four and a half times more likely to occur in harbor seals sampled in SFB when the turbidity was greater than 66 NTU with a classification accurracy of 65% (Table 2). *Vibrio* in free-ranging seals from ES was best predicted by turbidity ($P = 0.003$) and salinity ($P = 0.090$) with a classification accuracy of 72% (LR, $P = 0.002$; HL, $P < 1.000$). In contrast to SFB, seals from ES were twenty-six times more likely to carry *Vibrio* when turbidity was less than 7 NTU and four and a half times more likely when salinity measured less than 33 ppt (Table 2).

Table 2. Results from multivariate backwards stepwise logistic regression testing environmental variables as predictors of the presence or absence of *Vibrio* in free-ranging seals sampled from Elkhorn Slough in 2010 and then San Francisco Bay from 2007 to 2011.

Predictors	*n*	n_1[a]	Prevalence (%)	*P* value	Odds Ratio (OR)	CI for OR
Elkhorn Slough						
Salinity (ppt)	47	29	61.70	-	-	-
<33	21	12	50.00	0.09	**4.67**	0.78-28.05
≥33[b]	26	17	65.38	-	-	-
Turbidity (NTU)	47	29	61.70	-	-	-
≤ 7	17	15	88.23	**0.003**	**26.25**	3.04-226.6
> 7[b]	30	14	46.67	-	-	-
San Francisco Bay						
Turbidity (NTU)	71	32	45.07	-	-	-
≤ 66[b]	26	6	23.07	-	-	-
> 66	45	26	57.78	**0.008**	**4.51**	1.49-13.64

[a] *Vibrio* +, [b] Reference Category

Vibrio *prevalence in stranded seals*

The overall prevalence of *Vibrio* in stranded seals from 2007 to 2011 was 17% (n = 189). Stranded adults and subadults were dropped from further analysis because of small sample size (n = 5). Differences in *Vibrio* prevalence were detected among years (Cochran's $\chi^2_{0.05,1}$ = 10.31, P = 0.016) with the greatest percentage of seals positive for *Vibrio* (33 %, n = 43) in 2011 (Fig. 3). There was no effect of season (Cochran's $\chi^2_{0.05,1}$ = 0.08, P = 0.782), or sex (females 20 %, n = 49, males 21 %, n = 53; Cochran's $\chi^2_{0.05,1}$ = 0.002, P = 0.966) for the presence or absence of *Vibrio* in stranded seals.

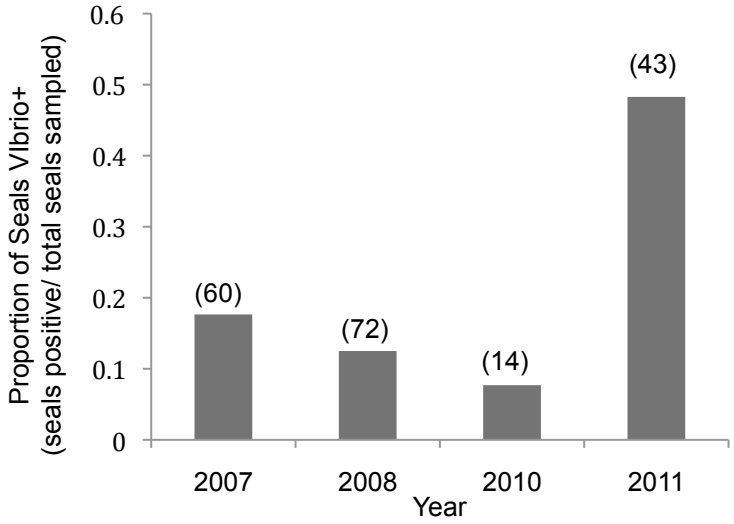


Figure 3. Proportion of stranded pups and yearlings admitted to The Marine Mammal Center with *Vibrio*, 2007 to 2011. Sample sizes are indicated in parentheses.

