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Salmonella spp., *Vibrio* spp., *Clostridium perfringens,* and *Plesiomonas shigelloides* in marine and freshwater invertebrates from coastal California ecosystems.

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Abstract

The coastal ecosystems of California are highly utilized by humans and animals, but the ecology of fecal bacteria at the land-sea interface is not well understood. This study evaluated the distribution of potentially pathogenic bacteria in invertebrates from linked marine**,** estuarine, and freshwater ecosystems in central California. A variety of filter-feeding clams, mussels, worms, and crab tissues were selectively cultured for *Salmonella* spp., *Campylobacter* spp., *Escherichia coli*-O157, *Clostridium perfringens, Plesiomonas shigelloides*, and *Vibrio* spp. A longitudinal study assessed environmental risk factors for detecting these bacterial species in sentinel mussel batches. Putative risk factors included mussel collection near higher risk areas for livestock or human sewage exposure, adjacent human population density, season, recent precipitation, water temperature, water type, bivalve type, and freshwater outflow exposure. Bacteria detected in invertebrates included *Salmonella* spp., *Clostridium perfringens, Plesiomonas shigelloides, Vibrio cholerae, Vibrio parahaemolyticus*, and *Vibrio alginolyticus*. Overall, 80% of mussel batches were culture-positive for at least one of the bacterial species, though the pathogens *Campylobacter, E. coli*-O157, and *Salmonella* were not detected. Many of the same bacterial species were also cultured from upstream estuarine and riverine invertebrates. Exposure to human sewage sources, recent precipitation, and water temperature were significant risk factors for bacterial detection in sentinel mussel batches. These findings are consistent with the hypothesis that filter-feeding invertebrates along the coast concentrate fecal bacteria flowing from land to sea, and show that the relationships between anthropogenic effects on coastal ecosystems and the environmental niches of fecal bacteria are complex and dynamic

Introduction

The ecology of fecal bacteria entering nearshore aquatic environments along the California coast is not well understood, but is gaining in importance as human populations utilizing these ecosystems grow (8, 37, 40, 43). Environmental loading of fecal byproducts from humans and their associated animals is significant and can affect the quality of water and food resources in coastal ecosystems (13, 24). Terrestrial sources of fecal waste entering aquatic ecosystems include animal-derived fecal matter carried in storm runoff, and human sources such as sewage outfalls, leaky septic tanks, and boat discharges. Many of the fecal parasites, bacteria, and viruses that have been detected in freshwater, estuarine, and marine environments are potential pathogens to humans and animals, and their environmental niches are quite diverse (6, 36).

Our previous studies have investigated the epidemiology of the fecal protozoan parasites *Cryptosporidium, Giardia*, and *Toxoplasma* species in coastal California ecosystems (8, 28-30, 32). All of these parasite life cycles involve fragile zoite stages that develop in the digestive tracts of animal hosts, including in some cases humans, and sturdy oocyst or cyst stages that are shed in the feces and can remain viable in the environment for prolonged periods of time (12, 39). Investigation into the ecology of these parasites in aquatic ecosystems revealed that detection of *Cryptosporidium* and *Giardia* spp. in bivalve shellfish was associated with exposure to freshwater sources and was greatest during the wet season in California (30, 32). *Toxoplasma gondii* in a marine mammal, the Southern sea otter (*Enhydra lutris nereis*), has also been correlated with exposure to areas of high freshwater outflow into the nearshore marine environment (28). While the life cycles of fecal bacteria are quite different than fecal protozoa, some of the same risk factors may apply to the transport of fecal bacteria from land to sea.

Fecal bacteria entering aquatic ecosystems in coastal California may have a more complex ecological niche because unlike the protozoans that are only known to multiply within animal cells, bacteria can multiply in the environment as well as within animal hosts (18, 44). This suggests that once fecal bacteria enter the aquatic environment, they may be able to establish long-term reservoirs, depending on each species' ability to acclimate and tolerate a range of environmental conditions. The bacteria in this study include primarily terrestrial fecal bacteria such as *Salmonella* and *Campylobacter* spp., as well as fecal bacteria well-adapted to aquatic environments such as *Vibrio* and *Plesiomonas* spp., all of which can act as pathogens under the appropriate host, microbe, and environmental conditions (2, 5, 18, 40, 44).

Filter-feeding invertebrates play an important role in the ecology of aquatic pathogens because they concentrate microbes from the surrounding waters (4, 16, 27). As a result, filter-feeders can act as bioindicators of the microbial diversity present in aquatic ecosystems, as well as food items that provide concentrated microbial doses to the animals and humans that consume them. The goal of this study was to investigate the distribution of potentially pathogenic bacteria among aquatic invertebrates in marine, estuarine, and freshwater ecosystems along the central California coast. Our primary hypothesis was that if fecal pollution is flowing from land to sea, then detection of fecal bacteria in invertebrates may be greatest near sites designated as higher risk for livestock runoff or human sewage exposure, compared to sites designated as lower risk for these sources. Based on our previous protozoal studies, we also hypothesized that high freshwater outflow exposure and wet season sampling would be associated with greater detection of fecal bacteria in bivalves.

