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Marine biofilms on submerged surfaces are a reservoir for *Escherichia coli* and *Vibrio cholerae*

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The enteric bacterium and potential human pathogen, *Escherichia coli*, is known to persist in tropical soils and coastal waters. *Vibrio cholerae* causes the disease cholera and inhabits marine environments including microbial films on submerged surfaces. The abundances of *E. coli* and *V. cholerae* were quantified in biofilm and water-column samples from three harbors in Honolulu, Hawai'i, which differ in their local and international ship traffic. *E. coli* and, in some cases *V. cholerae*, occurred in relatively high abundances in marine biofilms formed on abiotic surfaces, including the exterior hulls of ships. The community fingerprints of the biofilms and the water harboring these pathogens were further analyzed. The community compositions of biofilms from different locations were more similar to each other than to water-column communities from the same locations. These results suggest that biofilms are an overlooked reservoir and a source of dissemination for *E. coli* and *V. cholerae*.

Keywords: *Vibrio cholerae*; *Escherichia coli*; biofilms

Introduction

Biofilms are surface-associated communities of microorganisms and the extracellular substances they produce. Biofilms are ubiquitous on damp and aquatic surfaces in all environments, from the most benign to the most extreme (Hall-Stoodley et al. 2004). When a clean surface is submerged in the sea, it is quickly colonized by bacteria and other microorganisms forming a biofilm (Huggett et al. 2009) which may achieve great complexity in terms of bacterial composition and three-dimensional structure (Davey and O'Toole 2000; Molino et al. 2009). Because of their importance in medicine, biofilms on human tissues and implanted prostheses have been the subject of intense investigation in the last decade (Costerton and Stewart 2001). Pathogenic bacteria may thrive in biofilms in water reclamation systems (Hu et al. 2003) and drinking water filtration systems (Emtiazi et al. 2004). Once bacteria produce and become embedded in the extracellular matrix characteristic of biofilms they may become resistant to attack by many antibiotics (Costerton et al. 1999). Typically, the occurrence and abundance of pathogenic bacteria in bays, harbors, and near-beach waters are determined by filtering water or using biomonitors (eg clams or oysters) (Colwell and Spira 1992). However, the extent to which marine biofilms on surfaces like ships' hulls serve as a reservoir and means of dissemination for pathogenic bacteria has yet to be fully explored.

Escherichia coli and *Vibrio cholerae* have become model organisms in biofilm research (Pratt and Kolter 1998; Watnick and Kolter 1999). Both species have been shown to survive in estuarine sediments in laboratory studies (Hood and Ness 1982). *E. coli* persists in both soil and stream water in tropical environments (Hardina and Fujioka 1991; Byappanahalli and Fujioka 1998; Winfield and Groisman 2003), and *V. cholerae* thrives in marine habitats as free floating aggregates in surface water (Faruque et al. 2006), on biotic surfaces including the exoskeletons of zooplankton (Huq et al. 1983; Tamplin et al. 1990; Huq et al. 1995), on the surfaces of marine algae (Islam et al. 1989), and on abiotic surfaces including the inner surfaces of the ballast tanks of ships (Drake et al. 2007; Islam et al. 2007).

The question asked was whether marine biofilms on abiotic surfaces in coastal harbors, including glass microscope slides and the wetted hulls of ships, are reservoirs for *E. coli* and *V. cholerae*. Because most monitoring for pathogenic bacteria in harbors and coastal areas is done on water samples, bacterial abundances in the water column were also compared with those on nearby surfaces. A further question asked was whether microbial community fingerprints harboring these pathogens differ among harbors that

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are affected by differences in local and international boat traffic and freshwater input.

