FINAL REPORT

Molecular, Fluorometric and Confocal Microscopy Analysis of Microbial Community Composition and Structure in Sites Around Oahu

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Problem and Research Objectives

Biofilms are generally described as surface-associated bacterial communities surrounded by extracellular polymeric substances (EPS) [1] in aquatic or marine environments [2]. Most marine biofilms are composed of several different species that are probably arranged within the biofilm structure. It is generally known that EPS plays an important role in the adhesion of bacteria to surfaces and acts as "glue" and functions at cellular and intercellular levels [3,4]. Thus, EPS provide a nutrient-rich environment where bacteria can grow in complex biofilm communities. The diversity of marine microorganisms plays key roles in marine food webs and the cycling of nutrients. Marine biofilms have been shown to be associated with the settlement and marine sessile organisms such as invertebrates and algae [5].

Submerged surfaces are quickly covered by glycoconjugates and by microorganisms such as bacteria, diatoms and protozoa that form a biofilm [6]. The microbial communities in marine biofilms can have a strong influence on diatoms, algae and benthic invertebrates [7]. Indeed, marine bacteria can have stimulatory or inhibitory effects on the marine algal growth through the production of substances or lack of nutrients [8].

PCR-based molecular genetic techniques are very useful in studying microbial diversity, and DGGE, which separates PCR-amplified community 16S rDNA sequences. DNA bands from DGGE gels can be excised, and sequenced to identify community members. Thus, DGGE can be used to describe overall microbial diversity to identify individual community members from biofilm [9]. However, studies of the structure of biofilms require the intact biofilm remain on the surface. The biofilm structure can be studied three-dimensionally by Confocal Laser Scanning Microscopy (CLSM). This technique allows optical sectioning of the intact and undisturbed biofilm, and CLSM has been used in several studies for characterization of biofilm components and structure [10].

WRRC-sponsored research in 2012–2013 has allowed implementation of denaturing gradient gel electrophoresis (DGGE) to characterize bacteria in biofilms, with microscopic and fluorometric analyses. Biofilms are easily collected following immersion of substrates in shallow water sites; collection method in this study is easily modified to include deeper sites. Current efforts compare bacteria at shallow water eutrophic and oligotrophic field sites on Oahu via DGGE analysis, DNA sequence analysis, multidimensional scaling (MDS) and confocal microscopy characterizations to identify specific bacteria (e.g., fecal associated *Enterococcus* species and *Staphylococcus aureus*).

Applying methods in this study could allow to assess the microbial community associated with outfalls or sites with sewage spills on Oahu [11]. In an assessment of the microbial activity at the outfall, Novitsky and Karl [12] conclude "Although the activity of the microbial

populations does not seem to have been affected drastically by the input of sewage effluent, we have no indication of its effect on the microbial community structure. The fate of the large microbial community on the effluent itself is unknown." This is a serious short-coming, as a shift in the microbial community structure can be pathological and has serious ecological implications [13], especially in regions where ocean activities are so popular.

The objective of this research was to examine biofilm communities from select sites on Oahu, including sites which have potentially elevated nutrients or impaired water inputs (e.g., Ala Wai Canal) and invasive alien algae (Waikiki and Maunalua Bay), as well as reference sites, to characterize if and how quickly microbial communities shift from impacted to healthy states. Study of microbial community composition within the biofilm can directly aid in the understanding of the health of our coastal marine ecosystem. Research in coastal regions complements those of CMORE with their more open ocean research themes (www.hawaii.edu/cmore).

Methodology

Study Sites. The experimental arrays on Oahu were deployed in a less-developed Hawaii Kai site (pristine site), at Waikiki alongside the Natatorium—a site dominated by invasive weed *Gracilaria salicornia*, at the McCully bridge crossing the Ala Wai, and at Hawaii Kai reef which is impacted with *Avrainvillea amadelpha*. The arrays of three replicate microscope slides (10 cm x 4 cm) were vertically positioned and suspended at 1 m depth.

Sampling. Biofilm samples on each array were scraped by a sterile razor blade after 7 days immersion and kept in a 2 ml microfuge tube. Samples were stored in a -80°C freezer for future analysis. For image analysis, a glass slide was kept in a coplin jar with seawater collected from each study site, and observed or preserved in 2% formalin for microscopy.

Genomic DNA Extraction and PCR Amplification. Total genomic DNA were extracted from the scraped biofilm samples using a Powerbiofilm DNA kit (MB BIO Laboratories, Inc.). The extracted DNA (dissolved in 10 mM Tris–HCl, 1 mM EDTA, pH 7.5) were stored at -80°C until further analysis. PCR mixtures (50 µl total volume) contained 200 µM deoxynucleoside triphosphates (dNTPs), 1 µM of each primer, 0.05 unit/µl Taq DNA Polymerase, and 100 ng genomic DNA as template (negative controls with water). Partial 16S rDNA gene amplifications for DGGE analysis were performed using the universal primer sets for bacteria [14, 15]. The PCR program included an initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 57°C for 1 min, and extension at 72°C for 1 min, followed by 25 cycles. The PCR

products were visually analyzed by electrophoresis on 2% agarose gels (w/v) and run in 1 x TAE buffer stained with ethidium bromide (0.5 mg/l).

DGGE Analysis. DGGE analysis was performed with a DGGE-2000 system (C.B.S. Scientific Co.). The condition of DGGE was performed with 0.75-mm-thick 6% polyacrylamide gels (ratio of acrylamide to bis-acrylamide, 37.5:1) submerged in 0.5 x TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA; pH 7.4) at 60°C. The PCR samples were mixed with 6 µl of dye solution (0.1% bromphenol blue [w/v], 70% glycerol [v/v]) and applied to the gels. Electrophoresis was performed during 12 h at 30 V in a linear 20 to 60% denaturant agent gradient. That 100% denaturant agent was defined as 7 M urea with 40% formamide. The gels stained for 30 min in 1 x TAE buffer with SybrGold nucleic acid stain (Molecular Probes) and visualized with UV radiation used by Bio Photometer (Eppendorf) and Kodak Scientific Imaging Systems. The distinct and dominant 16S rDNA bands from the results of DGGE were excised from multiple lanes of the gels by a sterilized razor blade. Excised DNA bands were re-suspended in 0.5 ml of MilliQ water, and stored at 4°C overnight [16]. Re-suspended DNA re-amplified PCR reaction and duplicate amplifications of DGGE for DNA sequence analysis is described above.

Nucleotide Sequencing. All re-amplification products of the DGGE bands were sequenced directly in both directions using the respective amplification primers and the ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, USA). Sequencing reactions were run on a ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

Phylogenetic Analysis. The partial 16S rDNA sequences obtained from DGGE analysis were checked for chimeras with the program CHECK_CHIMERA of the Ribosomal Database Proposal and added to the MEGA-3 software program. Each set of sequences was grouped into phylotypes, (operational taxonomic units [OTUs]), based on a > 97% identity cutoff. The closest phylogenetic relatives of each phylotype were identified by comparison to the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) analysis tools (www.ncbi.nlm.nih.gov/BLAST/).