Methods

Study Design. Bacteria selected for this study included *Salmonella* spp., *Campylobacter* spp., *Escherichia coli*-O157, *Clostridium perfringens*, *Vibrio* spp., and *Plesiomonas shigelloides*. Three approaches were taken to evaluating their distribution among invertebrates along the central California coast. First, a longitudinal study was set up to deploy and collect sentinel mussel batches for quarterly testing during the dry (June-November) and wet (December-May) seasons along the central California coast. Figure 1 shows the various mussel sites sampled over the course of the three year study, 2001-2004. Mussels were initially collected from a pristine site near Bodega Bay, California, from which the target bacteria were not detected at any point during the study, and were deployed at three sites considered at higher risk for human sewage exposure, three sites at higher risk for livestock runoff, and three sites distant to these fecal sources (32). Mussels were collected quarterly as batches of 30 and when transplanted mussels were not available, resident mussels from the study sites were collected instead. Data on environmental risk factors that could be associated with fecal pathogen pollution flowing from terrestrial to aquatic ecosystems were also collected for each study site and sampling time.

Second, a comparative study was conducted of multiple invertebrate species collected from muddy and sandy habitats near Moss Landing (Monterey Bay) and Morro Bay, California. Clams, innkeeper worms, and mussels were sampled from a muddy estuarine habitat on the same day that clams, sand crabs, and mussels were collected from a nearby sandy habitat just outside the estuary during the wet season of 2002-2003. The target sample size was again 30 per species.

Third, freshwater clams (*Corbicula fluminea*) were deployed in the San Lorenzo and Salinas Rivers, two rivers with high historic coliform counts along the central coast (http://www.waterboards.ca.gov/centralcoast/BasinPlan/ Documents/3BactiObjsStaffReport05-04-04.doc), both of which feed into the Monterey Bay as shown in Figure 1. Clams were collected in batches of 30,

once during the dry season and again during the wet season for two consecutive years, 2002-2004. Bivalve collection and deployment methods were in accordance with long-term coastal monitoring protocols (30).

Invertebrate Sampling. Surf mussels (*Mytilus californianus*) and bay mussels (*Mytilus edulis*) were used in the longitudinal mussel study. Invertebrate species sampled from the estuaries near Moss Landing and Morro Bay, CA included fat innkeeper worms (*Urechis caupo*), gaper clams (*Tresus nuttalli*), Washington clams (*Saxidomus nuttalli*), bent-nose clams (*Macoma* spp.), and mussels (*Mytilus* spp.). Sampling from the sandy habitats outside the estuaries included Pismo clams (*Tivela stultorum*), mussels (*Mytilus* spp.) and two types of sand crabs (*Emerita analoga* and *Blepharipoda occidentalis*). Freshwater clams (*Corbicula fluminea*) were deployed for invertebrate testing in the San Lorenzo and Salinas Rivers.

Invertebrates were collected as batches of 30, of which six individuals were pooled per culture batch from each site and timepoint. Invertebrates were transported on ice to laboratories at the University of California, Davis (UCD), or to the Marine Wildlife Veterinary Care and Research Center in Santa Cruz, CA, for dissection within 24 hours of collection. Hemolymph, gill, and digestive gland tissues were collected as described (30, 32), and cultured for bacteria using selective media at UCD. The best invertebrate tissue to culture was determined by comparing the results of culturing hemolymph, gill and digestive gland tissues from the same invertebrates in the first year, with only digestive gland cultured in subsequent years.

Bacterial Culture. A 1-2 g aliquot of invertebrate tissues was macerated with a sterile swab and saline in a microcentrifuge tube before plating onto selective media according to established

protocols (34). Cultures were enriched for *Salmonella* and *Vibrio* spp. for 24 hrs using selenite broth and alkaline peptone water before plating onto Xylose Lactose Tergitol 4 (XLT4) and Thiosulfate Citrate Bile Sucrose (TCBS) agar, respectively. *Campylobacter* cultures utilized selective media containing Cefoperazone Vancomycin and Amphotericin B (Campy CVA) in a microaerophilic environment. *Escherichia coli*-O157 cultures were performed on Cefixime Potassium Tellurite Sorbitol MacConkey (CT SMAC) plates. *Clostridium perfringens* was identified using Egg Yolk Agar (EYA) under anaerobic conditions. *Plesiomonas* was identified from MacConkey plates. All incubations were performed at 37°C. Morphologic appearance and biochemical testing was used to initially identify the isolates, with further characterization using specific antiserum, API 20E strips, serotyping, and sequence analysis of the 16S rRNA gene.

Risk Factors. Environmental factors that could be associated with bacterial detection in mussels were identified and the data assimilated for all study sites and timepoints. Categorical data on fecal risk category was defined as lower risk for fecal pollution if sites were more than 5 km away from known fecal sources, as higher risk for livestock fecal sources if sites were less than 5 km from livestock runoff, and as higher risk for human sewage exposure if sites were less than 5 km from a sewage outfall or historic septic leakage. The distance of 5 km was selected based on biologic plausibility in a previous study (32) and in consultation with local resource managers who identified known fecal input sources within a 5 km radius of the sampling site. Season was categorized by dry (June-November) or wet (December-May) season for each collection. Recent precipitation was recorded as present or absent for the past day and past week. Water type at each collection site was defined as estuarine or marine. Bivalve type was recorded as resident or transplanted for each mussel batch. Freshwater outflow exposure data, defined as

low = <10 million L, medium = 10-100 million L, high = >100 million L of freshwater exposure in the past day, were available for each study site and mussel collection date from our previous *Cryptosporidium* study (32). In addition, human densities of the adjacent coastline were estimated using 2000 census data (http://www.census.gov/main/www/ cen2000.html) and water temperatures for each mussel collection date were obtained from National Oceanic and Atmospheric Administration records (http://co-ops. nos.noaa.gov/).