Materials and methods

Locations and methods of sample collection

Biofilm samples were collected on microscope slides suspended in the Ala Wai Harbor, Kewalo Basin, and Pearl Harbor, on the island of Oahu, Hawai'i. Biofilms were also collected from the hulls of three ships in each of the following harbors: Ala Wai Harbor, Kewalo Basin, and Honolulu Harbor. The Ala Wai Yacht Harbor is home to a large fleet of Hawai'i-based yachts and is a port-of-call for pleasure craft from throughout the Pacific. It receives a large load of urban runoff from several valleys and a substantial area of Honolulu. Kewalo Basin is home to a small fishing fleet and tourist-cruise boats. The major fresh-water runoff into this harbor is from urban storm sewers. Multiple international commercial shipping companies are based in Honolulu Harbor, in addition to a fleet of local fishing boats and pleasure cruise vessels. Fresh-water inputs include several streams and urban storm-sewer drainage. Pearl Harbor is a major naval base, where ships returning from ports throughout the world are moored for various periods of time. It receives fresh-water from at least 10 streams, mostly draining large agricultural areas.

Biofilms were collected on glass microscope slides at one inner and one outer harbor site near the fresh and salt water inputs of each harbor, respectively. Sterile slides were submerged near harbor walls or pilings approximately 1 m below the water surface for 1 week. Six replicate microscope slides were spaced approximately 10 cm apart and individually suspended in the water-column using plastic cable ties, monofilament fishing line, and a plastic frame. Slides were submerged and biofilms collected over the same period of time to reduce temporal variance between sites. Upon removal from the harbor, three replicate slides were placed into individual sterile plastic bags, 1 ml of autoclaved 0.22 μm filtered seawater (FSW) was added to each bag, and biofilms were manually dislodged from the slides by rubbing the exterior of the bag. Three replicate samples of biofilms (38 cm^2 areas per replicate) were collected from the hulls of each of three ships in each harbor either directly after the boats were hauled out of the water for routine maintenance, or by scraping the submerged hull with a 3.8 cm wide plastic edge and collecting the loose biofilm with a siphon. Three replicate 100–200 ml water-column samples were collected in sterile plastic bags from the ambient water near the biofilms at each location. Planktonic bacteria were concentrated into 1 ml FSW by passing the water sample through a 0.22 μm pore-size filter. Half of the liquid was analyzed for viable cell abundances by plating dilutions on selective medium while the other half was used for real-time Polymerase Chain Reaction (PCR)–Denaturing Gradient Gel Electrophoresis (DGGE) analysis. Total genomic DNA was extracted from biofilm and water samples and eluted in 50 μl nuclease-free water using a Microbial DNA Extraction Kit (MoBio) according to the manufacturer's instructions.

Quantification of bacteria in biofilm and water-column samples

To quantify viable bacterial cells of enteric-like and *Vibrio*-like species from biofilm and water-column samples, a selective media plate-count method was employed. Serial dilutions of bacteria from three replicate samples collected from known amounts of surface area or water volume from each sampling location (as described above) were plated onto MacConkey Sorbitol Agar (Remel) and Thiosulphate Citrate Bile Salt Sucrose (TCBS) Agar (Difco) to determine viable cell abundances of enteric-like and *Vibrio*-like species, respectively. Isolates were tested by PCR for species identification and the possession of pathogenicity genes by growing each strain overnight in Luria–Bertani (LB) broth, and extracting DNA from 1 ml of culture using a DNeasy Blood and Tissue Kit (Qiagen). PCR was then performed, as described previously, with primers specific for the detection of *V. cholerae* Intergenic Spacer Regions (ISR) (Chun et al. 1999), *ompW* (Nandi et al. 2000), *ctxA, ctxB* and *tcpA* (Vital Brazil et al. 2002) genes and *E. coli sfmD* (Kaclikova et al. 2005), 16S rRNA (Sabat et al. 2000) and LTI and LTII heat labile enterotoxin (Kong et al. 1995) genes.