Sequence analysis of cultured biofilm bacteria. Bacteria from the Ala Wai biofilm samples were inoculated on Tryptic Soy Agar (TSA) media and grown at room temperature for 48 h. DNA was extracted from isolated colonies and amplified 16s rDNA as in 'genomic DNA extraction and PCR amplification' protocol. Amplified 16s rDNA samples were identified by nucleotide sequencing and compared to the NCBI GenBank database using the BLAST analysis tools.

Pulse Amplitude Modulated Fluorometry (PAM). PAM (using the Junior PAM device, Waltz, Germany) was used to assess the photosynthetic characteristics of autotrophic organisms

attached to glass slides at the four research study sites. We examined if and to what extent the study site were changing the microbial community by measuring the differences in photosynthesis. PAMs rapid light curves (RLC) were performed to determine the maximum electron transport rate (ETRmax) of photosystem II (PS II) of the sampled organisms [17]. All samples were continuously immersed in seawater after collection. RLCs were performed in shade immediately after collection. For each of the four study sites, three replicate RLCs were performed on each of three randomly selected slides (9 RLCs/site). The mean ETRmax for each site was calculated.

Microscopy. Biofilms were visualized with light and CLSM. The data presented here focused on biofilms from the Ala Wai site that were grown on glass slides in the same manner as for DGGE analysis. To support this work we inoculated biofilm scrapings onto microbial media including TSA, EMB, and/or MSA; incubated for 48 h at room temperature; and isolated colonies for study. Gram and/or capsule stains were carried out directly after isolating the colonies.

To visualize the cells and EPS/biofilm matrix on the glass slides, we stabilized glycoconjugates and stained biofilms with fluorescently-labeled lectins after the methods of Michael and Smith 1995 [18]. Lectins were chosen for their affinities in complementary binding with mannose/glucose moieties (Concavalan A [Con A]) or N-acetylglucosamine and sialic acid residues (Wheat Germ Agglutinin [WGA]). Commercially prepared lectins with fluorescein isothiocyanate (FITC) were obtained from Vectra Laboratories (Vectra Laboratories Inc., Burlingame, CA, USA). The FITC-labeled lectins were dissolved in a stock solution containing 10 mM HEPES, 0.15 M NaCl, pH 7.5, 0.1 mM Ca++, and 0.08% sodium azide (0.01 mM Mn++ for Con A). Microbes were visualized with the use of 4',6-diamidino-2-phenylindole (DAPI) to localize DNA. Labeled biofilms were examined with an Olympus FV1000 Laser Scanning Confocal Microscope with digital image capture. Exciting lasers were 488 nM for FITC channel, 543 nM for red autofluorescence (Rhodamine channel), and 405 nM for the DAPI channel (Blue Diode).

Water Quality Analysis. Water samples from each site were collected in a 50 ml test tube by 0.2 μm filtration. The samples were analyzed using an Exeter Elemental Analyzer for nitrogen and sulfur analysis (CNS), via the new analytical laboratory in SOEST.

Principal Findings and Significance

To analyze changes of marine microbial communities on the effect of bacteria at impacted urban reefs compared to a pristine site (Hawaii Kai), the DGGE fingerprint technique was

applied using the universal primer sets (GM5F-GC clamp 341F and 907R). This was done in order to obtain a partial 16S rDNA gene, to analyze the 16S rDNA sequence from the pristine site, and finally to compare that community with suspected impacted sites (Waikiki, Ala Wai Canal, and Hawaii Kai in the *Avrainvillea amadelpha* meadow) on Oahu (Figure 1).

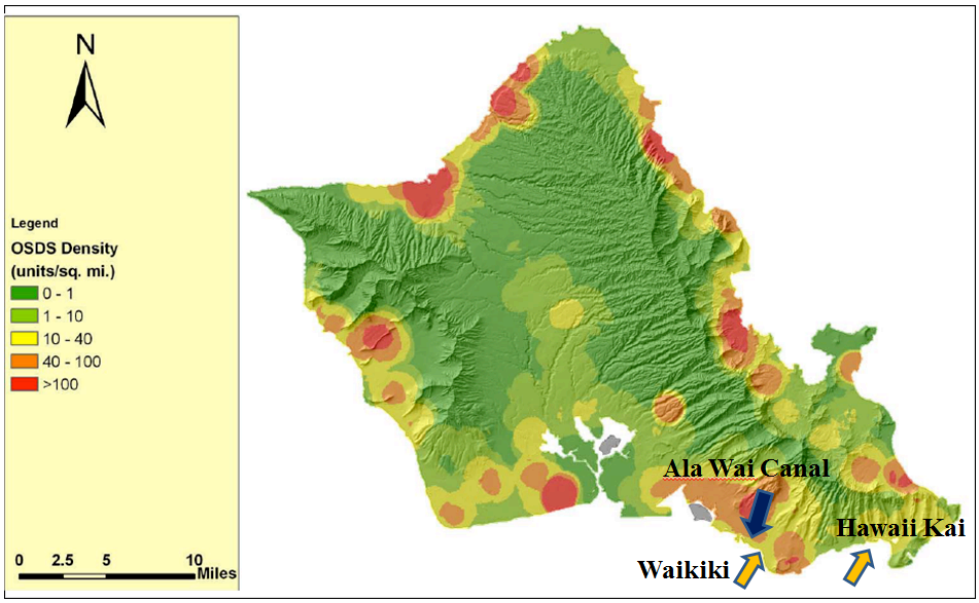

Figure 1. Study sites on Oahu based on Onsite Sewage Disposal System (OSDS) report [from 19].

DGGE results indicated that a total of 57 different 16S rDNA patterns were detected from the study sites (Figure 2). The dominant and distinct DGGE fragments were excised and identified for sequence analysis (Figure 4). To investigate the intensity matrix from the DGGE bands study sites, the present study used MDS. Different DGGE fingerprints are shown in Figure 3. Encircled areas indicate that the compositions of the microbial communities were significantly distinct at each of the study sites. The compositions of microbial communities from Hawaii Kai reef were clearly distinguished from the other study sites within 12.8% of similarity (Figure 3A and B). On the other hand, the compositions of microbial communities between Waikiki and Ala Wai Canal showed 37.7% similarity; the study areas that are geographically close share aspects of the environment that similarly influence the microbiota.

To assess differences of microbial community per study site, a pair-wise test was used for an analysis of similarities function (ANOSIM). The results showed R significance level (%) for the composition difference in microbial communities based on the different sites (Table 1). The present study interpreted that R-values > 0.75 as well distinguished among groups; R > 0.5 as overlapping, but clearly different among groups; and R < 0.25 as barely separable at all, in accordance with the Primer 6 manual [19]. According to results of our study, global R value

0.796 indicated that the compositions of microbial communities showed significantly different results based on the different sites during the experimental period. In addition, the stress values in MDS (Figure 3A) showed 0.1 that indicates an excellent representation with no prospect of misinterpretation within 0.5 levels [20]. Therefore, the results strongly indicate the compositions of microbial communities are distinct among the marine microbial community at these different study sites. While the community composition has largely unknown effects on upper trophic levels, they are likely to be very important to overall ecosystem health. Further, this tool could lead to rapid assessment of the different microbial consortia that could be found in other fine scale sampling, such as regions adjacent to outfalls or sites with chronic sewage spills as in Ala Wai Canal.