Statistical Analysis. McNemar's χ^2 test for paired data was used to evaluate whether significant differences in culture success could be detected among hemolymph, gill, and digestive tissues from the same bivalves, as well as comparing culture results from mud and sand invertebrates collected at the same estuaries. For the mussel risk factor analysis, univariate and then multivariate logistic regression models were created to quantify the strength of association between environmental variables and detection of the enteric bacteria of interest. Logistic regression is a powerful tool for simultaneously assessing the relative contributions of multiple risk factors that may be associated with a dichotomous outcome of interest such as the presence or absence of a bacterial species. The odds ratio produced for each risk factor is interpreted as the probability that the outcome of interest will occur in samples with the risk factor, relative to the probability of the outcome occurring in samples without the risk factor (or referent category). To minimize sparse data problems, models were only created for bacteria that were detected more than five times over the three year study (19). No study sites were within 5 km of each other, and only one site (the most downstream site) per estuary was included. A cluster effect was used in all logistic regression models, to adjust for repeated sampling at the same sites over

time. All statistical analyses were performed using Stata software (Stata Corp, College Station, TX, USA). Significant P values were defined as P<0.1.

Results

Tissue Comparison Study. During the first year of bivalve collections, the hemolymph, gill, and digestive gland tissues from the same animals (n=110) were cultured to determine the optimal tissue for long-term study. There was no significant difference between the frequency of isolation of enteric bacteria from hemolymph or gill tissues (P=0.4). However, digestive gland tissues significantly outperformed both hemolymph and gill tissues (P<0.001). Based on these results, only digestive gland was cultured for the remainder of the study.

Mussel Study. The three year mussel study involved deploying and collecting mussels quarterly from sites ranging south from Bodega Bay to Morro Bay, CA as shown in Figure 1. Forty-six mussel batches met the study inclusion criteria for statistical analysis, with each study site sampled at least three times during the study. Table 1 shows the bacterial prevalence among the 46 batches in the dry season, wet season, and in total. Overall, 80% of mussel batches during the dry and wet seasons were positive for at least one of the bacterial species, though no *Campylobacter, E.coli*-O157, or *Salmonella* spp. were detected. *Plesiomonas shigelloides* was detected in 4% of batches collected during the dry season but not during the wet season. *Vibrio parahaemolyticus, V. cholerae* non-O1, and *V. alginolyticus* were detected in up to 48% of mussel batches collected during the dry season and up to 26% of mussel batches collected during the wet season. In contrast, *C. perfringens* was detected more often in the wet season (68%) than in the dry season (41%).

Univariate analyses evaluated the strength of association between individual environmental variables and a mussel batch culturing positive for each bacterial group. The odds ratios and associated P values for factors significantly associated with detection of *V. cholerae, V. alginolyticus, C. perfringens*, and 'Any bacterial species' outcomes are given in Table 2. The other individual bacterial species were not included due to the sparse nature of the data. *Vibrio cholerae* was not significantly associated with any of the environmental variables. A mussel batch was significantly less likely to test positive for *V. alginolyticus* during the wet season or if there was precipitation in the day or week preceding mussel collection. A batch was more likely to have *V. alginolyticus* isolated if the water temperature was greater than 12°C at the time of sampling or if the study site was marine as compared to estuarine. Detecting *C. perfringens* in mussel batches was significantly more likely to occur during the wet season, if there was not precipitation in the day preceding mussel collection, or if the water temperature was less than 12°C. Mussel batches in which any of the bacteria were cultured were significantly associated with the higher risk category for exposure to human sewage, and with not having precipitation in the day or week preceding mussel collections. Human density and bivalve type were not significantly associated with any of the bacterial outcomes.

Forward-stepping multivariate logistic regression was used to examine multiple environmental variables simultaneously against the outcome of a batch culturing positive for the bacteria of interest. Table 3 shows the three models for which significant explanatory variables were found. Culturing *V. alginolyticus* from mussel batches collected within 24 hours of a precipitation event was 5 times less likely (odds ratio = 0.2) than mussel batches in which no precipitation occurred in the preceding day (P<0.01). *Clostridium perfringens* was also less likely to be detected in mussel batches collected within a day following a precipitation event

(P=0.07), and was associated with water temperatures less than 12°C (P=0.02). Detecting any of the targeted bacterial species in a mussel batch was significantly associated with no precipitation in the day preceding mussel collection (P<0.01), and was 39 times greater if the mussel batch was collected from a site considered higher risk for human sewage exposure compared to sites considered lower risk for human sewage or livestock waste exposure (P=0.01).