Pups and yearlings with *Vibrio* had slightly greater masses at admission ($\bar{X}$ = 8.9 kg, SE = 0.49), and a slightly greater BCI ($\bar{X}$ = 0.03, SE = 0.22) compared with individuals without *Vibrio* (mass: $\bar{X}$ = 8.5 kg, SE = 0.30; BCI: $\bar{X}$ = -0.01, SE = 0.11), however, these differences were not statistically significant (mass t (100) = -0.613, P =

0.541; BCI t (99) = -0.174, P = 0.862). Since none of the demographic factors were significantly related to *Vibrio* prevalence in stranded seals, no further analyses were performed.

Prevalence of *Vibrio* in stranded seals varied by stranding county and year; however, data were too sparse to test for statistical significance. In general, San Luis Obispo (SLO), Monterey, Marin, and Mendocino counties had the greatest percentage of stranded seals positive for *Vibrio* from 2007 to 2011, although it varied among years (Fig. 1). The greatest prevalence of *Vibrio* observed per county occurred in 2011 for stranded seals sampled from SLO (67 %, n = 3), followed by Monterey (42 %, n = 12), Marin (33 %, n = 9), Mendocino (33 %, n = 9), and San Mateo (25 %, n = 4). Animals that stranded in Alameda and Santa Cruz counties were negative for *Vibrio* in all study years.

Comparison of Vibrio *prevalence, species distribution, and virulence profiles between free-ranging and stranded seals*

Because there were very few stranded adults and subadults, only data from pups and yearlings were used to compare *Vibrio* prevalence between free-ranging and stranded seals among sample locations. *Vibrio* prevalence observed in stranded and free-ranging pups and yearlings increased from 2007 to 2011 in all locations and varied among sample year (Table 3). *Vibrio* was not detected until 2011 in free-ranging pups and yearlings sampled from TB (17 %, n = 6). *Vibrio* prevalence was greater in free-ranging pups and yearlings compared with stranded pups and yearlings from SFB and ES. The opposite

trend was observed for TB in 2011. All free-ranging pups and yearlings sampled from ES in 2010 were positive for *Vibrio*, whereas all stranded individuals were negative.

Table 3. Prevalence of *Vibrio* in free-ranging and stranded pups and yearlings positive for *Vibrio* per location from 2007 to 2011.

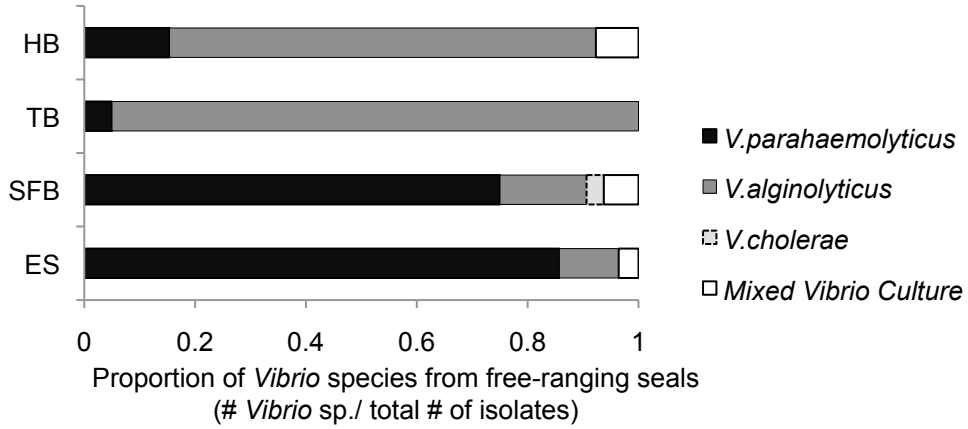
	Vibrio Prevalence % (*n*)			
Location	2007	2008	2010	2011
Tomales Bay				
Free-ranging	0 (4)	0 (12)	0 (1)	17 (6)
Stranded	0 (4)	0 (6)	100 (1)	25 (4)
San Francisco				
Free-ranging	64 (11)	47 (19)	67 (6)	0 (2)
Stranded	11 (19)	17 (23)	0 (5)	60 (5)
Monterey Bay				
Free-ranging[a]	NA	NA	100 (4)	NA
Stranded	13 (8)	0 (13)	0 (3)	57 (7)