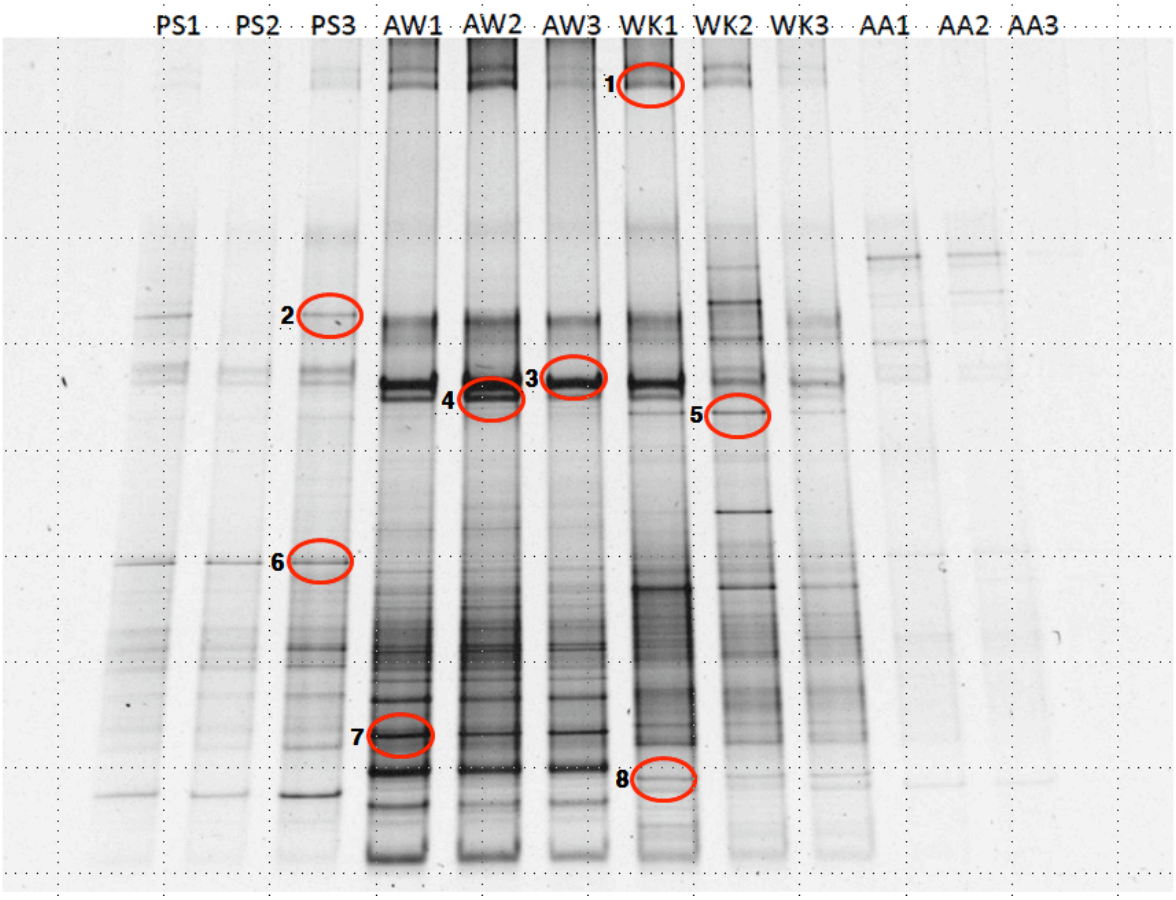

Figure 2. Images of DGGE fingerprint profiles containing 16S rDNA fragments which were amplified with universal primer sets (GM5F-GC clamp 341F and 907R) from the Hawaii Kai pristine site and the three impacted sites (Ala Wai Canal, Waikiki, and the *Avrainvillea* meadow in Hawaii Kai reef) on Oahu. Microbial samples with three replicates were scraped by a sterile razor blade after 7 days. The numbered red circles refer to the excised and identified bands for DNA sequence analysis (Figure 4).

Table 1. Pairwise test by the analysis of similarities function (ANOSIM) among groups. The sample statistic (Global R) for all pairwise data was 0.796.

Pairwise Field Sites	R Significance Level
Pristine, Ala Wai	1
Pristine, Waikiki	1
Pristine, Hawaii Kai	0.833
Ala Wai, Waikiki	1
Ala Wai, Hawaii Kai	1
Waikiki, Hawaii Kai	0.648

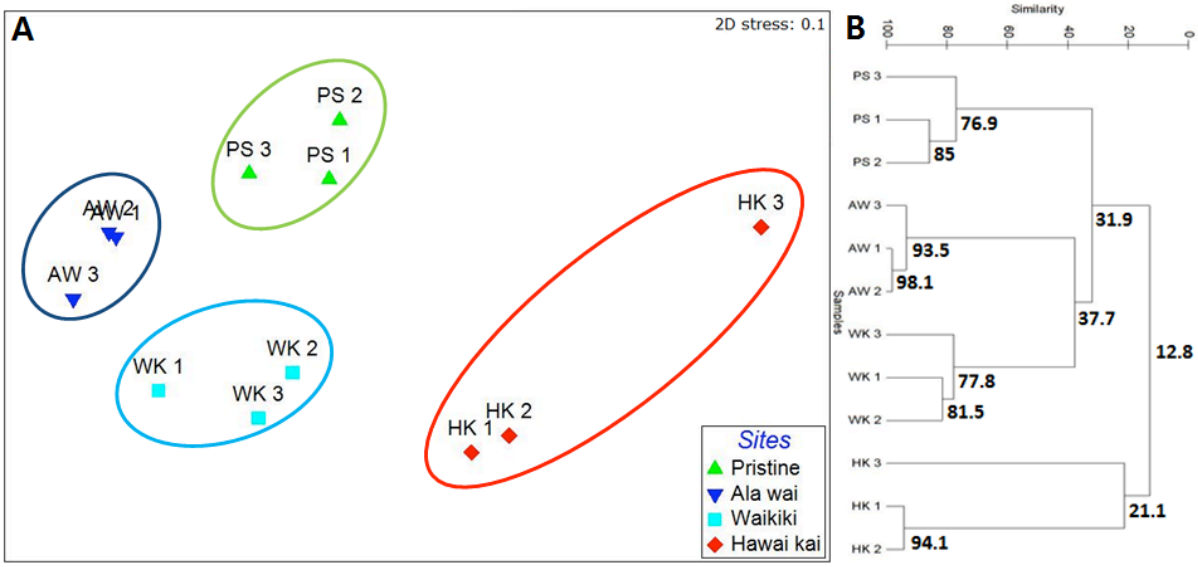

Figure 3. (A) Multidimensional scaling (MDS) diagram representing the changes in bacterial community structure at impacted urban reefs compared to a pristine site. Encircled areas indicate groups that showed high similarity among samples. (B) The scale bar indicates the percent of similarity among groups.