Comparative Invertebrate Study. The results of culturing pools of six invertebrates from muddy and sandy habitats near two fecal-impacted estuaries in the wet season of 2002-2003 are shown in Table 4. Bacterial pathogens were detected in the innkeeper worms, bivalves, and sand crabs that live in the sediment, as well as in mussels that were suspended higher up in the water column. *Salmonella* Typhimurium was detected in Pismo clams and *S.* Heidelberg in fat innkeeper worms at the Moss Landing site. *Vibrio parahaemolyticus* was cultured from Pismo clams and from at the Morro Bay site. *Vibrio alginolyticus* and *Clostridium perfringens* were detected in a variety of invertebrate species at both sites. Invertebrate pools (n=44) from the muddy sites inside the estuaries were more often positive for enteric bacteria than invertebrates collected at the sandy sites just outside the estuaries (P<0.01).

Freshwater Clam Study. The prevalence of each bacterial species cultured from pools of six freshwater clams collected during the 2002-2004 dry and wet seasons is shown in Table 5. *Vibrio cholerae V. alginolyticus,* and *C. perfringens* were only detected during the dry seasons. Downstream clam batches were more often culture-positive for bacteria than upstream batches. No *Campylobacter, E.coli*-O157, *Plesiomonas, Salmonella*, or other *Vibrio* spp. were detected. During the first wet season sampling, no clams were found alive at the lower San Lorenzo River

site and both Salinas River sites. Clams were repeatedly deployed at a third Salinas River site but the clam batches were consistently missing at each sampling timepoint. Sites and time periods for which no clams were cultured were excluded from further analysis.

Discussion

The goal of this study was to undertake the first multiyear investigation of the distribution of potentially pathogenic bacteria among aquatic invertebrates in linked marine, estuarine, and freshwater ecosystems along the Pacific coast of North America. *Campylobacter* and *E.coli*-O157 were not detected in the study, *Salmonella* and *Plesiomonas shigelloides* were only rarely detected, and *Clostridium perfringens* and *Vibrio* spp. were commonly detected. We had hypothesized that if biologic pollution due to fecal bacteria is flowing from land to sea, then detection of these organisms in invertebrates would be greatest near sites at higher risk for exposure to livestock or human feces, compared to sites designated as lower risk for these sources. This hypothesis was supported by our risk factor analysis, in which detection of any of these bacteria was greatest in invertebrates collected near areas at higher risk for exposure to human sewage. However, no significant association was observed between detecting the targeted bacteria and invertebrates collected from areas at higher risk for exposure to livestock runoff, nor were either of the higher risk fecal categories significant risk factors in the logistic regression models for individual bacteria. Study sites considered at higher risk for human sewage exposure included sites near human sewage treatment outfalls, as well as sites influenced by leaking septic tanks. In addition, other non-point sources such as urban runoff could be transporting human and animal fecal bacteria from land to sea in these higher risk areas but could not be assessed in this study. Detecting potentially pathogenic bacteria in invertebrates exposed

to human sewage could be due to increased pathogen input into the marine ecosystem with human waste, but could also be due to increased nutrient inflow that could make environmental conditions more favorable for bacteria to grow. Other studies have previously detected associations between compromised water quality in nearshore marine ecosystems and human sewage inputs (24, 25, 35).

Based on our recent studies, we also hypothesized that high freshwater outflow exposure and wet season sampling would be associated with detection of fecal bacteria in bivalves (11, 28, 32). In this study, high freshwater outflow exposure was not significantly associated with detecting bacteria in mussels, and while C. *perfringens* was detected most often in the wet season, *Vibrio* spp. and *P. shigelloides* were detected most often in the dry season. These findings suggest that the ecology of bacteria in coastal ecosystems is quite different than the ecology of fecal-borne protozoa. All of the bacteria in this study can be shed in the feces of humans and animals, but unlike the protozoa, many bacteria also survive and grow in environmental reservoirs when the conditions are favorable (9, 18). In other studies, *Vibrio* and *Plesiomonas* spp. have been detected most often during the warmer temperatures of summer and under certain salinity ranges (6, 18, 44). Furthermore, *Vibrio* spp. have been shown to play a role in chitin degradation, biofilm formation, and microbial competence in aquatic ecosystems, again highlighting their specialized ecologic niche in aquatic ecosystems (2).

Clostridium perfringens was not associated with the same environmental parameters as the other bacteria targeted in our study or others (4, 9). Several studies have detected *C. perfringens* throughout the year under a variety of conditions, and it has been utilized as an indicator for fecal source tracking studies (1, 25, 35). *Clostridium perfringens* is an anaerobic, spore-forming bacteria shed in the feces of a variety of animals and humans, with some genotypes associated

with clinical disease (34). The finding that both *Vibrio* spp. and *C. perfringens* were significantly associated with mussels collected when no precipitation had occurred in the previous day suggests that rain may influence the bacteria-invertebrate interactions, possibly by altering the bacterial concentration in surface waters, and/or the invertebrate feeding and depuration dynamics (33).

Several types of *Vibrio* spp. were detected in this study. *Vibrio cholerae* is the most infamous of the *Vibrios*, and while none of the epidemic-causing O1 strains were detected in this study, even non-O1 *V. cholerae*s have been associated with clinical disease in humans in California and elsewhere (18, 22, 36). *Vibrio parahaemolyticus* is another potentially pathogenic strain that can cause enteritis, and *V. alginolyticus* is the most widespread environmental strain, causing gastroenteritis or wound infections under opportune conditions (3, 18). The current study did not test bacterial isolates for virulence-associated factors to more definitively evaluate their potential pathogenicity, nor did it quantitate bacterial concentrations, another important consideration when specifically evaluating microbial disease potential (5).