[a]Sampled in Elkhorn Slough
NA = did not sample

Vibrio parahaemolyticus, *V. alginolyticus,* and *V. cholerae*, were isolated from seals, although the proportions of these species were not equal in free-ranging and stranded seals among locations (Fig. 4). Isolates from free-ranging seals in ES were predominantly *V. parahaemolyticus*, whereas greater proportions of all three *Vibrio* species were observed in seals that stranded in MB. All three *Vibrio* species also were isolated from free-ranging and stranded seals from SFB, although greater proportions of *V. parahaemolyticus* and *V. alginolyticus* were isolated than *V. cholerae* in free-ranging seals. *Vibrio cholerae* was isolated primarily from stranded pups and yearlings except it was found in one free-ranging adult from ES and one free-ranging pup from SFB. The majority of isolates from free-ranging seals sampled in TB and HB were *V. alginolyticus*, with the exception of three isolates of *V. parahaemolyticus* (TB, $n = 1$; HB, $n = 2$). One

stranded seal in TB was positive for *V. paraheamolyticus*, and one for *V. cholerae.* Three isolates (SFB, *n* = 2; ES, *n* = 1) could not be identified to species level due to mixed biochemical and genotypic results.

A.



B.

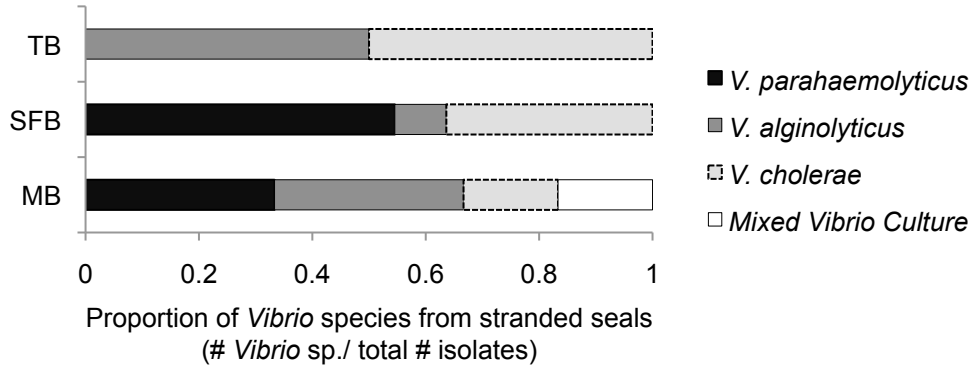


Figure 4. Proportions of *Vibrio* species isolated from (A) free-ranging harbor seals sampled in Humboldt Bay (HB, *n* = 13), Tomales Bay (TB, *n* = 20), San Francisco Bay (SFB, *n* = 32) and Elkhorn Slough (ES, *n* = 28), and (B) harbor seals stranded in TB (*n* = 2), SFB (*n* = 11), and Monterey Bay (MB, *n* = 6). Mixed *Vibrio* Culture are isolates that could not be identified to species level, and samples with more than one species of *Vibrio* detected.