Real-time PCR using a LightCycler system (Roche), and TaqMan probes (Integrated DNA Technologies) were used to detect and quantify *E. coli* (pathogenic and non-pathogenic) and *V. cholerae* (O1, non-O1, O139, and non-O139 strains). A TaqMan probe specific to the *sfmD* gene, encoding a putative outer membrane export usher protein (Kaclikova et al. 2005), was used to detect *E. coli*. Quantification of *V. cholerae* was conducted using a TaqMan probe specific to the *hlyA* gene, encoding a non-classical hemolysin gene (Lyon 2001). *Vibrio mimicus* ATCC 33653, possessing the closest known match to the *V. cholerae hlyA* homolog, *vmhA* (Kim et al. 1997), was used as a negative control in all *V. cholerae* detection experiments.

Standard curves for real-time PCR were established by first extracting DNA from three replicate samples of 1 ml of overnight grown culture of *E. coli* strain CC118 and *V. cholerae* strain N16961 grown in LB broth using a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturers' instructions. DNA concentration was measured using a SpectraMax spectrophotometer (Molecular Devices). The number

of genome copies per nanogram DNA was determined by calculating the molecular weight of one genome of each species, given that 660 g mol^{-1} bp^{-1} (1.096×10^{-21} g bp^{-1}) and the size of each genome is 4835 kb for *E. coli* (Daniels 1990) and 4033 kb for *V. cholerae* (Heidelberg et al. 2000). One nanogram of DNA is therefore equivalent to 1.89×10^5 and 2.26×10^5 genomes of *E. coli* and *V. cholerae*, respectively (Hein et al. 2001). Real-time PCR reactions were carried out as described previously (Lyon 2001; Kaclikova et al. 2005) except High-fidelity PCR Master (Roche) was used for PCR reactions. Three replicates of seven 10-fold dilutions were used to create the standard curves, which showed a linear range of detection of 5.18 to 5.18×10^6 genome copies (Average C_T of 36.27 to 17.97, respectively) for *E. coli* ($r^2 = 0.99$) and 4.64×10 to 4.64×10^7 genome copies (Average C_T of 37.65 to 16.26, respectively) for *V. cholerae* ($r^2 = 0.99$).

DNA from three replicate biofilm samples from glass microscope slides or ships' hulls from each location and three replicate water column samples from each location were assayed with real-time PCR for the detection of *E. coli* and *V. cholerae*. Genome number per biofilm area was determined by calculating the total genome copies per DNA prep and dividing by the area occupied by the biofilm on each slide (1.875 cm^2) or ship-hull surface (38 cm^2 assayed per replicate). Genome number per volume water column sample was determined by calculating the total genome copies per DNA preparation of each water-column sample and dividing by the total volume of water assayed (100–200 ml). LightCycler Software (Version 3.5) was used to calculate C_T values and apply standard curves to determine the genome number per experimental sample.

Laser Scanning Confocal Microscopy (LSCM)

Four representative 1 week old biofilms formed on microscope slides at Kewalo Basin were collected and fixed in 3.7% paraformaldehyde in seawater. Biofilms were stained overnight at 4°C with propidium iodide (5 μg ml^{-1}). Five image stacks were acquired from each slide using a Zeiss 510 Laser-Scanning Confocal Microscope (LSCM) and a 40 × objective. Three-dimensional images of biofilms were reconstructed using Imaris software (Bitplane).

Biofilm and water-column community analysis

PCR–DGGE analysis, initially described by Muyzer et al. (1993), was conducted as previously described (Shikuma and Hadfield 2006), except domain-bacteria-specific forward primer PRBA338f (5′-ACTCCTAC GGGAGGCAGCAG-3′) and conserved reverse primer PRUN518r with a 40 bp GC clamp (italicized) 5′-*CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC ACGGGGGG*ATTACCGCGGCTGCTGG-3′) (Ovreas et al. 1997) were used to amplify a roughly 238 bp fragment of the 16S ribosomal gene of bacterial biofilm and water column communities and analyzed using DGGE. Three replicate DGGE analyses were performed with separate biological replicates to analyze community fingerprints.

A Non-metric Multi-Dimensional Scaling (NMDS) analysis was performed on the resulting bacterial community fingerprints as described by van Hannen et al. (1999). Briefly, the presence or absence of bands appearing in adjacent lanes, which have run similar distances down the gel in different community fingerprints, were recorded creating a binary matrix. The binary matrix was entered into the statistical program SPSS (Student Version 10.0), and a NMDS analysis was performed on the resulting distance matrix, using a stress value of <0.1.