Vibrio cholerae was first reported along the California coast in the 1980's (21-23). The Kenyon et al. (22, 23) studies were undertaken as a follow-up to a human case of *V. cholerae* non-O1 gastroenteritis, and the highest *V. cholerae* levels were detected in water samples during the summer, at times when coliform counts exceeded the legal limit of 1,000 per 100 ml seawater. The Kaysner et al. (21) study tested water, sediment, and shellfish samples for *V. cholerae* from 24 estuaries along the U.S. Pacific coast. *Vibrio cholerae* non-O1 was detected in Washington, Oregon, and California, with *V. cholerae* O1 Inaba reported in three water samples from Morro Bay, CA. Predominantly non-O1 *V. cholerae* strains have also been reported on the gulf and east coasts of the United States (20, 46).

Two serotypes of *Salmonella* were detected in invertebrates during the wet season collections. *Salmonella* Heidelberg was cultured from fat innkeeper worms living in the muddy estuary floor and *S.* Typhimurium was detected in Pismo clams from the sandy beach just outside the estuary mouth. Both of these *Salmonella* serotypes have been associated with clinical disease and are shed in the feces of a variety of hosts (17, 38, 45). Because *Salmonella* spp. were not cultured from mussels collected at the same time as the benthic invertebrates, it is uncertain whether the *Salmonella* were passing through in the water or were already present in the benthic sediment when filtered by the invertebrates. For most of the other bacterial species, suspended mussels and adjacent benthic invertebrates were culture-positive at the same sampling point, suggesting that the bacteria were passing through in the water column.

The comparative invertebrate and freshwater clam surveys showed that many of the same bacterial species isolated from marine mussels were also detectable in upstream invertebrates, though increased sample sizes and source tracking are needed to evaluate these associations statistically. Both estuaries in the study are at the interface between human activities and natural ecosystems, draining watersheds impacted by a wide range of agricultural and urban activities. Both have had problems with pollution and water quality, though invertebrates from both areas are still consumed by humans and marine mammals, such as the federally-listed threatened Southern sea otter (http://www.waterboards.ca.gov/centralcoast/BasinPlan/Documents/3Bacti ObjsStaffReport05-04-04.doc). More invertebrates were positive for bacteria from the muddy sites inside the estuaries than from the sandy sites just outside the estuaries. This finding is consistent with sources of bacteria coming from inland waterways and draining out into the ocean. However, it is also possible that the different environmental conditions inside the estuary are simply more favorable for bacterial survival than outside the estuary, allowing for bacteria

that enter the estuary on the incoming tide to find environmental niches in muddy habitat that aren't available in sandy habitat.

Mussels (*Mytilus* spp.) and freshwater clams (*Corbicula* spp.) were used for outplanting as sentinel bivalves for specific reasons. First, they already existed in the ecosystems of interest along the California coast, and they had been used for years by coastal monitoring programs to study pesticide and metal pollutants in coastal ecosystems (41). Second, the inherent filter-feeding activities of bivalves make them a natural concentrating mechanism that is easier to process in the laboratory than large volumes of water. Other invertebrates worth considering for sentinel studies include fat innkeeper worms in muddy habitats, that were recently shown to retain much higher levels of the algal neurotoxin domoic acid than other invertebrates along the California coast (http://seafloor. csumb. edu/publications/capstones/goldbergthesis.pdf), and sand crabs that live on sandy beaches where mussels are not always found, and have been shown to retain high levels of domoic acid in recent studies (14).

The culture methods used in this study were chosen because they are widely accepted and cost-efficient compared to molecular methods. The use of selective media allowed for efficient sample screening of a large number of samples, followed by further biochemical and molecular confirmation techniques on a more limited sample set. As has been shown in prior studies (10, 30, 31), digestive gland was the most sensitive tissue for screening purposes. It is possible that some samples falsely tested negative by culture in our study due to differences between the selective culture conditions and natural environmental conditions, or if the bacteria were in a viable but non-culturable state (15).

In conclusion, this study assessed a number of invertebrate species for potentially pathogenic bacteria present in coastal California ecosystems. The increased detection of bacteria in

invertebrates exposed to human sewage sources suggests that anthropogenic changes to the nearshore marine ecosystem may have significant effects on the ecology of fecal bacteria at the land-sea interface. Other studies support this association (11, 13, 26), making research on methods to minimize the impacts of humans and their associated animals on nearshore ecosystems important for long-term sustainability and health (1, 7, 42).

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References

1. Barrett, EC, Sobsey, MD, House, CH, White, KD (2001) Microbial indicator removal in onsite constructed wetlands for wastewater treatment in the southeastern U.S. Water Sci Technol 44:177-182.

2. Bartlett, DH, Azam, F (2005) Chitin, cholera, and competence. Science 310:1775-1777.

3. Blake, PA (1983) Vibrios on the half shell: what the walrus and carpenter didn't know. Ann Intern Med 99:558-559.

4. Burkhardt, W, Watkins, WD, Rippey, SR (1992) Seasonal effects on accumulation of microbial indicator organisms by *Mercenaria mercenaria*. Appl Environ Microbiol 58:826-831.