V. parahaemolyticus and *V. parahaemolyticus*-like species (API 20E confirmed, *ToxR*-) were detected in free-ranging and stranded seals at all locations. Ninety percent of *V. parahaemolyticus* isolates (*n* = 53) contained the *tl* gene, whereas 67% contained both *trh* and *tl* genes (Table 4). Five *V. parahaemolyticus* isolates were positive for all three hemolysin genes (*tl*, *trh*, and *tdh*), and were collected from free-ranging seals sampled in SFB (*n* = 2) and ES (*n* = 3). Overall, 77% percent of *V. parahaemolyticus* (*n* = 53) isolates contained one or more virulence genes (*trh*, *tdh,* or both), with the majority of virulent isolates from free-ranging adults and subadults in SFB and ES. Two isolates of *V. parahaemolyticus* (*ToxR*+), and three isolates biochemically similar to *V. parahaemolyticus (ToxR*-) were lacking all three target genes. One free-ranging seal sampled from ES was carrying *V. cholerae* (*ToxR*+) and potentially virulent *V. parahaemolyticus* (*trh*+, and *tl*+) simultaneously. All *V. cholerae* (*ToxR*+) isolates (*n* = 3) collected from seals in 2010 and 2011 demonstrated hemolytic activity on blood agar media, although they were negative for *tcp* and *ctx* virulence target genes. Target amplicon identity was confirmed by comparing sequences from a subset of *V. parahaemolyticus* and *V. cholerae* isolates with reference sequences in Genbank (Appendix C).

Table 4. Virulence gene profiles for *V. parahaemolyticus* isolates collected from free-ranging seals from Elkhorn Slough (ES), San Francisco Bay (SFB), Tomales Bay (TB), and Humboldt Bay (HB), and stranded seals (TMMC) in 2010 and 2011. Sample sizes represent the number of isolates tested.

	Target Genes			Location				
Species	*tdh*	*trh*	*tl*	ES (*n*=27)	SFB (*n*=12)	TB (*n*=2)	HB (*n*=5)	TMMC (*n*=7)
V.parahaemolyticus	+	+	+	3	2	0	0	0
	-	-	-	0	2	0	0	0
	-	+	+	20	7	1	2	1
	-	-	+	4	1	0	0	2
Vibrio spp. (Vp like)	-	-	-	0	0	1	1	1
	-	+	+	0	0	0	2	3

Four stranded pups sampled in 2010 and 2011 were carriers of potentially virulent *V. parahaemolyticus* (*trh*+) and *V. parahaemolyticus*-like (*trh*+) isolates, and three of those seals died in treatment. Evidence of enteritis was observed during necropsy for two of the three deceased seals carrying potentially virulent *V. parahaemolyticus*, and one carrying hemolytic *V. cholerae*.

Discussion

Pacific harbor seals off the coast of California were carriers of potentially virulent isolates of *Vibrio*. All free ranging seals appeared healthy at the time of sampling, so were asymptomatic carriers, whereas stranded seals were all underweight for their age, thus could potentially have been impacted by the *Vibrio* infections. Similar species of *Vibrio* were observed among free-ranging and stranded seals, with the exception of *V. cholerae. Vibrio cholerae* were primarily detected in non-weaned stranded pups and may

be of greatest concern to harbor seal health. Stranded seal pups with potentially virulent *V. parahaemolyticus* and hemolytic *V. cholerae* presented symptoms of enteritis. Future clinical studies should focus on assessing the impact of virulent *Vibrio* strains on stranded seal health.

Vibrio likely contribute to the natural microbial flora of coastal areas used by harbor seals, and population dynamics of *Vibrio* may be influenced by both oceanographic and terrestrial contributions to water quality. Changes in temperature and salinity commonly relate to the seasonal abundance and distribution of *Vibrio* in the water column [17, 19, 39, 45, 52, 55]. However, turbidity and/or salinity best predicted *Vibrio* occurrence in harbor seals, although the relationship differed with location. The differences in related environmental factors suggest that *Vibrio* exposure in seals may be related to other factors not measured in this study. Furthermore, the lack of seasonal variation for *Vibrio* prevalence in seals may indicate that seals may frequent reservoirs of *Vibrio* that are decoupled from seasonally influenced environmental factors measured here [27, 55, 86].

Differing land-use practices may influence the variation in *Vibrio* prevalence observed in seals among locations. *Vibrio* prevalence in seals from TB was the least among all the locations. Rural land surrounding TB is typically used for agriculture where the dominant enterprise is livestock farming [5]. Movement patterns and habitat use of seals from TB are unknown making it difficult to make inferences about dynamics of *Vibrio* in seals sampled at this location. However, we can conclude that it is unlikely that dairy cattle near TB are a source of *Vibrio* because TB had the greatest concentration

of dairies yet the lowest prevalence of *Vibrio* compared with ES or SFB. Furthermore, no cattle sampled near ES were confirmed with *Vibrio* during years we sampled harbor seals [65].