For DNA sequence analysis based on the excised DGGE bands (Figure 2), a total of 8 distinct DGGE fragments were excised and identified from the study sites (Figure 4). Among diversely revealed bacterial communities during the experimental period, *Synechococcus* sp. (CC9311, Figure 2, Band #1) was detected on all study sties except at Hawaii Kai reef. *Synechococcus* sp. is one of oxygenic photoautotrophs of the genera and widely accepted as the most abundant members of the picophytoplankton that contributes significantly to primary production in the ocean [21].

Two distinct DGGE fragments were excised and identified from the Hawaii Kai's pristine site: *Shewanella denitrificans* (OS217, Figure 2; Band #2) and *Saprospira grandis* (str. Lewin, Figure 2; Band #6). *S. denitrificans* (OS217, Figure 2; Band #2) is a diverse group of marine gamma-proteobacteria. It is an important species because *S. denitrificans* contributes significantly to the total denitrification of the system in the ocean [22]. *S. grandis* (str. Lewin, Figure 2; Band #6) is a gram-negative marine bacterium that is free-living in marine littoral sand or coastal zones. *S. grandis* contributes to consumption of algae in the prevention of algal blooms [23, 24]. Additionally, Sangkhobol et al. [25] reported *S. grandis* also preys on other bacteria and protists.

Three distinct DGGE fragments were excised and identified from Ala Wai Canal: *Marivirga tractuosa* (DSM 4126, Figure 2, Band #3), *Flavobacterium johnsoniae* (UW101, Figure 2, Band #4), and *Vibrio vulnificus* (CMCP6, Figure 2, Band #7). *M. tractuosa* (DSM 4126, Figure 2, Band #3) is a gram-negative bacterium that is non-spore forming. *M. tractuosa* (DSM 4126) is found in a variety of places such as soil, freshwater or beach sand. *F. johnsoniae* (UW101, Figure 2, Band #4) is an aerobic gram-negative bacterium that is commonly found in soil and freshwater and rapidly digests chitin and many other macromolecules [26]. The last distinct DGGE fragment from Ala Wai Canal is *V. vulnificus* (CMCP6, Figure 2, Band #7) which is a gram-negative bacterium and found in salty or coastal waters. It can thrive in molluscan shellfish such as oysters, thereby causing health risks for those who ingest raw or undercooked seafood. Thompson el al. [27] reported *V. vulnificus* can be a virulent bacterium associated with about 95% of all seafood related deaths.

From Waikiki, two distinct DGGE fragments were excised and identified which were *Synechococcus* sp. (PCC7002, Figure 2, Band #5) and *Pseudomonas mendocina* (ymp, Figure 2, Band #8). *Synechococcus* sp. was found in a DGGE gel band from the Hawaii Kai pristine site, as well as in Ala Wai Canal, and in Waikiki. It seems to indicate that the equal 16S rDNA sequences could present at different positions on the DGGE gel. On the other hand, Boon et al. [28] and Vallaeys et al. [29] reported that a single DGGE fragment could be interpreted by numerous species with partial 16S rDNA sequences. *P. mendocina* is a gram-negative bacterium that can be found in many different environments such as soil, marshes, and coastal marine habitats. *P. mendocina* can cause opportunistic nosocomial infections, such as infective endocarditis [30].

DNA sequence analysis of 4 colonies isolated from Ala Wai samples indicate that *Shewanella baltica* (homology: 89%), *Vibrio cholerae* IEC224 (homology: 91%), *Pseudomonas syringae* B728 (homology: 97%) and *Escherichia coli* MG1655 (homology: 100%) were found as biofilm components at this site. Notably, *Shewanella* sp., *Vibrio* sp., *Pseudomonas* sp. had

similar DGGE fingerprint results. These results indicate that the microbial community structure from the study sites can be pathological and could have serious ecological implications.

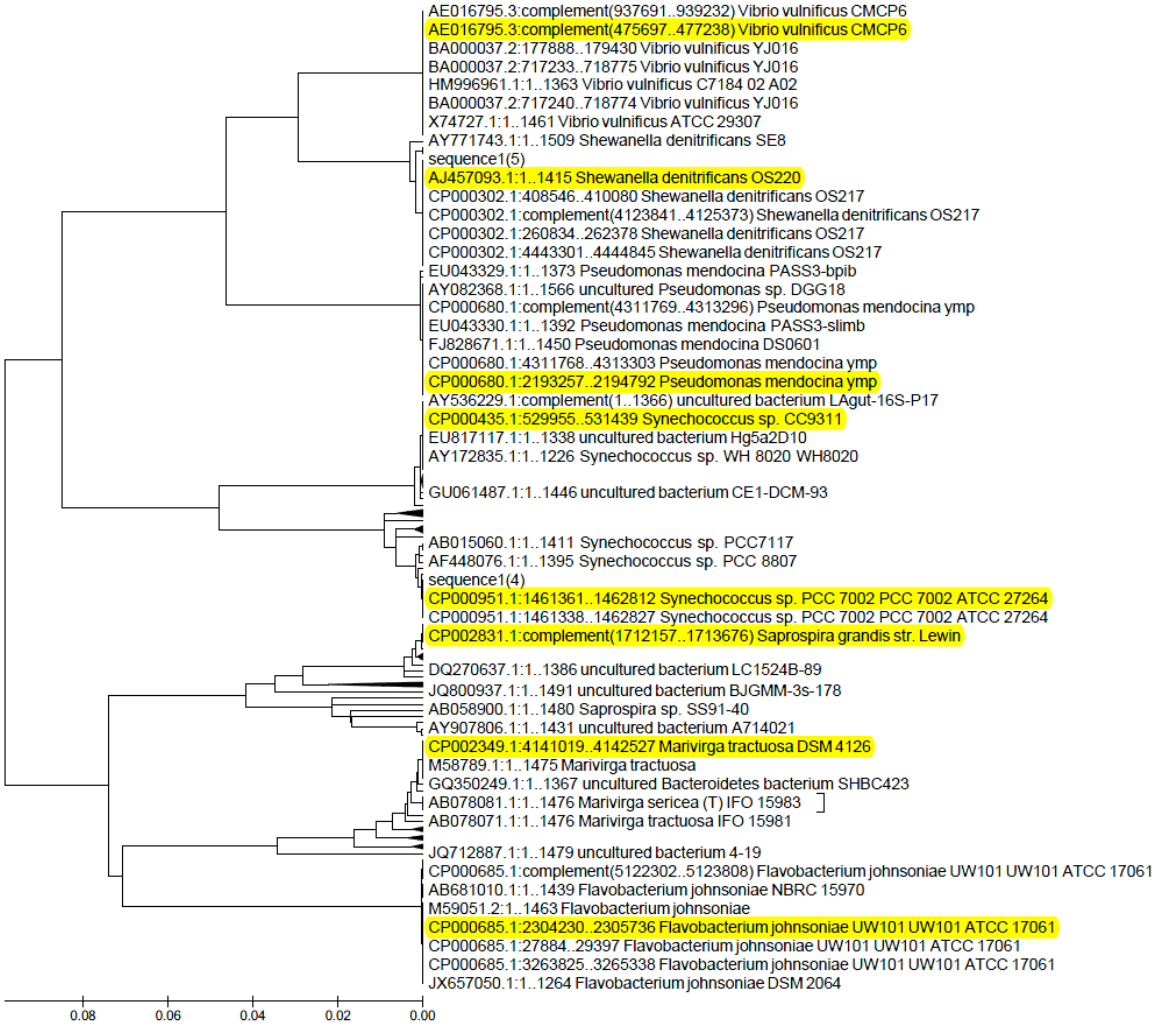

Figure 4. Phylogenetic tree based on DGGE fingerprint profiles from the microbial community in the test sites. The yellow bands indicate organisms we identified within our study.