5. Butt, AA, Aldridge, KE, Sanders, CV (2004) Infections related to the ingestion of seafood, Part I: viral and bacterial infections. Lancet 4:201-212.

6. Chowdhurry, MA., Miyoshi, S, Yamanaka, H, Shinoda, S (1992) Ecology and distribution of toxigenic *Vibrio cholerae* in aquatic environments in a temperate region. Microbios 72:203-213.

7. Collins, R, Rutherford, K (2004) Modelling bacterial water quality in streams draining pastoral lands. Water Res 38:700-712.

8. Conrad, PA, Miller, MA, Kreuder, C, James, ER, Mazet, J, Dabritz, H, Jessup, DA, Gulland, F, Grigg, ME (2005) Transmission of *Toxoplasma*: clues from the study of sea otters as sentinels of *Toxoplasma gondii* flow into the marine environment. Int J Parasitol Sep 10 [Epub ahead of print].

9. Desmarais, TR, Solo-Gabrielle, HM, Palmer, CJ (2002) Influence of soil on fecal indicator organisms in a tidally influenced subtropical environment. Appl Environ Microbiol 68:1165-1172.

10. Dore, WJ, Lees, DN (1995) Behavior of *Escherichia coli* and male-specific bacteriophage in environmentally contaminated bivalve molluscs before and after depuration. Appl Environ Microbiol 61:2830-2834.

11. Dwight, RH, Semenza, JC, Baker, DB, Olson, BH (2002) Association of urban runoff with coastal water quality in Orange County, California. Water Environ Res 74:82-90.

12. Fayer, R, Ed (1997) *Cryptosporidium* and Cryptosporidiosis, CRC Press, Boston.

13. Fayer, R, Dubey, JP, Lindsay, DS (2004) Zoonotic protozoa: from land to sea. Trends Parasitol 20:531-536.

14. Ferdin, ME, Kvitek, RG, Bretz, CK, Powell, CL, Doucette, GJ, Lefevre, KA, Coale, S, Silver, MW (2002) *Emerita analoga* (Stimpson)-possible new indicator species for the phycotoxin domoic acid in California coastal waters. Toxicon 40:1259-1265.

15. Garcia-Armison, T, Servais, P (2004) Enumeration of viable *E. coli* in rivers and wastewaters by fluorescent in situ hybridization. J Microbiol Methods 58:269-279.

16. Graczyk, TK, Conn, DB, Marcogliese, DJ, Graczyk, H, De Lafontaine, Y (2002) Accumulation of human waterborne parasites by zebra mussels (*Dreissena polymorpha*) and Asian freshwater clams (*Corbicula fluminea*). Parasitol Res 89:107-112.

17. Guerin, MT, Marin, SW, Darlington, GA, Rajic, A (2005) A temporal study of *Salmonella* serovars in animals in Alberta between 1990 and 2001. Can J Vet Res 69:88-99.

18. Holmberg, SD (1988) *Vibrios* and *Aeromonas*. Infect Dis Clin North Am 2:655-676.

19. Hosmer, DW, Lemeshow, S (2000) Applied Logistic Regression, 2nd Ed, Ch. 8, John Wiley & Sons, New York, New York.

20. Kaper, J, Lockman, H, Colwell, RR, Joseph, SW (1979) Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay. Appl Environ Microbiol 37:91-103.

21. Kaysner, CA, Abeyta, C, Wekell, MM, DePaolo, A, Stott, RF, Leitch, JM (1987) Incidence of *Vibrio cholerae* from estuaries of the United States west coast. Appl Environ Microbiol 53:1344-1348.

22. Kenyon, JE, Gillies, DC, Piexoto, DR, Austin, B (1983) *Vibrio cholerae* (non-O1) isolated from California coastal waters. Appl Environ Microbiol 46:1232-1233.

23. Kenyon, JE, Piexoto, DR, Austin, B, Gillies, DC (1984) Seasonal variation in numbers of *Vibrio cholerae* (non-O1) isolated from California coastal waters. Appl Environ Microbiol 47:1243-1245.

24. Kim, JH, Grant, SB, McGee, CD, Sanders, BF, Largier, JL (2004) Locating sources of surf zone pollution: a mass budget analysis of fecal indicator bacteria at Huntington Beach, California. Environ Sci Technol 38:2626-2636.

25. Lipp, EK, Farrah, SA, Rose, JB (2001) Assessment and impact of microbial fecal pollution and human enteric pathogens in a coastal community. Mar Pollut Bull 42:286-293.

26. Mallin, MA, Williams, KE, Esham, EC, Lowe, RP (2000) Effect of human development on bacteriological water quality in coastal watersheds. Ecol Appl 10:1047-1056.

27. Marino, A, Lombardo, L, Fiorentino, C., Orlandella, B, Monticelli, L, Nostro, A, Alonzo, V (2005) Uptake of *Escherichia coli, Vibrio cholerae* non-O1 and *Enterococcus durans* by, and depuration of mussels (*Mytilus galloprovincialis*). Int J Food Microbiol 99:281-286.

28. Miller, MA, Gardner, IA, Kreuder, C, Paradies, DM, Worcester, KR, Jessup, DA, Dodd, E, Harris, MD, Ames, JA, Packham, AE, Conrad, PA (2002) Coastal freshwater runoff is a risk factor for *Toxoplasma gondii* infection of southern sea otters (*Enhydra lutris nereis*). Int J Parasitol 32:997-1006.