San Francisco Bay is the largest urbanized estuary on the eastern Pacific, and is greatly impacted by industrial and residential inputs from the Sacramento and San Joaquin rivers. Compared with ES and TB, the SFB estuary is deeper on average and comprised of a larger watershed [14]. Tidal mixing and run-off from rivers, tributaries, treatment plant effluent, industry, and groundwater contribute to changes in turbidity throughout the bay. Turbidity best predicted *Vibrio* in seals from SFB. Bacterioplankton in SFB are generally evenly distributed, and areas of greater flow near the delta facilitate conditions of optimal bacterial growth [41, 42]. No differences were detected for *Vibrio* prevalence in seals sampled from north and south SFB. Freshwater run-off from the delta also may facilitate optimal conditions for *Vibrio* growth in SFB. The turbidity maximum zone of SFB where freshwater inputs from the delta meet seawater is dominated by aggregations of particle-associated bacteria and plankton [41, 42]. *Vibrio* can associate with plankton [45, 55, 86], therefore blooms following nutrient loading from the delta may relate to the abundance of *Vibrio* in SFB. It is unknown whether *Vibrio* in SFB are free-living, or form aggregations with plankton or particulate matter in the sediment. However, if *Vibrio* aggregate near the maximum turbidity zone in SFB, seals ingesting contaminated prey or sediment in these areas may acquire greater concentrations of *Vibrio*.

Environmental dynamics relating to *Vibrio* prevalence in free-ranging seals from ES were opposite to those of SFB. It is unknown whether ES is a source for *Vibrio* in seals, yet free-ranging seals in ES had the greatest prevalence of virulent *Vibrio* overall. If ES is a source of *Vibrio* for seals, conditions that are unique to ES may relate to the observed differences. Water quality and hydrography in ES have been altered by intensive agriculture cultivation and dairy farming [14, 15]. Pesticides are continuously used for crop production and are introduced into ES from irrigation run-off, erosion, and groundwater [70, 72]. The effects of these inputs likely are magnified due to the small size and shallow bathymetry of ES compared with SFB and TB [14]. Less turbid and less saline waters were related to *Vibrio* occurrence in seals of ES. Previous studies indicated the greatest concentrations of bacteria in ES were observed near areas with the greatest freshwater inputs [72]. Tidal relaxation events coupled with continuous freshwater inputs from crop irrigation and groundwater seepage may be the driving factor promoting *Vibrio* proliferation in this location. If conditions in ES allow *Vibrio* populations to flourish, this small estuary could act as a reservoir for dense aggregations of these bacteria. Furthermore, zooplankton or fish that occur in ES may acquire *Vibrio* and become vectors when recruiting to habitat in MB [76, 86]. Data from seals in ES were limited to one sample year, therefore it is possible the greater *Vibrio* prevalence observed in these seals was an anomaly.

Alternatively, the different relationships between environmental factors and *Vibrio* prevalence in seals in ES and SFB may be explained by differences in habitat use and foraging behavior. Harbor seals that haul-out in ES generally use this habitat to rest

after foraging bouts [23, 64, 85]. Seals sampled in ES may forage in ES, although they spend the majority of their time exploiting habitat and foraging on benthic and pelagic prey (e.g. octopus, flatfish, and cusk-eel) in MB [67]. *Vibrio parahaemolyticus* was detected most often in seals from ES, and can be pathogenic in fish and humans. *Vibrio parahaemolyticus* was shown to occur less frequently than other species of *Vibrio* from water and invertebrates sampled from ES [59], and may persist in greater concentrations in sediment, offshore zooplankton, and fish [19, 45, 55, 86]. Therefore, resources and habitat in MB may be a source of greater concentrations of *Vibrio parahaemolyticus*. Depending on the source of *Vibrio* in seals from ES, environmental data from ES may not represent *Vibrio* dynamics in seals sampled here. Further research is needed to determine inter-annual variability, population dynamics, and host-vector interactions of *Vibrio* in ES and MB.