Biofilm Microscopy for Community Structure

Light microscopy of biofilm slides from the Ala Wai showed gram positive and negative bacteria with notable components including marine yeasts (to be confirmed), diverse capsulated microbes (Figures 5 and 6), and endospores or endospore forming bacteria (Figure 7).

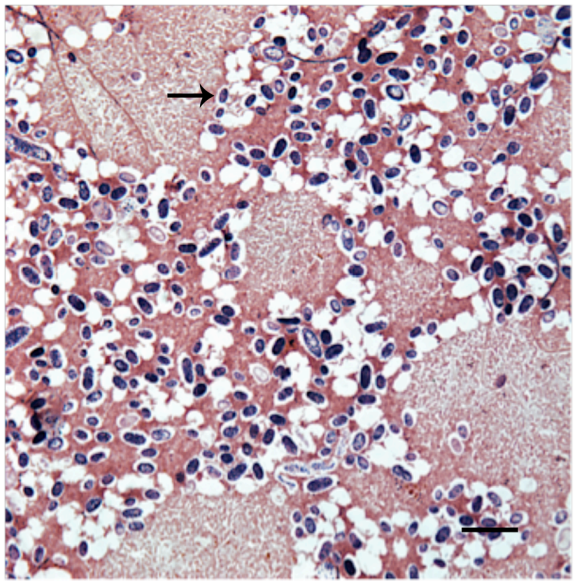

Figure 5. Gram-negative bacteria that grew on an EMB medium but did not ferment lactose (non-coliforms). These bacteria were interspersed with large, capsulated (arrow) eukaryotic cells on the glass substrate we speculate are marine yeasts. Scale 100 μm.

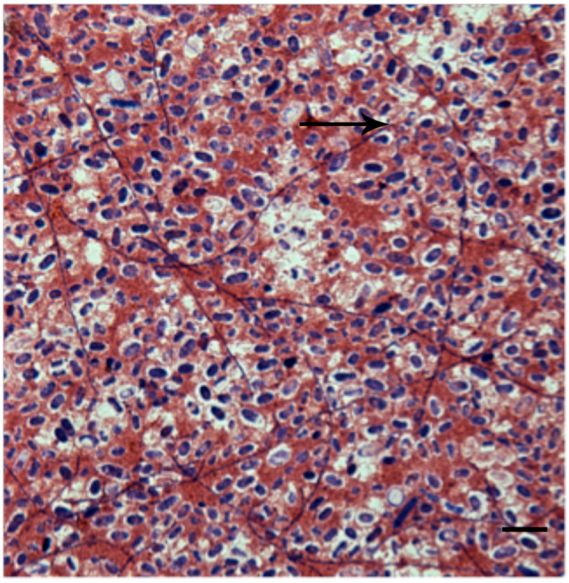

Figure 6. The "yeast" and gram-negative bacteria were consistently associated with filamentous bacteria (arrow) that were also evident in this biofilm community. Scale 50 μm.

Biofilms were inoculated onto TSA plates, grown at room temperature for 48 hours and colonies were isolated. A gram-positive bacterium was found to produce endospores during this incubation time (Figure 7). While capsules in marine microbes are common and diverse, the presence of capsules for several bacteria isolated in our study, withstood gram stain conditions. The capsules as well as endospores and/or endospore-forming organisms in the biofilms from Ala Wai could be considered as pathogenic potential for these organisms. The overall dimensionality of a biofilm is shown by Differential Interference Contrast Microscopy (DIC) (Figure 8) and further evaluated with confocal microscopy.

To evaluate the community structure we used lectins to stabilize the biofilms that were conjugated with FITC for visualization. Biofilms were additionally labeled with DAPI for visualization of biofilm microbes.

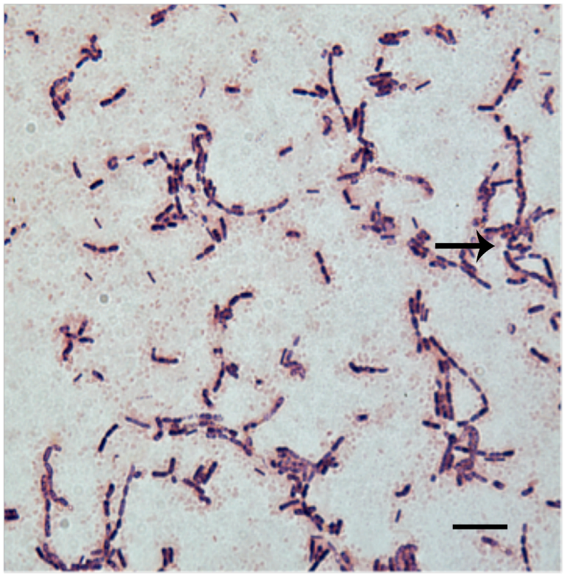

Figure 7. Gram positive bacteria and endospores (arrow) following inoculation onto Ala Wai biofilm organisms TSA and incubation at room temperature. Scale 10 μm.

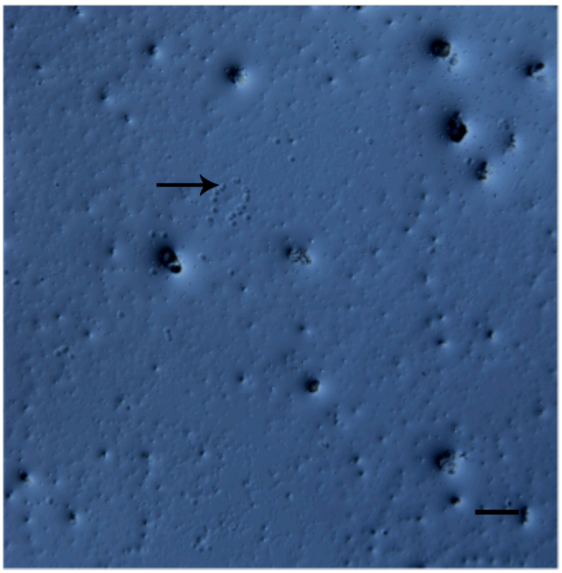

Figure 8. DIC overview of an unstained biofilm showed scattered cocci (small dots) and larger eukaryotic cells (arrow) that were determined to be photosynthetic with epifluorescence excitation (green). Scale 50 μm.