Results

Quantification of E. coli *and* V. cholerae *within marine biofilms*

The hypothesis was that biofilms in tropical marine harbors are a reservoir for *E. coli* and *V. cholerae*. To determine whether *E. coli* resides within marine biofilms, real-time PCR and primers specific to the *sfmD* gene of *E. coli* were employed. *E. coli* occurred in relatively high abundance within biofilms from all harbors sampled (Figure 1A). The mean biofilm abundances of *E. coli* ranged from 6.43 (SD, 8.99) to 1.25×10^5 (SD, 1.28×10^5) genome copies cm^{-2} while abundances in the water-column ranged from 2.3×10^{-1} (SD, 2.0×10^{-1}) to 1.4×10^4 (SD, 1.8×10^4) genome copies ml^{-1}. In most cases, mean *E. coli* abundance estimates using real-time PCR were greater than total enteric-like bacterial counts using plate-count methods (Figure 1B).

Because it was found that biofilms formed on glass microscope slides contained considerable abundances of *E. coli*, a further question asked was whether biofilms formed on the hulls of marine ships could be reservoirs for this microbial species. Indeed, *E. coli* also occurred in high abundances (between 2.52×10^3 SD, 2.02×10^3 to 9.61×10^2 SD, 8.90×10^2 genome copies cm^{-2}) within biofilms on the hulls of ships from each harbor (Figure 2). These results suggest that biofilms that formed both on glass microscope slides and on the hulls of ships are reservoirs for *E. coli*.

The facultative human pathogen *V. cholerae* is well known to form biofilms, which are thought to facilitate environmental survival (Faruque et al. 1998). It was therefore asked whether *V. cholerae* occurred within biofilms in marine harbors. *V. cholerae* was detected in

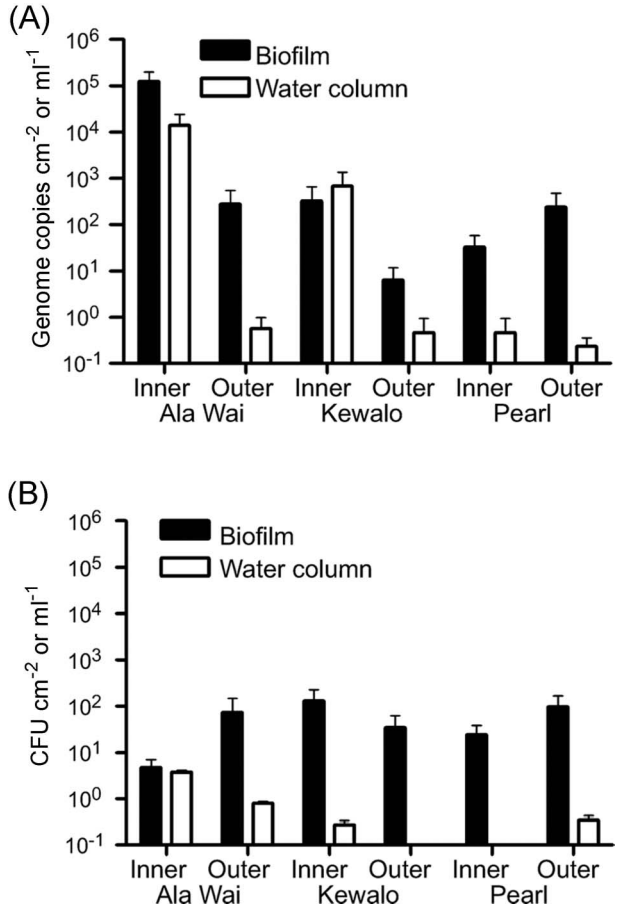


Figure 1. Mean *E. coli* abundances quantified by real-time PCR and plate-count methods. Genome copies of *E. coli* and CFU of enteric-like bacteria were enumerated using real-time PCR (A), and MacConkey Sorbitol agar (B), respectively. Abundances were enumerated using real-time PCR used a TaqMan probe specific to the *sfmD* gene. Samples were collected from two sites in each harbor. Bars indicate mean genome copies cm^{-2} or ml^{-1} or CFU cm^{-2} or ml^{-1} of biofilm and water column samples, and error bars indicate SDs ($n = 3$).

