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Suppressing Biodiversity in the World's Waterbodies: Ballast Biofilms are the Dental Plaque of the Oceans

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Abstract

Ships that have ballasted and de-ballasted in common world harbors, visited by numerous other ships, acquired a similar dominant population of biofilm-dwelling microbiota at their ballast tank walls and sediments. Secondary spread of the microorganisms to receiving waters has been demonstrated to occur by seeding from the biofilm to those waters and bounding surfaces, as well as by bioaerosol generation from ballast discharge plumes. This work was facilitated by installing, and subsequently analyzing, material test coupons within Ballast Organic Biofilm (BOB) samplers in the ships' ballast tanks. Supplementary data were acquired from shipboard-mounted flow cells during the voyages. Five specific "benchmark" bacterial species were common in these retained biofilms in spite of the different substratum materials of coatings, tank walls, and re-suspendable sediments.

Introduction

An effect reminiscent of the well-known strong similarities of dental plaques in the world's population, despite differences in home location, tooth repair materials, and personal habits, has been noted in ballast water biofilms—the equivalent of dental plaques of the oceans. In addition to now-required bulk ballast water exchange, this suppression of the biodiversity of the world's harbors and water bodies may still occur if there is not, also, required biofilm cleaning and conversion to recoverable biowaste at designated shore facilities. In addition, application of easy-release, corrosion-resistant tank-wall coatings can facilitate this process without the risk of toxic ingredients.

Increasing attention is finally being paid to needed research on the hygienically important bacteria that are sequestered in water biofilms [1] and how the biogeographical patterns of marine bacteriophytoplankton, especially Vibrio species, are distributed around the world [2]. An interest of particular concern is the likely suppression of worldwide diversity of this biomass, as ships acquire, carry, and distribute the biota from port to port around the globe. It is not yet known whether suppression of bacterial biodiversity is taking place in distributed worldwide ports and waterbodies, or whether newly introduced species will be harmless or pathogenic. This research focused on the dental plaque analogy of dominant resident species by examining biofilms formed on various material coupons within the ballast tanks of transglobal, multiply ballasted ships travelling from ports in Israel through the Mediterranean Sea to the United States (US) east coast, through the Panama Canal, to the USWest coast, then on to Japan, South Korea, and China in multiple round trips.

Methods and Materials

Beginning in the 1980's, Ballast Organic Biofilm (BOB) units and flow cells [3, 4] were developed and deployed in ships visiting a total at least 16 consecutive world ports in the Atlantic, Pacific, and Mediterranean Seas. The carried coupons were examined for dominant species of microbes and particles as they were built up from station-to-station and in total [5,6]. Biofilms were examined by light and scanning electron microscopy, and by fluorescence light microscopy using special stains developed for five "consensus" species that always appeared as benchmark microbes [7], and by multiple attenuated internal reflection infrared (MAIR-IR) spectroscopy [8] for qualitative and quantitative analyses of biofilm constituents. The five "benchmark" bacteria were originally identified and isolated from naturally formed biofilms on materials from the Atlantic Ocean and stored in Atlantic Ocean water aquaria [7]. The bacteria were noted to be quite cosmopolitan in regard to the volumetric habitats they normally thrived in: water (fresh and salt), food, animal sources, soil, sewage, and air. Each species was a known opportunistic human pathogen, and so presents the concern that biofilm-induced invasion by these and more serious pathogenic species might someday occur. Specifically, *Vibrio alginolyticus* has been isolated from infections of the ear (otitis externa), eye (conjunctiva), gastro-intestinal system, and wounds [9]. *Pseudomonas putrefaciens* (*Shewanella putrefaciens*) has been isolated from human skin, soft tissue, and intra-abdominal infections, and may cause sepsis, bacteremia, and meningitis. *Vibrio alginolyticus* and *Pseudomonas putrefaciens* (*Shewanella putrefaciens*), members of normal fish micro-fauna, are also bacteria that cause spoilage of harvested fish-flesh and, thereby, are microbes of health concern to the food industry [10].