29. Miller, MA, Grigg, ME, Kreuder, C, James, ER, Melli, AC, Crosbie, PR, Jessup, DA, Boothroyd, JC, Brownstein, D, Conrad, PA (2004) An unusual genotype of *Toxoplasma gondii* is common in California sea otters (*Enhydra lutris nereis*) and is a cause of mortality. Int J Parasitol 34:275-284.

30. Miller, WA, Atwill, ER, Gardner, IA, Miller, MA, Fritz, HM, Hedrick, RP, Melli, AC, Barnes, NM, Conrad, PA (2005) Clams (*Corbicula fluminea*) as bioindicators of fecal contamination with *Cryptosporidium* and *Giardia* spp. in freshwater ecosystems in California. Int J Parasitol 35:673-684.

31. Miller, WA, Gardner, IA, Atwill, ER, Leutenegger, CM, Miller, MA, Hedrick, RP, Melli, AC, Barnes, NM, Conrad, PA (2005) Evaluation of methods for improved detection of *Cryptosporidium* spp. in mussels (*Mytilus californianus*). J Microbiol Methods, In Press.

32. Miller, WA, Miller, MA, Gardner, IA, Atwill, ER, Harris, M, Ames, J, Jessup, D, Melli, A, Paradies, D, Worcester, K, Olin, P, Barnes, N, Conrad, PA (2005) New genotypes and factors

associated with detection of *Cryptosporidium* detection in mussels (*Mytilus* spp.) along the California coast. Int J Parasitol 35:1103-1113.

33. Mourino-Perez, RR, Worden, AZ, Azam, F (2003) Growth of *Vibrio cholerae* O1 in red tide waters off California. Appl Environ Microbiol 69:6923-6931.

34. Murray, P.R., Ed (1995) *Manual of Clinical Microbiology*. ASM Press, Washington D.C.

35. Paul, JH, Rose, JB, Jiang, S, Kellogg, C, Shinn, EA (1995) Occurrence of fecal indicator bacteria in surface waters and the subsurface aquifer in Key Largo, Florida. Appl Environ Microbiol 61:2235-2241.

36. Rippey, S (1994) Infectious diseases associated with molluscan shellfish consumption. Clin Microbiol Rev 7:419-425.

37. Schlundt, J, Toyofuku, H, Jansen, J, Herbst, SA (2004) Emerging food-borne zoonoses. Rev Sci Tech 23:513-533.

38. Seepersadsingh, N, Adesiyun, AA, Seebaransigh, R (2004) Prevalence and antimicrobial resistance of *Salmonella* spp. in non-diarrhoeic dogs in Trinidad. J Vet Med B Infect Dis Vet Public Health 51:337-342.

39. Sinski, E (2003) Environmental contamination with protozoan parasite infective stages: biology and risk assessment. Acta Microbio Pol 52S:97-107.

40. Skelly, C, Weinstein, P (2003) Pathogen survival trajectories: an eco-environmental approach to the modeling of human campylobacteriosis ecology. Environ Health Perspect 111:19-28.

41. Stephenson, MD, Martin, M, Tjeerdema, RS (1995) Long-term trends in DDT, polychlorinated biphenyls, and chlordane in California mussels. Arch Environ Contam Toxicol 28:443-450.

42. Tate, KW, Pereira, MDGC, Atwill, ER (2004) Efficacy of vegetated buffer strips for retaining *Cryptosporidium parvum*. J Environ Qual 33:2243-2251.

43. Turbow, DJ, Osgood, ND, Jiang, SC (2003) Evaluation of recreational health risk in coastal waters based on enterococcus densities and bathing patterns. Environ Health Perspect 111:598-603.

44. Venkataswaren, K, Takai, T, Navarro, IM, Nakano, H, Hashimoto, H, Siebeling, RJ (1989) Ecology of *Vibrio cholerae* non-O1 and *Salmonella* spp. and role of zooplankton in their seasonal distribution in Fukuyama coastal waters, Japan. Appl Environ Microbiol 55:1591-1598.

45. Vugia, DJ, Samuel, M, Farley, MM, Marcus, R, Shiferaw, B, Shallow, S, Smith, K, Angulo, FJ, Emerging Infections Program FoodNet working Group (2004) Invasive *Salmonella* infections in the United States, FoodNet, 1996-1999: incidence, serotype distribution, and outcome. Clin Infect Dis 38:S149-S156.

46. Weber, JT, Levine, WC, Hopkins, DP, Tauxe, RV (1994) Cholera in the United States, 1965-1991, risks at home and abroad. Arch Intern Med 154:551-556.

Table and Figure captions:

Table 1. Prevalence of fecal bacteria in mussel batches, 2001-2004.

Table 2. Univariate analysis of factors significantly associated with detection of enteric bacteria in mussel batches.

Table 3. Multivariate analysis of factors significantly associated with detection of enteric bacteria in mussel batches.

Table 4. Bacteria cultured from invertebrate species living in two muddy and sandy estuarine habitats in the wet season, 2002-2003.

Table 5. Bacteria cultured from freshwater clams in two coastal California Rivers, 2002-2004.[a]

Figure 1. Map of the central California coast showing invertebrate field sampling sites.