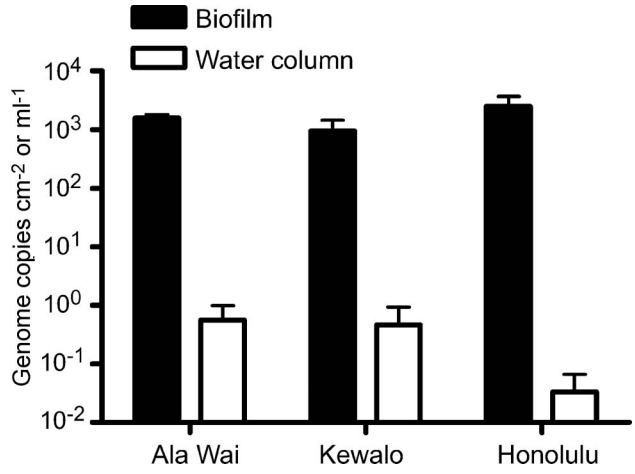


Figure 2. Comparison of *E. coli* abundances between biofilms from the hulls of ships and water-column samples collected nearby. Samples were collected from the hulls of three ships in each of the following harbors: Ala Wai Yacht Harbor, Kewalo Basin, and Honolulu Harbor in Honolulu, Hawai'i ($n = 3$ per ship). Abundances were enumerated using real-time PCR and a TaqMan probe specific to the *sfmD* gene. Bars indicate mean genome copies cm^{-2} or ml^{-1} of biofilm or water column samples, respectively, and error bars indicate SDs ($n = 3$).

biofilms in both the Ala Wai Yacht Harbor and Kewalo Basin. Quantification, using real-time PCR, revealed a high abundance of *V. cholerae* in biofilms formed on microscope slides from the inner site of Ala Wai Yacht Harbor (3.15×10^3 SD, 1.53×10^3 genome copies cm^{-2}), while abundances in water-column samples were less from the same location (1.56×10^1 SD, 4.61 genome copies ml^{-1}). At the same location, abundances of viable *Vibrio*-like species, enumerated using TCBS Agar, reached 3.44×10^3 Colony Forming Units (CFU) cm^{-2} (SD, 1.01×10^3) in biofilms compared to 5.20×10^1 CFU ml^{-1} (SD, 3.70) in water-column samples. *V. cholerae* was also detected in the biofilm of one replicate ($n = 3$) on one ship ($n = 3$) in both the Ala Wai Harbor and Kewalo Basin using real-time PCR, while it was undetected in water-column samples collected nearby.