Similarly, *V. parahaemolyticus* was observed most often in seals from SFB, although seals in SFB primarily forage on benthic prey (e.g. gobies, staghorn sculpin, plainfin midshipman) within the estuary. They also forage offshore on pelagic schooling fish like northern anchovy, although these prey comprise a smaller portion of their diet [28, 62, 84]. Given *V. parahaemolyticus* can associate with sediment and zooplankton, transmission in seals from SFB may occur while foraging on benthic prey. Although total *Vibrio* prevalence was greater for seals from ES, similar proportions of *Vibrio parahaemolyticus* were observed in seals from SFB and ES. Seals from ES spend majority of their time exploiting habitat and resources in MB [23, 67, 85] therefore the

offshore life cycle of *Vibrio parahaemolyticus* may be important component for *Vibrio* dynamics in seals.

In general, *Vibrio* prevalence in free-ranging seals was greater than that in stranded seals. If *Vibrio* were acquired by ingesting contaminated prey or sediments while foraging, differences in foraging behavior between weaned and non-weaned pups may explain the observed differences in *Vibrio* prevalence. The majority of stranded seals were non-weaned pups that were abandoned or separated from their mothers that are dependent on mother's milk [32]. Stranded pups that were weaned may have foraged, although, not as successfully as healthy individuals. Weaned pups also may consume different types of prey in lesser quantities compared with older seals because of limited foraging experience and diving capacity [71]. Stranded individuals may suffer from other ailments or trauma, thereby, further hindering their abilities to successfully forage [50].

Free-ranging pups and yearlings may be at greater risk of acquiring *Vibrio* if they frequent habitat and resources in ES and SFB with greater *Vibrio* burden because they have greater home ranges than adults [50, 62, 64]. Age was a poor predictor of *Vibrio* prevalence for seals in SFB, however, the transient behavior of pups and yearlings may explain the greater *Vibrio* prevalence observed when compared with adults and subadults. If resources and space are limited near preferred habitat, juveniles may forage in alternative habitats to avoid competition with adults [25]. Transient pups and yearlings also may transmit *Vibrio* among locations if they are shedding the bacteria. Age could not be tested as a predictor of *Vibrio* in seals from ES or TB because the majority of free-ranging seals sampled in ES and TB were adults.

When comparing pups and yearlings, a greater *Vibrio* prevalence was observed in free-ranging seals than in stranded seals. All free-ranging pups and yearlings sampled were weaned, in better body condition, and likely were ingesting contaminated prey or sediment while foraging. In general, stranded weanlings and yearlings with *Vibrio* had greater body condition indices than those without. A few non-weaned and stranded pups in poor body condition were observed with *V. parahaemolyticus* and *V. cholerae*. The etiology of *Vibrio* in seals was not examined, and alternative transmission routes should be considered. Transmission also may occur via gestation, lactation, or contact with other seals shedding *Vibrio* at haul-out sites [90]. Additionally, *Vibrio* prevalence varied among locations and years for free-ranging and stranded pups and this was likely a result of poor sample sizes, or, the transient behavior of pups and yearlings. Prey abundance and distribution also may be a source of variation if pups and yearlings leave natal haul-out sites to locate prey. Regardless, it is apparent from our data that *Vibrio* prevalence in stranded pups and yearlings is increasing. This increase may be due to increased susceptibility to *Vibrio* acquisition or mother-to-pup transmission before separation. It is unlikely that the increase in prevalence reflects increased ability to culture the organisms during the study, as all isolations were performed at diagnostic laboratory with consistent practices.