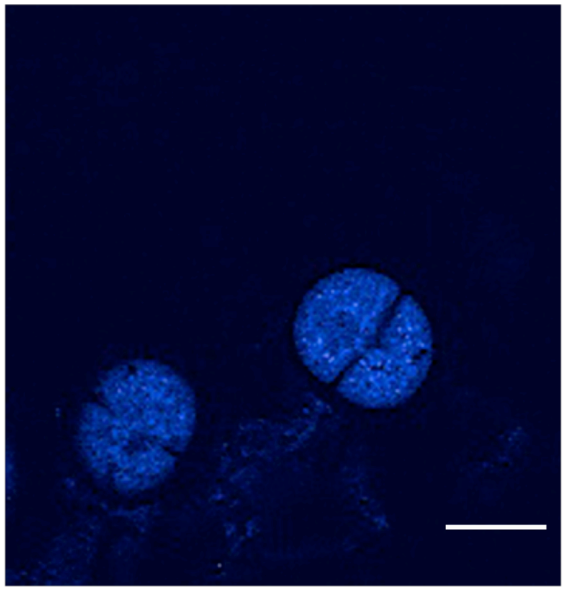

Figure 9. DAPI-stained photosynthetic cells in the biofilm were associated with EPS. Scale 25 μm.

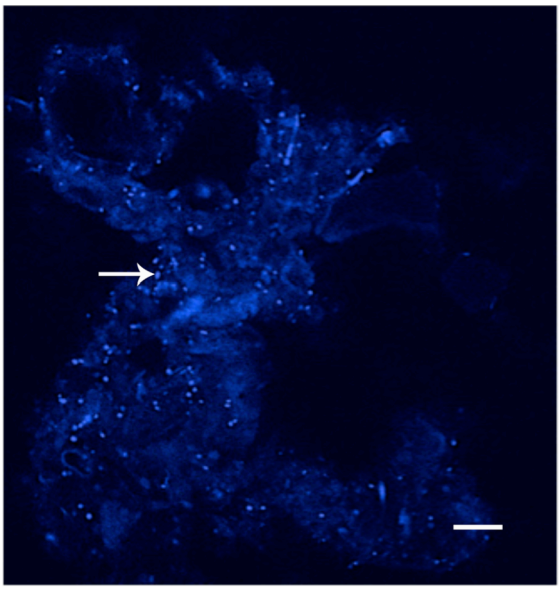

Figure 10. DAPI-stained bacteria (arrow) were within EPS of the biofilm. Scale 50 μm.

The consistent associations of biofilm cells and EPS (Figures 9 and 10) allow us to see glycochemistry sources and distribution, and we further visualized the glycochemistry of the biofilms with the use of lectins.

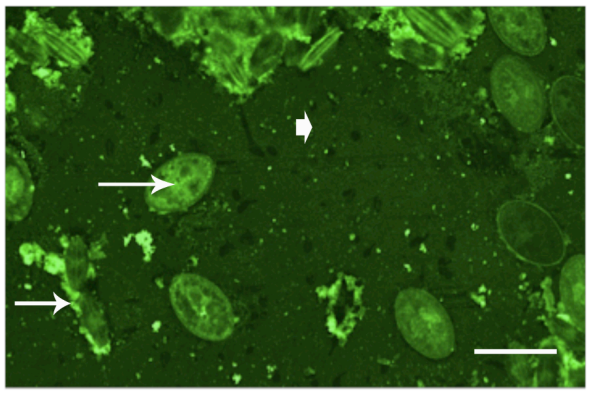

Figure 11. Con A-labeled biofilm shows mannose/glucose-rich glycoconjugates from diatom EPS (arrows) and non-cellular matrix (arrowhead). Scale 50 μm.

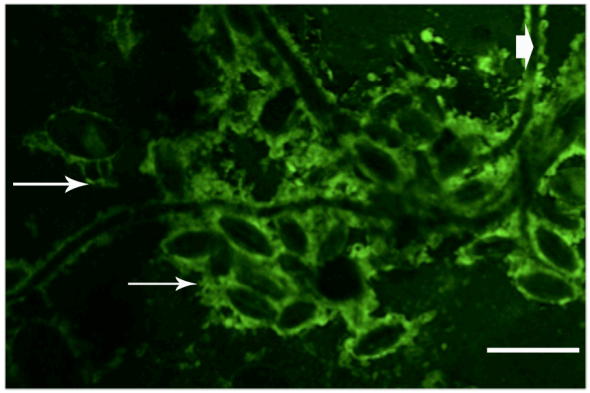

Figure 12. WGA-labeled EPS from diatoms (arrows) and cyanobacteria (arrowhead) localized glycoconjugates with N-acetylglucosamine residues external to the cells as EPS. Scale 50 μm.

Our results indicate complex chemistry of the EPS from diatoms and bacterial cells/EPS. By the specificity of the lectin binding we concluded that the composition of the biofilm matrix contains glycoconjugates with mannose/glucose moieties which is also localized at bacterial cells and EPS (Figure 11). The glycoconjugates with sialic acid residues are most concentrated in the diatom and bacterial EPS (Figure 12). The community structure of Ala Wai biofilms has been visualized with diverse organisms that include photoautotrophs (Figures 13 and 14). Specific glycoconjugates reveal remarkable complexity in the biofilm matrix and EPS (Figures 11–14). The distribution of specific cellular and EPS glycoconjugates has significance in substrate colonization as well as future community development in this coastal region [31].

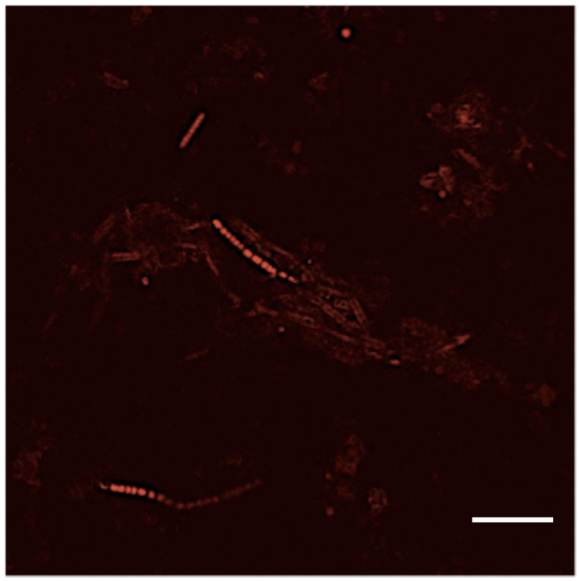

Figure 13. Photosynthetic components of the biofilm were evident as red autofluorescence following green laser excitation. Scale 100 μm.