Antibody Generation: With approval of the university's Animal Care and Use Committee, antibodies against the bacteria were developed by inoculations of New Zealand White Rabbits and were deliberately of the polyclonal type. Polyclonal antiserum raised against any individual antigen consists of an assortment of antibodies of a variety of classes binding to different epitopes on the antigen with a diverse range of affinities, the proportion varying from animal to animal and within the one animal from bleed to bleed [11,12]. The chances of having cross-reactions with other non-targeted bacteria that might be found in natural biofilms, using polyclonal antibodies,

are – of course -- greater than if monoclonal antibodies were utilized. In monoclonal antibody (mAb) production, the cell (B-lymphocyte) producing a single specific antibody to a specific epitope is isolated and propagated in large amounts [11,12]. Monoclonal antibodies decreased the chances of cross-reactions with non-targeted bacteria. On the other hand, the problem with monoclonal antibodies was that they may only have targeted a certain strain of a species, thereby being too selective for general surveys. The genus-specific Ab preparation resulting from polyclonal Abs was judged to be a more useful tool for the general survey studies reported here. Antigenicity or reactivity of the antibodies used was — in every case -- relatively weak, with titers ranging from 13:1 to 30:1, where antibodies with strong antigenicity would be in the range of 100's:1.

Immunofluorescence Monitoring: The examination of immunofluorescent-stained bacteria associated with biofilms on surfaces by the technique of fluorescence microscopy utilized incident lighting for observation and enumeration of the biofilm-sequestered bacteria formed on flat test coupons within the ballast tanks. The technique also allowed for detection of immuno-stained bacteria associated with biofilms on sediments and other particles re-suspended within those tanks. If only transmission lighting had been used, the opaque properties of the sediments or particles would not have uniformly allowed for detection of stained bacterial biofilms on these opaque, irregular bits of matter.

Scanning Electron Microscopy – Energy Dispersive X-ray Analysis: General scanning electron microscopic and coordinated energy-dispersive X-ray surveys at low magnifications were used to confirm silicon as the major element of the biofilms' trapped particles, mostly silica as in beach sand. Corresponding MAIR-IR spectroscopic analysis also confirmed the presence of silica as found in clay and other soil minerals of usually re-suspended harbor sediments.

MAIR-IR Spectroscopy: Sterile cotton swabs were utilized to sample the biofilms from the coupons deployed in the flow cells and BOB samplers. The material removed from each coupon was transferred to a germanium prism for analysis by MAIR-IR spectroscopy. Spectra were evaluated for the presence of carbonates and silica-based inorganic particles associated with the organic constituents of biofilms from the transglobal ships.

Light Microscopy: Normal transmission light microscopy was used to reveal larger particles associated with the biofilms and was useful in visual comparisons among different samples. Light microscopy also was useful in observing protists and occasional small crustaceans in-situ, grazing on the primary biofilms (see video at www.wings.buffalo.edu/iucb/video.html).

Results

The results were surprisingly clear and uniform. For example, Figure 1 is an infrared spectrum that is representative of the spectra obtained from all the acquired biofilms. From left to right, the major absorption bands in the spectrum indicate the presence of inorganic and organic hydroxyl groups, hydrocarbons, carbonates, sulfates and silicates. Figure 2 is a 200x scanning electron micrograph of a typical biofilm, showing the variable distributions of microbes and inorganic particles associated with all such films on all substrata that were examined. The biofilms were present on all substrata, but they were the least diverse and most easily detached from the low-energy substrata known to be associated with "easy-release" of biomass [13]. Figure 3 is a 500X fluorescence light micrograph of the typical

distribution of *Pseudomonas putrefaciens* species in one such biofilm; fluorescence micrographs of the other species are shown elsewhere [3].
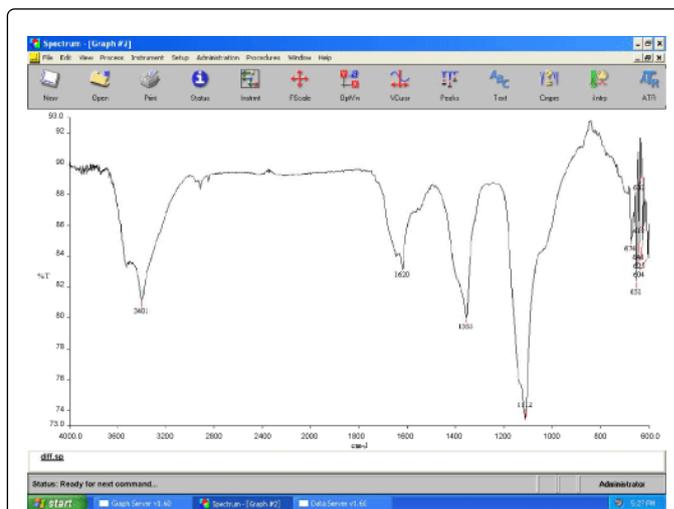