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524 Table 1

Bacterial species	*Seasonal prevalence(%)*		*Overall prevalence (%) (n=46)*
	Dry (n=27 batches)	*Wet (n=19 batches)*	
Campylobacter	0	0	0
E.coli-O157	0	0	0
Salmonella	0	0	0
Plesiomonas shigelloides	4	0	2
Vibrio parahaemolyticus	11	5	9
Vibrio cholerae	26	11	20
Vibrio alginolyticus	48	26	39
Clostridium perfringens	41	68	52
Any of the above bacteria	81	79	80

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Table 2

	Vibrio cholerae		*Vibrio alginolyticus*		*Clostridium perfringens*		*Any bacterial species*	
Univariate risk factors	*Odds ratio*	*P value*	*Odds ratio*	*P value*	*Odds ratio*	*P value*	*Odds ratio*	*P value*
Fecal risk category - lower	1	-	1	-	1	-	1	-
higher - livestock sources	3	0.2	0.5	0.4	1.4	0.7	2.6	0.4
higher - human sources	3.7	0.3	2.4	0.3	2.1	0.4	13.6	0.08[a]
Season: Dry (June-November)	1	-	1	-	1	-	1	-
Wet (December-May)	0.3	0.2	0.4	<0.01[a]	3.2	0.04[a]	0.9	0.7
Precipitation in the past day: no	1	-	1	-	1	-	1	-
yes	0.4	0.3	0.3	0.1[a]	0.3	0.1[a]	0.1	0.01[a]
Precipitation in the past week: no	1	-	1	-	1	-	1	-
yes	0.6	0.3	0.2	<0.01[a]	0.9	0.8	0.3	0.02[a]
Water temperature over 12°C: no	1	-	1	-	1	-	1	-
yes	1.7	0.5	3.2	0.07[a]	0.3	0.1[a]	0.8	0.6
Water type: estuarine	1	-	1	-	1	-	1	-
marine	0.5	0.5	3.8	<0.01[a]	0.9	0.8	0.3	0.3
Freshwater outflow exposure: low	1	-	1	-	1	-	1	-
medium	1.8	0.4	1.6	0.5	1	1	0.5	0.4
high	1.8	0.4	0.5	0.5	1.7	0.5	0.5	0.5

[a]P value <0.1 considered significant.

Table 3

Bacterial species	*Significant risk factor*	*Odds ratio*	*P value* [a]
Vibrio alginolyticus	Precipitation in the past week: no	1	-
	yes	0.2	<0.01
Clostridium perfringens	Precipitation in the past day: no	1	-
	yes	0.15	0.07
	Water temperature over 12°C: no	1	-
	yes	0.17	0.02
Any bacterial spp.	Fecal risk category: lower risk	1	-
	higher risk - livestock sources	6.9	0.2
	higher risk - human sources	39.4	0.01
	Precipitation in the past day: no	1	-
	yes	0.04	<0.01

[a]P value <0.1 considered significant.

Table 4

Site	*Host species*	*Salmonella* [b]	*Vibrio parahaemolyticus* [b]	*Vibrio alginolyticus* [b]	*Clostridium perfringens* [b]
Moss Landing (mud)	**Innkeeper worms**	**+**	**-**	**+**	**+**
	Gaper clams	**-**	**-**	**+**	**+**
	Washington clams	**-**	**-**	**+**	**+**
	Bent-nose clams	**-**	**-**	**-**	**+**
	Mussels	**-**	**-**	**+**	**+**
Moss Landing (sand)	**Pismo clams**	**+**	**-**	**-**	**-**
	Blepharipoda crabs	**-**	**-**	**-**	**-**
	Emerita crabs	**-**	**-**	**+**	**-**
Morro Bay (mud)	**Innkeeper worms**	**-**	**-**	**-**	**-**
	Bent-nose clams	**-**	**-**	**+**	**+**
	Mussels	**-**	**+**	**+**	**+**
Morro Bay (sand)	**Pismo clams**	**-**	**+**	**+**	**+**
	Blepharipoda crabs	**-**	**-**	**+**	**-**
	Emerita crabs	**-**	**-**	**-**	**-**
	Mussels	**-**	**-**	**+**	**+**

[a]No *Campylobacter* spp., *E.coli* O157:H7, *Plesiomonas shigelloides,* or other *Vibrio* spp. were detected.

[b] **+** = culture positive batch; **-** = culture negative batch.

549 Table 5

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Riverine Study Site	*Vibrio cholerae*			*Vibrio alginolyticus*			*Clostridium perfringens*		
	D1 [b]	*D2*	*W2*	*D1*	*D2*	*W2*	*D1*	*D2*	*W2*
San Lorenzo Upper	-	-	-	-	-	-	-	-	-
San Lorenzo Middle	-	-	-	+	-	-	+	-	-
San Lorenzo Lower	-	+	-	+	-	-	-	-	-
Salinas Upper	-	+	-	-	-	-	-	-	-
Salinas Lower	-	+	-	+	-	-	-	-	-

[a] No *Campylobacter, E.coli* -O157, *Plesiomonas, Salmonella* , other *Vibrio* spp. were detected.

[b] D = Dry season, W = Wet season; 1 = Year 1, 2 = Year 2.

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