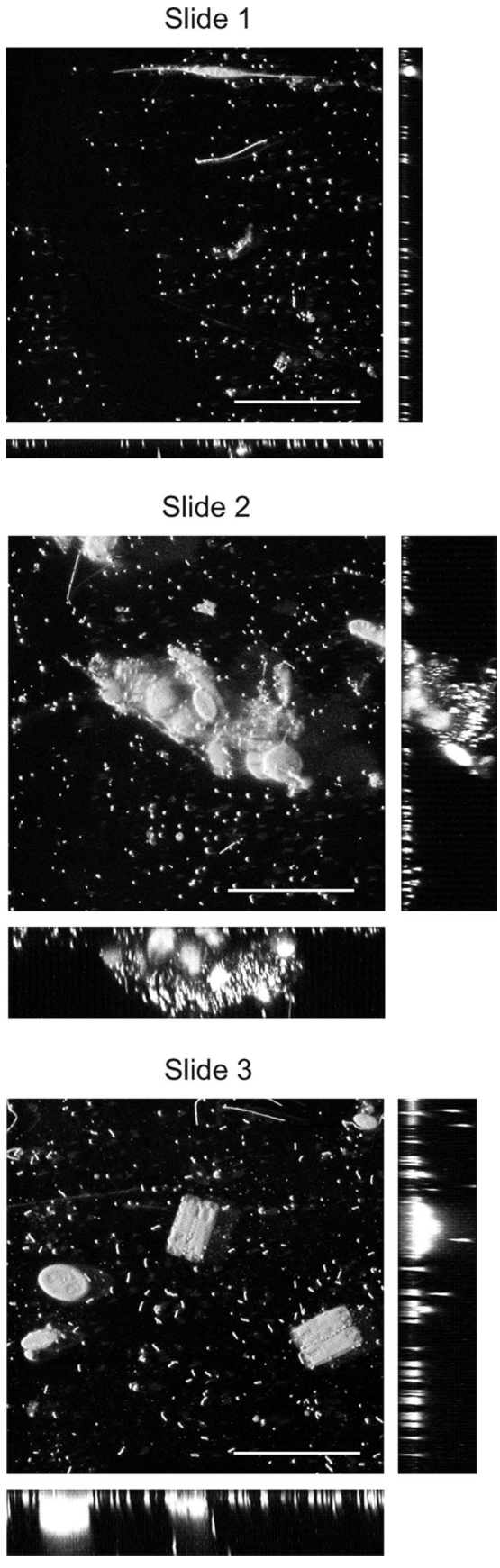
Isolates of *E. coli* and *V. cholerae* from marine biofilms were further characterized by species confirmation and detection of genes associated with pathogenicity. From the 1-week old biofilm samples, 20 isolates were selected from each type of selective media, 4 from each inner-harbor site, and 4 each from the outer-harbor sites of the Ala Wai Yacht Harbor and Kewalo Basin. Species identity was confirmed with PCR and primers specific to the 16S rRNA and *sfmD* genes of *E. coli* and intergenic spacer regions (ISR) and *ompW* gene of *V. cholerae*. Sixteen of 20 enteric-like isolates were confirmed to be *E. coli*, where 2 *E. coli*-negative isolates were isolated from the outer-site of the Ala Wai Yacht Harbor, and 2 from the inner-site of Pearl Harbor. One isolate from the inner-site of the Ala Wai Yacht Harbor was found to be enteropathogenic, harboring a type II heat-labile toxin gene (LTII). Two of 20 *Vibrio*-like isolates isolated from the inner-site of the Ala Wai Yacht Harbor were confirmed to be *V. cholerae*, however, neither harbored genes encoding for the Toxin-Coregulated Pilus (TCP) (*tcpA*) or cholera toxin subunit A or B (*ctxA* and *ctxB*).

Biofilm complexity and community fingerprinting

To visualize the complexity of biofilms harboring these microbial pathogens, representative biofilms on

microscope slides submerged for 1 week at Kewalo Basin were visualized using LSCM. Biofilm thickness appeared heterogeneous between replicate slides and between different points on the same slide (representative images are shown in Figure 3). Single cells, cell aggregates, and diatom-like structures are apparent in each image.

Once abundances of *E. coli* and *V. cholerae* were quantified from different harbors, the question was asked whether the bacterial community 'fingerprints' of biofilm and water-column communities were different between harbors. The hypothesis was that microbial community compositions from different harbors would be different, since each harbor is subject to differences in both coastal runoff and boat traffic. To analyze bacterial community fingerprints from each harbor site, PCR–DGGE was employed. DNA extracted from a portion of the biofilm and water-column samples used in plate-count and real-time PCR analyses above were used for DGGE analysis. Multiple bands were found to be exclusive to either biofilm or water-column communities (Figure 4A). The DGGE gels were analyzed using a NMDS analysis and depicted using a distance matrix where differences between communities are graphically represented by distances between points (van Hannen et al. 1999). NMDS indicated that biofilm communities were more similar to other biofilm communities from different harbors than to water-column communities from the same harbor (Figure 4B). Moreover, biofilm communities from two sites in the same harbor were more similar to each other than to biofilm communities from different harbors. Some bands appeared in both biofilm and water-column samples, while many bands appeared exclusively in one or the other. These results suggest that some members of the bacterial composition might differ between biofilm and water-column communities.