Figure 1: Typical infrared spectrum of ballast tank biofilms, removed by a cottom swab and transferred to a germanium internal reflection prism. Absorption bands shown are for the hydroxyl, carboxyl, and sulfate groups of dried seawater, along with a modest fraction of organic matter and a large fraction of siliceous sediments.
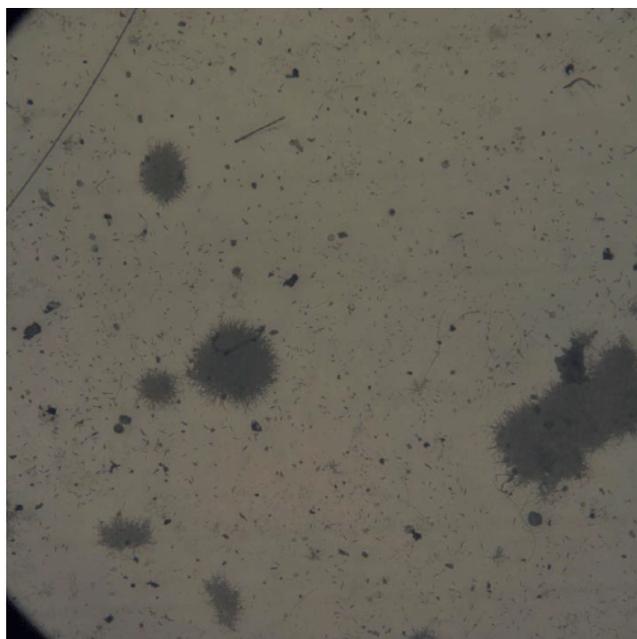

Figure 2: Biofilm from BOB sampler, revealing bacterial clusters and sediment particles of typical oceanic biofilm on test coupon (200X original magnification, SEM).
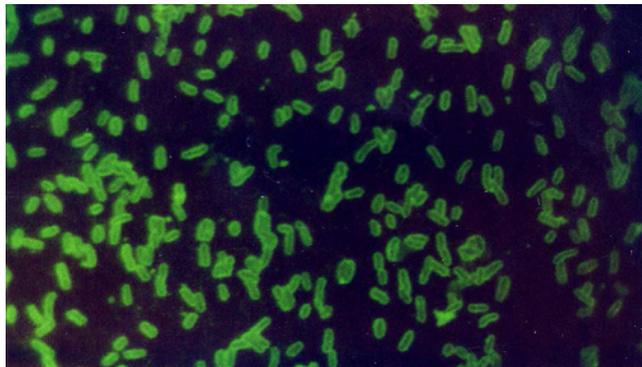
Figure 3: *Pseudomonas putrefaciens* distribution within a typical oceanic biofilm (500X original magnification, fluorescence).

All of the acquired biofilms on all of the various materials tested contained the same five characteristic organisms previously specified, but in different abundances. Light microscopic images showed that the different ships' biofilms were similar in particle loads, but different in relative composition, which was also confirmed by MAIR-IR. Statistically significant relationships have not yet been developed among the different environments so far sampled, but it has been very clear and statistically significant that retained the biofilm masses were lowest on surfaces with CrticalSurface Tension (CST) values between 20 and 30 mN/m. Many of the sedimentary and other trapped particles present within the biofilms were too small to be resolved by light microscopy. SEM images revealed many particles that were not seen by light microscopy or obviously associated with the biofilms when observed by fluorescence microscopy. The higher magnifications attained by SEM and observation of dehydrated biofilms (biofilms were "drawn-down" on particles or objects that otherwise may have been hidden in the bulk biofilms) revealed objects that could have been missed by the other techniques. Specifically, SEM images and energy-dispersive X-ray spectroscopy revealed "crater-like" features or depressions in the biofilms that were identified to be associated with FeMn spheres. The craters, some not very deep into the biofilms, indicated an arrival and departure phenomenon where FeMn spheres were in a constant state of turnover. It is possible that some spheres may have been dislodged during sample handling and preparation. The origin of these spheres was taken to be evidence of underfilm corrosion of the steel ballast tank walls.

Comparison of the voyages' settled bottom sediment compositions versus each voyage's biofilm-associated sediment compositions showed that all biofilm-associated sediment particles were nearly identical in elemental composition to their respective ballast-bottom sediment compositions. Only one sediment sample composition was different, with calcium being the element in greatest abundance. Again, statistically significant compositional relationships with harbors have not yet been developed, but as later described the FeMn particles did reveal special associations with individual ships—suggesting differential corrosion states. Results did reveal a trend indicating increased bacterial population densities occurring when high numbers of biofilm-associated particles were present. A possible scenario is that this may have occurred upon ballast exchange: the sediment particles at the bottom of the ballast tank were re-suspended into the water column of the ballast tank and a portion of these particles (with their own attached bacterial biofilms) was incorporated into the biofilms on the hard surfaces of each ballast tank. Voyages having the greatest number of ballast water exchange events had biofilms with higher populations of bacteria species and particles. Again, statistical significance could not be developed with the limited samples available.