Free-ranging and stranded seals not only differed by prevalence, but also the species of *Vibrio*. The proportions of *Vibrio* species differed among locations, and general trends between free-ranging and stranded seals were similar with the exception of *V. cholerae*. *Vibrio cholerae* was detected almost exclusively in non-weaned stranded

pups, and may be of greatest concern to harbor seal health. *Vibrio cholerae* are freshwater tolerant compared with other species of *Vibrio* and some strains are highly pathogenic in mammalian hosts [18, 24]. It is possible that stranded individuals may not have enough energy reserves to leave the shore and may accidentally ingest *V. cholerae* contaminated water or sediment near freshwater run-off sites. Sea otters (*Enhydra lutris*) using similar habitat consumed invertebrates near freshwater run-off sites and had similar prevalence of *V. cholerae* as stranded seals in this study [58]. Additionally, different species of *Vibrio* may associate with different prey or substrate types among regions [20, 45, 66]. This also may explain the differences in the proportions of species observed among locations for free-ranging and stranded seals.

Potentially virulent *V. parahaemolyticus* and hemolytic *V. cholerae* were observed in seals from ES and SFB. Direct mechanisms that contribute to virulence of *Vibrio* in seals have yet to be identified, although it is possible that virulence relates to conditions that are location specific [22]. Virulence expression may occur in response to environmental stressors, although few researchers have adequately tested this hypothesis [57, 79]. For example, iron is an important factor for *Vibrio* growth [80], therefore, hemolytic activity may be an adaptation selected for iron-limited oceanographic conditions. This hemolytic stress response may relate to increased virulence in the host however, *in situ* research is needed to test this hypothesis [80, 89].

The virulence gene regions targeted in this study can be up-regulated in human epidemic strains following experimental manipulation [22]. Environmental stressors introduced in *Vibrio* cell culture (e.g. pH, temperature, salinity, bicarbonate) can up-

regulate toxin production [1, 87]. Virulence expression and adhesion also increased in experimental hosts when isolates were subjected to certain growth conditions before infection [79]. Industrial contaminants and pesticides also may induce a virulence related stress response for some species of *Vibrio,* although this has not been tested *in situ* [26]. Seals suffering from contaminant burden also may become immuno-compromised, therefore, more susceptible to virulent pathogens [53, 61].

Vibrio alginolyticus was detected infrequently in free-ranging and stranded seals from all sample locations. This species is considered a pathogen of invertebrates, although it is rarely associated with disease in mammals [58]. In some cases, *V. alginolyticus* can have deleterious affects on mammalian hosts [7], although its role in the health of marine mammals is unknown. Given the low prevalence of *V. alginolyticus*, this species of *Vibrio* likely has the least impact on the health of harbor seals.

Environmental conditions resulting from climate variability may alter *Vibrio* ecology [17, 39, 49, 66, 75]. Because of this, real-time data on pathogen and host interactions relating to environmental perturbation are needed to better understand what induces virulence in the marine environment [57, 80, 88]. Furthermore, it is imperative to identify risks of potential pathogens like *Vibrio* to the health of marine mammals. In this study, we demonstrated that seals using habitat and resources near urbanized watersheds ES and SFB may have the greatest risk of acquiring potentially virulent *Vibrio*. Considering that humans share the environment and food resources with seals, potentially virulent *Vibrio* observed in seals also may be of concern to human health. Impaired watersheds like ES and SFB may be further perturbed as human populations increase

along the coast [43], and *Vibrio* may serve as a bioindicator for monitoring changes to regional ecosystem stability. It is critical to identify mechanisms of pathogen proliferation and associated risks of infection so we can forecast how aquatic pathogens may impact the health of marine mammals and the ecosystem they inhabit.

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Appendix A. Descriptive statistics, and bin criteria used for categorizing environmental predictor variables to be used in multivariate logistic regression analysis for SFB, from 2007 to 2011. Presence and absence of *Vibrio* were tested between categories for each predictor using a Pearson's chi-squared test, and associated p-values are reported.