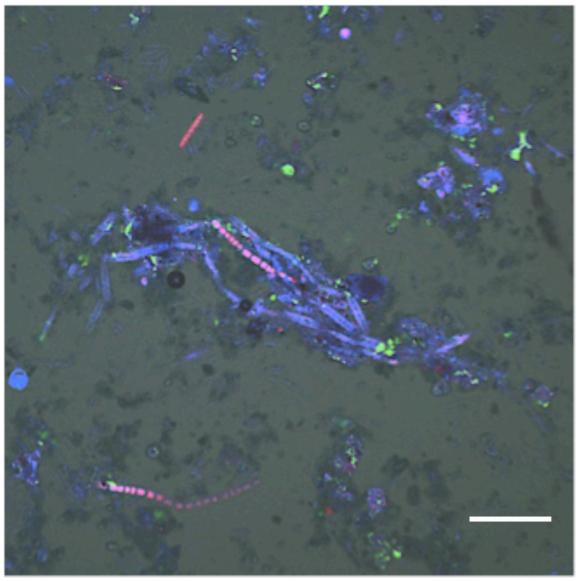

Figure 14. This composite image from excitation with blue, green and UV lasers as well as white light, illustrates cellular and acellular components of the biofilm structure with heterogeneous distribution of mannose/glucose specific glycoconjugates. The autofluorescent cyanobacteria (red) and diatoms and bacteria (blue) as well as mannose/glucose-rich glycoconjugates (green) in the matrix and EPS structure the biofilm community. Scale 100 μm.

Water Quality Analysis

The results of the inorganic nutrient levels for the water quality analysis are shown in Table 2. Ala Wai Canal has high levels of inorganic nutrients among the study site. In contrast, the pristine site shows the lowest levels of inorganic nutrients among the study site. The Ala Wai Canal is eutrophic via inputs of impaired water or with sewage spills and this has been a public health concern.

Table 2. Results of the inorganic nutrient level from the study sites (unit: µmol/L).

Site	Nitrate	Nitrite	Inorganic P	Silicate	Ammonia	Nitrate
Pristine reef	1.76	0.067	0.014	3.21	1.22	1.70
Ala Wai Canal	7.86	0.463	0.390	74.54	1.62	7.40
Waikiki reef	1.17	0.111	0.122	2.11	0.327	1.06
Hawaii Kai reef	2.90	0.050	0.074	2.23	4.10	2.85

To examine the photosynthetic characteristics of autotrophic organisms attached to the glass, PAM was used to measure the differences of photosynthesis in the microbial community among the four study sites (Figure 15). The results indicate that Ala Wai Canal showed the highest level of ETRmax at 35.9, while Hawaii Kai reef showed the lowest level at 16.7. The data indicates the population of autotrophic organisms in the Ala Wai Canal is higher than other study sites. In addition, the results of the ETRmax t-test showed P = 0.044089 and that the data of ETRmax are significant at < 0.05 (Excel).

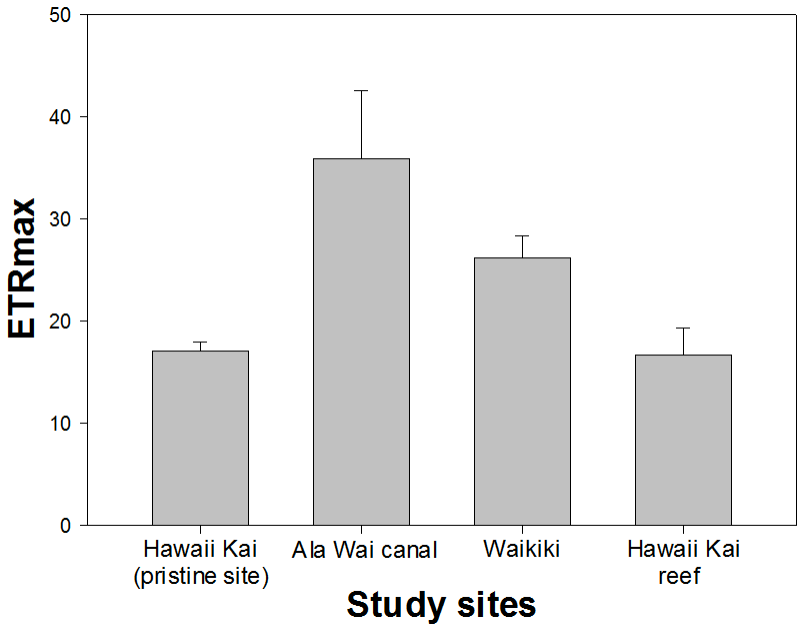

Figure 15. Mean values (+ standard deviation) of ETRmax for biofilms at the four study sites.

Our examination of Oahu's biofiolm microbial communities including those that have potentially elevated wastewater inputs, especially for Ala Wai Canal and Waikiki, showed pathological and potentially serious ecological implications in the findings with organisms such as *Vibrio vulnificus* and *Pseudomonas mendocina.* How microbial communities quickly shift from impacted invasive alien algae to healthy states are still unknown. The results of this study provide information on the microbial community composition within the biofilm that could become one of research priorities in the understanding of marine ecosystems and effects of elevated wastewater inputs.

Conclusion and Potential Significance of this Work

To analyze changes in marine microbial communities, bacteria at impacted urban reefs were compared to a pristine site with DGGE analysis. Our results indicated that a total of 57 different 16S rDNA patterns were detected from the study sites. The compositions of microbial communities from Hawaii Kai reef were clearly distinguished from the other study sites within 12.8% of similarity. On the other hand, the compositions of microbial communities between Waikiki and Ala Wai Canal showed 37.7% similarity. Therefore, the results strongly indicate the compositions of microbial communities are distinct among the marine microbial community at these different study sites, and at relatively fine scales.

For DNA sequence analysis based on the excised DGGE bands, a total of 8 distinct DGGE fragments were excised and identified from the study sites. A diverse group of bacterial communities was also found in the study sites during the experimental period (*Synechococcus* sp. CC9311, *Shewanella denitrificans* OS217, *Saprospira grandis*, *Marivirga tractuosa* DSM 4126, *Flavobacterium johnsoniae* UW101, *Vibrio vulnificus* CMCP6, *Synechococcus* sp. PCC7002, and *Pseudomonas mendocina* ymp). *S. denitrificans* OS217, *V. vulnificus* CMCP6, and *P. mendocina* ymp indicate that the microbial community structure from the study sites can be pathological and could have serious ecological implications.

The biofilm microscopy for community structure results indicated complex chemistry of the EPS from diatoms and bacterial cells/EPS. The specificity of the lectin binding allows us to conclude that the composition of the biofilm matrix contains glycoconjugates with mannose/glucose moieties that is also localized at bacterial cells and EPS. The glycoconjugates with sialic acid residues are most concentrated in the diatom and bacterial EPS.

Results of water chemistry and PAM indicated that Ala Wai Canal showed high levels of inorganic nutrients and the highest algal photosynthesis level (ETRmax) among the study sites.

Therefore, we could conclude Ala Wai Canal is eutrophic via inputs of impaired water or with sewage spills, and the populations of autotrophic organisms are higher than at other study sites.