In most cases, swab samples from high-surface-energy polystyrene coupons showed higher Si-O (silica) absorbance values in the IR spectra, indicating a surface-energy effect. High-surface-energy polystyrene (e.g. tissue culture polystyrene prepared by gas plasma treatment) is more polar, has a higher critical surface tension, and therefore is more adhesive, than the much less polar, bacterial-grade polystyrene. Hygroscopic organic carbonates and hydrated sea salt were abundant on biofilm swab samples transferred to the germanium MAIR-IR prisms. Surprisingly, proteins, lipids, carbohydrates, and other biological substances were not detected in the swab-transfers from any of the biofilms. Bacterial exudates, which one would expect to be present in the bulk of the biofilms of slime-producing bacteria, were also not readily detected, although immunofluorescence microscopy showed that the bacteria were abundantly present. From similar studies of comparable biofilms formed directly on the surfaces of germanium internal-reflection prisms, MAIR-IR spectra revealed large infrared absorptions from protein bound to prism surfaces prior to bacterial attachment [14]. It is certain that the proteinaceous films were present on the ballast biofilm coupons, but were too thin and tightly bound to be successfully transferred by swabbing.

Discussion

Under the conditions studied here, "benchmark" bacteria species were present on all sample materials/surfaces on all voyages just as, similarly, "benchmark" bacteria (of different species) are present on human teeth around the globe. Few statistical differences have yet been found among the five tested oceanic "benchmark" species populations in ballast tank biofilms, indicating relatively homogenous populations of these species relative to one another. Biofilms from all of the voyages so far sampled were in the primary state of succession, indicating an only oligotrophic environment present in ballast tanks. Ballast tank sediment particles are often re-suspended (a consequence of ballast exchange operations), and incorporated into biofilms. The particles are now noted to be predominately silica or silicate in composition (common earth minerals), and may be present in the forms of sands, silts, or clays. Some particles incorporated into biofilms may be corrosion products originating from ballast tank metal, itself. Ballast tank biofilms with their bacteria and particles (with associated bacteria), may act as "seedbeds" equaling other seeding environments or ecosystems. Biofilm communities may inhibit further colonization with a stable community in place, preventing further species colonization. Evidence of less dense biofilms observed on silanized (low CST) materials may allow for lower-dose ballast water treatment (chemical), and easier mechanical removal. "Benchmark" species absence or replacement may be useful for the risk assessment of exotic or more pathogenic species colonizing surfaces. Because of their cosmopolitan distribution, an increase in species population or the absence of species could signal that a negative ecological event has occurred.

The use of self-tending biofilm samplers clearly gave the means to acquire in-situ biofilms on materials of choice while deployed in the ballast tanks of commercial shipping vessels, revealing the following risks.

Athough *Vibrio alginolyticus* and *Pseudomonas putrefaciens* (*Shewanella putrefaciens*) generally require at least a 1% NaCl concentration to thrive, some strains of *Pseudomonas putrefaciens* have been isolated from freshwater environments [15]. *Acinetobacter* does not require as high a saline concentration to thrive and consequently is found in both freshwater and marine environments, a useful trait for a further recommended study of freshwater ballast biofilms (e.g. from Great Lakes shipping) Interestingly, *Pseudomonas putrefaciens* (*Shewanella putrefaciens*) has been observed to be associated with iron-manganese nodules [15] and so may be an indicator, as well, of ballast tank protective paint failures and subsequent corrosion. More specialized bacteria found in the marine environment or even human-associated (e.g. *Enterobacteria*) and/or more geographically restricted species might yield more information in regard to colonization of material surfaces. A geographically exclusive or human-associated species could give insights into problem areas' origination and general knowledge of survival rates of bacteria in ballast biofilms.

Samples with the largest amounts of biofilm-associated sediments pose multiple analytical problems. When an aliquot of immunostain reagent is applied to these samples, there is a tendency for the drop to spread by capillary wicking between the sediment particles and to interfere with other sample areas. Also, the aliquot absorbed into the sediment would dry more quickly than if it were placed on a less sediment-rich sample area. Large amounts of sediment in some specimens completely obscured the primary bacterial biofilm that was associated with the base material's surface and, in extreme cases, inhibited focusing. Bacteria residing in the upper levels (water side) of the biofilm-supported sediment and in voids between particles were enumerated in these cases. The biofilm-coated sediments and suspended particles, whether sparse or great in quantity, did always react with the fluorescent conjugates, at least to some degree, the result being fluorescent sediment and particles. The source of this apparent "nonspecific" fluorescent protein binding was clearly that of the sediment-associated biofilms.