Discussion

Data obtained in this investigation reveal that relatively high abundances of *E. coli* and, in some cases, *V. cholerae*, occurred in biofilms adherent to abiotic surfaces in Hawai'i's sub-tropical marine harbors, including the hulls of ships. These results suggest that biofilms on abiotic surfaces may be an overlooked reservoir for pathogenic bacteria. Furthermore, biofilms might aid in the transport and introduction of non-native bacterial species *via* the hulls of ships.

Figure 3. LSCM images of biofilms produced on microscope slides at Kewalo Basin after submersion for 1 week. Each image is a representative biofilm formed from replicate slides 1–3. Images represent top-down (*xy*) and side-view (*xz* and *yz*) projections of each biofilm. Scale bars represent 50 μm.

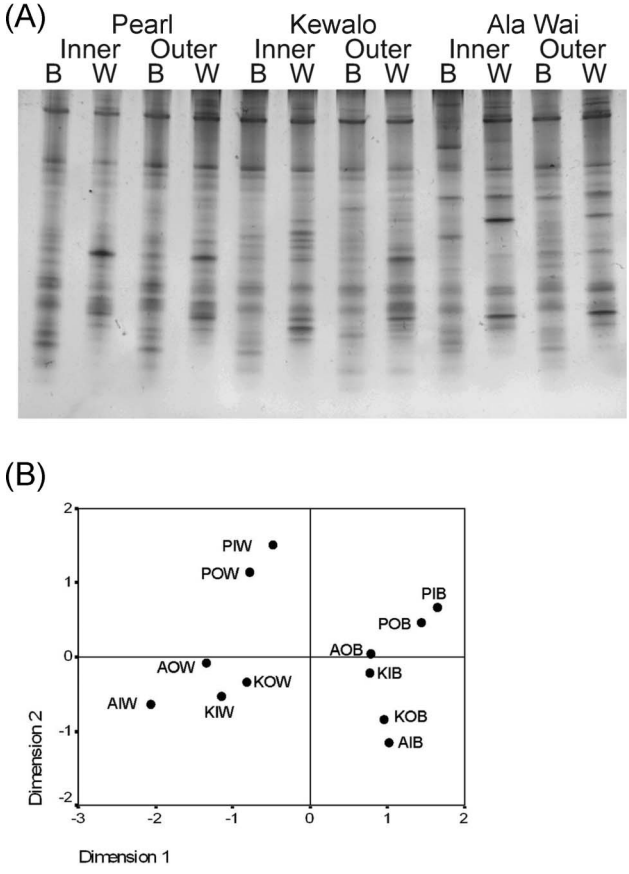


Figure 4. Bacterial community fingerprints from biofilm and water-column samples. (A) DGGE gel comparing bacterial biofilm (B) and water-column (W) communities from Ala Wai Yacht Harbor, Kewalo Basin, and Pearl Harbor. Samples were collected from two sites in each harbor, one inner site (Inner) near the freshwater inputs and one outer site (Outer), near the more saline harbor mouth ($n = 3$ biofilm and water-column samples at each location). One representative DGGE analysis of three biological replicates is shown. (B) Distance matrix analyzed with a NMDS analysis representing microbial community fingerprints of the DGGE gel. Relative distances between points represent similarities between community fingerprints.