On Oahu, including sites that have potentially elevated wastewater inputs, indications of pathological and serious ecological implications were found (e.g., *Vibrio vulnificus* and *Pseudomonas mendocina*) in the microbial community structure, especially at Ala Wai Canal and at Waikiki. However, how quickly microbial communities shift from impacted invasive alien algae to healthy states, remains unknown.

References Cited

[1] Costerton, J.W., Z. Lewandowski, 1995, Microbial biofilms, *Annual Review of Microbiology*, 49, 711–745.

[2] Kristensen, J.B., R.L. Meyer, B.S. Laursen, S. Shipovskov, F. Besenbacher, C.H. Poulsen, 2008, Antifouling enzymes and the biochemistry of marine settlement, *Biotechnology Advances,* 26, 471–481.

[3] Lawrence, J.R., D.R. Korber, B.D. Hoyle, J.W. Costerton, D.E. Caldwell, 1991, Optical sectioning of microbial biofilms, *Journal of Bacteriology*, 173, 6558–6567.

[4] Wetherbee, R., J.L. Lind, J. Burke, R.S. Quatrano, 1998, Mini review—The first kiss: establishment and control of initial adhesion by raphid diatoms, *Journal of Phycology*, 34, 9–15.

[5] Egan, S., T. Thomas, S. Kjelleberg, 2008, Unlocking the diversity and biotechnological potential of marine surface associated microbial communities, *Current Opinion in Microbiology*, 11, 219–225.

[6] Little, B.J., J.R. DePalma, 1998, Marine biofouling, in D.F. Hanson, C.R. Crowe, H. Herman, eds., *Treatise on Materials Science and Technology-Materials for Marine Systems and Structures*, New York, Academic Press, 89–119.

[7] Salvesen, I., K.I. Reitan, J. Skjermo, G Øie, 2000, Microbial environments in marine larviculture: impacts of algal growth rates on the bacterial load in six microalgae, *Aquaculture International*, 8, 275–287.

[8] McBain, A.J., 2009, *In vitro* biofilm models: an overview, in S. Sima, M.G. Geoffrey, eds., *Advances in Applied Microbiology*, vol. 69, Academic Press, 99–132.

[9] Welsh, A.K., R.J. McLean, 2007, Characterization of bacteria in mixed biofilm communities using denaturing gradient gel electrophoresis (DGGE), *Current Protocols in Microbiology*, Chapter 1:Unit 1E.1.

[10] Bjørkøy, A., L. Fiksdal, 2009, Characterization of biofouling on hollow fiber membranes using confocal laser scanning microcscopy and image analysis, *Desalination,* 245, 474–484.

[11] Hunt, C., 2007, Ground-water nutrient flux to coastal waters and numerical simulation of wastewater injection at Kihei, Maui, Hawaii, *U.S. Geological Survey Scientific Investigations Report* 2006–5283, 88.

[12] Novitsky, J., D. Karl, 1985, Influence of deep ocean sewage outfalls on the microbial activity of the surrounding sediment, *Applied and Environmental Microbiology*, 50, 1464–1473.

[13] Smith, J., M. Shaw, R. Edwards, D. Obura, O. Pantos, E. Sala, S.A. Sandin, S. Smriga, F. Rohwer, 2006, Indirect effects of algae on coral: algae-mediated, microbe-induced coral mortality, *Ecology Letters*, 9, 835–845.

[14] Muyzer, G., A. Teske, C.O. Wirsen, H.W. Jannasch, 1995, Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments, *Archives Microbiology*, 164, 165–172.

[15] Muyzer, G., 1999, DGGE/TGGE a method for identifying genes from natural ecosystems, *Current Opinion in Microbiology*, 28, 317–322.

[16] Di´ez, B., C. Pedro-Alio, T.L. Marsh, R. Massana, 2001, Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques, *Applied and Environmental Microbiology*, 67, 2942–2951.

[17] Schreiber, U., R. Gademann, P.J. Ralph, A.W. Larkum, 1997, Assessment of photosynthetic performance of *Prochloron* in *Lissoclinum patella in hospite* by chlorophyll fluorescence measurements, *Plant and Cell Physiology*, 38, 945–51.

[18] Michael, T., C.M. Smith, 1995, Lectins probe molecular films in biofouling: Characterization of early films on non-living and living surfaces, *Marine Ecology Progress Series*, 119, 229–236.

[19] Whittier, R.B., A.I. El-Kadi, 2009, Human and environmental risk ranking of onsite sewage disposal systems, Department of Geology and Geophysics, University of Hawai‘i at Manoa, Honolulu, HI. 57 pp.

[20] Clarke, K.R., R.M. Warwick, 2001, Change in marine communities: an approach to statistical analysis and interpretation, 2nd ed., PRIMER-E, Plymouth.

[21] Scanlan, D.J., N.J. West, 2002, Molecular ecology of the marine cyanobacterial genera *Prochlorococcus* and *Synechococcus*, *FEMS Microbiology Ecology*, 40, 1–12.

[22] Brettar, I., E.R.B. Moore, M.G. Höfle, 2001, Phylogeny and abundance of novel denitrifying bacteria isolated from the water column of the Central Baltic Sea, *Microbial Ecology*, 42, 295–305.

[23] Delk, A.S., C.A. Dekker, 2012, Characterization of rhapidosomes of *Saprospira grandis*, *Journal of Molecular Biology*, 64, 287–288.

[24] Lewin, R.A., 2012, *Sapropsira grandis*: a flexibacterium, *Microbial Ecology*, 34, 232–236.

[25] Sangkhobol, V., V.B.D. Skerman, 2012, *Saprospira* species-natural predators, *Microbiology*, 5, 169–174.

[26] Stanier, R.Y., 1947, Studies on non-fruiting myxobacteria, *Journal of Bacteriology*, 53, 297–315.

[27] Thompson, F., B. Austin, J. Swings, eds., 2006, The biology of *Vibrios*, Hemdon, Virginia, ASM Press, 349–354, 359–361.

[28] Boon, N., W.D. Windt, W. Verstraete, E.M. Top, 2002, Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants, *FEMS Microbiology Ecology*, 39, 101–112.

[29] Vallaeys, T., E. Topp, G. Muyzer, V. Macheret, G. Laguerre, A. Rigaud, G. Soulas, 1997, Evaluation of denaturing gradient gel electrophoresis in the detection of 16S rDNA sequence variation in rhizobia and methanotrophs, *FEMS Microbiology Ecology*, 24, 279–285.

[30] Aragone, M.R., D.M. Maurizi, L.O. Clara, E.J.L. Navarro, A. Ascione, 1992, *Pseudomonas mendocina*, an environmental bacterium isolated from a patient with human infective endocarditis, *Journal of Clinical Microbiology*, 30, 1583–1584.

[31] Michael, T.M., 2009, Glycoconjugate organization of *Enteromorpha (=Ulva) flexuosa* and *Ulva fasciata* (Chlorophyta) zoospores, *Journal of Phycology*, 45, 660–677.