Predictor Variable	*n*[a]	Min	Max	Mean	Median	S.E.	S.D.	Bin Criteria	Cochran's X^2, *P*
Temperature (*C)	873	7.19	23.88	16.51	17.22	0.13	3.92	<16, ≥16	0.640, *P* = 0.424
Salinity (ppt)	874	7.97	28.22	23.23	24.78	0.15	4.32	<23, ≥23	**2.22, *P* = 0.136**
pH (standard units)	837	7.65	9.08	8.03	7.98	0.01	0.20	≤8, >8	**3.82, *P* = 0.051**
Turbidity (NTU)	874	7.51	603.94	66.37	36.59	2.61	77.18	≤66, >66	**8.02, *P* = 0.005**

[a]Daily values collected for sampling duration

Appendix B. Descriptive statistics, and bin criteria used for categorizing environmental predictor variables to be used in multivariate logistic regression analysis for ES in 2010. Presence and absence of *Vibrio* were tested between categories for each predictor using a Pearson's chi-squared test, and associated p-values are reported.

Predictor Variable	*n*[a]	Min	Max	Mean	Median	S.E.	S.D.	Bin Criteria	Cochran's X2, P
Nutrients (N03- uM)	47	16.17	108.11	59.64	24.92	6.43	44.12	≤24.92, >24.92	0.01, P=0.901
Temperature (*C)	152	9.37	20.29	15.91	16.53	0.25	3.07	<16, ≥16	0.01, P=0.908
Salinity (ppt)	152	28.45	34.81	33.35	33.48	0.09	1.15	<33, ≥33	**0.33, P=0.563**
pH (standard units)	101	7.79	8.26	7.98	7.97	0.01	0.1	≤7.9, >7.9	**5.81, P=0.016**
Turbidity (NTU)	140	3.43	19.39	7.37	6.80	0.21	2.54	≤7, >7	**7.94, P=0.005**

[a]Daily values collected for sampling duration

Appendix C. Sequence confirmation for target genes for a subset of isolates collected from harbor seals.

Harbor Seal Isolate	Target Region	Observed Length	Expected	E-Value	% Pairwise Identity	Strain & Reference Accession #
V.parahaemolyticus tdh, trh, tl[a]						
Adult Female C	*tdh*	313	270	5.13E-130	98.2	*V.parahaemolyticus 03:K6 (Bangladesh), AY044114*
	trh	482	500	0	97.6	*V.parahaemolyticus TH3996, AB455531*
	tl	447	450	0	98.7	*V.parahaemolyticus, AY289609*
1972-1973	*tdh*	315	270	8.94E-128	97.9	*V.parahaemolyticus 03:K6 (Bangladesh), AY044114*
	trh	481	500	0	98.5	*V.parahaemolyticus, AY742213*
	tl	434	450	0	99.1	*V.parahaemolyticus ATCC 33846, GU971655*
1935-1936	*tdh*	309	270	4.04E-131	98.5	*V.parahaemolyticus 03:K6 (Bangladesh), AY044114*
	trh	481	500	0	97.9	*V.parahaemolyticus, AY742213*
	tl	434	450	0	98.8	*V.parahaemolyticus, AY289609*
1892-1893A	*tdh*	306	270	6.70E-129	97.8	*V.parahaemolyticus 03:K6 (Bangladesh), AY044114*
	trh	445	500	0	98.2	*V.parahaemolyticus, AY742213*
	tl	436	450	0	98.7	*V.parahaemolyticus, AY289609*
V.parahaemolyticus ToxR[b]						
2014-2015	*ToxR*	274	297	3.59E-133	98.5	*V.parahaemolyticus RIMD 2210086, AY527397*
1822-1823C	*ToxR*	246	297	2.56E-109	96.3	*V.parahaemolyticus RIMD 2210086, AY527397*
V.cholerae ToxR[b]						
1824-0491	*ToxR*	573	640	0	98.6	*V.cholerae 01 El Tor N16961, AE003852*

[a]Bej et al. 1999, [b]Bauer & Rorvik 2007