Iron-manganese (FeMn) spherical particles on the surfaces of both gas-plasma- treated polystyrene and the less polar bacterial grade polystyrene were ubiquitous on samples deployed in biosamplers installed in the ballast tanks of one vessel. As already noted, observations revealed that the high-surface-energy polystyrene had greater densities of these FeMn spheres than did the less-polar polystyrene samples in each biosampler.

Another interesting observation was the similar, but smaller, densities of the retained FeMn particles enumerated from two different biosamplers on two different voyages of the vessel. Differences in "up" versus "down" sample orientations also were noted. One biosampler tended to reveal greater densities of the FeMn particles than the other samplers; a result interpreted as an additive effect because that sampler was carried on two cruises of the same vessel. One of the major elements, second to iron, in carbon steel is manganese [16]. The origination of FeMn particles was taken to be corrosion products from the inner walls of the ballast tanks that settled or "seeded" (and were retained) on the sample surfaces. Densities of FeMn particles on one around-the-world voyage approximated the same density of FeMn particles found on the sum of all other voyages. Also, the densities of the FeMn particles approximated the same retention densities of many of the bacterial species that were enumerated by the immunofluorescence staining technique,

suggesting that the corrosion particles were also capable "seeding" biofilms on the hard surfaces of the ballast tank.

The voyage histories of some vessels indicated identical travel routes; yet the surface/biofilms'-associated particles were different. Ballast exchange in different ports, estuaries, or on more turbulent seas could possibly explain the different particle loadings in each vessels' ballast tanks, but it is more likely that their states of biocorrosion were different. Information provided by the ZIM Israel Shipping Co. via research collaborators (personal communication from Dr. Bella Galil) revealed the ships' ballast holds (where biosamplers were deployed) were coated with the same material – "Tamarin, a solventless modified epoxy in a 350 μm layer". One ballast hold, however, was coated approximately one year prior to the other's ballast hold. Possibly, this age disparity (one year extra of environmental exposure) degraded the coating enough to allow the ballast water to react more with the carbon-steel. First-hand evidence of the corrosive nature of ballast water was observed while obtaining ballast water samples from a ship in port on Chesapeake Bay. When a steel nut was used as a weight to submerge sampling vials into the ballast tank water, green corrosion was observed within minutes of use indicating a probable electro-chemical reaction (the vessel was equipped with zinc anodes for cathodic protection). These findings indicate that current ballast tank wall coatings are not sufficiently able to resist cracking and underfilm corrosion, and are probably not easily releasing attached biofilms to the ballast exchange process.

Conclusions

Ocean water ballast tank biofilms are considerably less diverse than their origins in different world ports might suggest, based on acquired data from surface-characterized test coupons of varying surface energies placed inside Ballast Organic Biosamplers and carried around the world.

Recognizing that adjacent ships in port are often simultaneously discharging or taking on ballast water, it is not unthinkable that the organisms preferring a sessile state will come to dominant different ships' equivalents of dental plaque, usually quite common in bacterial species for people over the globe.

The work done so far has demonstrated that there is a parallelism between the common contents of bacterial biofilms (plaques) otherwise given as many as 16 opportunities for reflecting a port's diversity of species. The concern is, valuing world-wide population diversities, that such diversity may be suppressed. Conversely, the worry is that a pathogenic or invasive organism may come to dominate ships' biofilms and thus be inadvertently dispersed to new world ports. It remains for future work to determine if the diversity of all benign oceanic biofilms will match the known-to-be-limited diversity of the healthy oral flora, and if similar risks to health are associated with dominance of invasive species, as in periodontal disease.

Recommendations for Future Work

Another approach to enumerating bacteria for the study of biodiversity is the technique known as Fluorescence In Situ Hybridization (FISH) with rRNA oligonucleotide probes. In recent years, molecular methods based on the comparative analysis of 16s rRNA sequences have yielded new insights into the diversity of marine and freshwater bacterioplankton communities [17,18]. One great advantage of this method is the lack of need for culturing and isolation

of the targeted bacteria species, thereby presenting a more accurate biodiversity picture with respect to the biofilm community, (non-culturable species could be represented). Testing the effectiveness of easy-release (CST= 20 – 30 mN/m), corrosion-resistant coatings applied to the inner surfaces of ballast tanks and the possible benefits these coatings offer to easier sediment/biofilm removal and chemical treatment is another area of research consideration [13,19]. One must remain vigilant, however, in preventing premature release that could enter ballast tank discharge plumes [20]. Cleaning and conversion to useful byproducts at central stations would be preferred [21, 22].

Acknowledgment

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