It is shown that *E. coli* was abundant in tropical marine biofilms using real-time PCR methods. In most cases, bacterial counts quantified by real-time PCR were greater than counts quantified by colonies grown on selective media. These results are not surprising, because real-time PCR methods may include dead or Viable But Non-Culturable cells (VBNC) (Roszak and Colwell 1987) that were not detected by traditional plate-count methods, especially within biofilms where cells might be unable to grow on selective media but retain their pathogenic potential (Lleo et al. 2001). Marine biofilms might serve as a source for fecal indicator bacteria in marine waters. It was previously recognized that tropical soils support the growth of *E. coli*, which skewed results of water quality standards (Hardina and Fujioka 1991; Byappanahalli and Fujioka 1998). Tropical soils may be washed through storm sewers into marine harbors and serve as a source of *E. coli* in marine biofilms. *E. coli* abundance was high, in comparison to *V. cholerae*, in biofilms from all harbors sampled and these results might be because *E. coli* can proliferate in tropical soils.

The distribution of *V. cholerae* differed from that found of *E. coli*. *V. cholerae* was found in high abundance in biofilms formed on microscope slides at the inner harbor site of the Ala Wai Harbor, and detected in low abundance on ships' hulls in the Ala Wai Harbor and Kewalo Basin, but undetected elsewhere. *V. cholerae* may have established itself at the inner harbor site of the Ala Wai Harbor because of its ability to tolerate fresh water in comparison to other *Vibrio* species (Thompson et al. 2004). Long et al. (2005) showed that multiple marine bacteria, isolated from pelagic particles, were able to impede the growth of *V. cholerae* in competition assays. It is likely that *V. cholerae* faces strong competition for space on submerged surfaces in the sea but might proliferate in waters of lower salinity (Singleton et al. 1982).

In this study, *E. coli* was detected and, in some cases *V. cholerae*, on the hulls of ships. The biofilm lifestyle may protect microbial pathogens when attached to the wetted hulls of ships while in transit overseas. Advantages to the biofilm state include protection from osmotic stress, dehydration, UV radiation (Flemming 1993), and refuge from protozoan grazing (Matz et al. 2005). Both *E. coli* and *V. cholerae* are known to enter a VBNC state (Roszak and Colwell 1987) where metabolism is slowed and unfavorable conditions, such as the oligotrophic environment in the open ocean, can be weathered (Hall-Stoodley et al. 2004).

The findings reported here also raise important questions about the transport and distribution of marine biofilms on ships' hulls when the ships are underway. Stoodley et al. (2001) described the detachment and dissemination of discrete cell clusters from mixed species biofilms in culture flow cells and noted that detaching biomasses range from single cells to aggregates with diameters up to 500 μm. While hull fouling has long been recognized as a major avenue of introductions of both plants and animals (see review by Piola et al. 2009), the importance of ballast water in this regard has only been recognized in the last 20 years (Carlton 1987). In the last 12 years, ballast water has also been implicated as a vector for the transport of plant and animal pathogens including virulent strains of *V. cholerae* (McCarthy and Khambaty 1994; Ruiz et al. 2000). Although commercial ships may treat or exchange ballast water in the open ocean before

arriving at port (Wonham et al. 2001), biofilms in sea chests and on the hulls of ships can harbor and transport microbes internationally. Many seafaring vessels, such as navy ships, do not contain ballast tanks. However, these vessels may still have upwards of 10^4 m^2 of wetted surface area capable of harboring invasive bacterial species.

In the present study, bacterial community compositions formed for 1-week were observed to be considerably different between biofilm and water-column samples based on PCR–DGGE gels. Biofilm community fingerprints were more similar to biofilms from different harbors, than to water column fingerprints from the same harbor. It will be informative in later studies to determine whether biofilms older than 1 week follow a similar trend. Community fingerprints of biofilms suggest that some microbial species preferentially reside within these 1-week-old biofilms, and that *E. coli* and *V. cholerae* may be among these species based on their abundances in biofilm *vs* water column samples. The results suggest that *E. coli* and *V. cholerae* may favor the biofilm lifestyle on submerged surfaces and ships' hulls in marine harbors and the transport of microbes *via* ships' hulls might be a viable means of dissemination between international ports. Furthermore, the abundance of pathogenic bacteria may be overlooked if only water-column samples are tested.

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