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Katie E. Vella
Nova Southeastern University Oceanographic Center, katevella1@gmail.com

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Microbial Effects on the Production and Transformation of Surfactants within the Microlayer and Subsurface Waters in Application to Remote Sensing Techniques

By
Katie E. Vella

Submitted to the Faculty of
Nova Southeastern University Oceanographic Center
in partial fulfillment of the requirements for
the degree of Master of Science with specialties in:
Marine Biology and Coastal Zone Management
Nova Southeastern University
9 November 2012

Thesis of

Katie E. Vella

Submitted in Partial Fulfillment of the Requirement for the Degree of

Master of Science:

Marine Biology and Coastal Zone Management

Nova Southeastern University
Oceanographic Center

November 2012

Approved:
Thesis Committee

Major Professor: _____
Alexander Soloviev, Ph.D.
Nova Southeastern University Oceanographic Center

Committee Member: _____
Silvia Matt, Ph.D.
Nova Southeastern University Oceanographic Center

Committee Member: _____
Mahmood Shivji, Ph.D.
Nova Southeastern University Oceanographic Center

Committee Member: _____
Aurelien Tartar, Ph.D.
Nova Southeastern University

ACKNOWLEDGEMENTS

I would like to thank some very important people without whom this research thesis project would not have been possible. First, a special thanks is due to my head advisor, Dr. Alexander Soloviev, for offering me the opportunity to work on this project and for seeing potential in me. His expert knowledge and mentoring throughout this process has attributed to my growth as a researcher and scientist. I am grateful to my committee members, Drs. Silvia Matt, Mahmood Shivji, and Aurelien Tartar. I sincerely thank Dr. Matt for her direction, discussions, and all her most valuable contributions on this work. I am grateful to Dr. Shivji for his efforts as part of my committee. I am indebted to Dr. Tartar for his expert microbial advice, for the use of his laboratory and equipment, and for his dedication to his work and his students.

This thesis project was initiated after one of the authors attended the COST Action 735 meeting "Surfactants and the Microlayer Gas Exchange" organized by Peter Liss (UEA). This work was supported initially as a part of the NSUOC project “Hydrodynamics and Remote Sensing of Far Wakes of Ships” and further supported from the ONR project “Characterization of Impact of Oceanographic Features on the Electromagnetic Fields in Coastal Waters”. I acknowledge support from the Kevin Kohler Memorial Scholarship Research Fund at NSUOC and the GoMRI project “Consortium for Advanced Research on Transport of Hydrocarbons in the Environment” via a subcontract to UM RSMAS.

I also acknowledge the Italian Space Agency for collecting COSMO SkyMed SAR satellite images and am grateful for David Moraga Amador and Ernesto Almira at the University of Florida, Genomics Lab for processing our samples with 454 pyrosequencing. I also extend a heartfelt thanks to my colleagues: Chris Maingot, Jenny Fenton and Brian Ettinger for their help with the sample collection. I would like to offer a special thanks to my lab mate, Naoko Kurata, for her guidance and unlimited supply of encouragement throughout my graduate school experience.

Most importantly, I give thanks to God, without whom none of this would have purpose or be nearly as fulfilling. To my family and friends, I would like to thank them all for their unending encouragement, support and love along my journey. And finally, this thesis is a special gift for my beloved mother.

ABSTRACT

The sea surface microlayer is a millimeter-scale interfacial layer between the atmosphere and the ocean. A number of studies have suggested that there is a unique ecosystem for marine bacteria in the sea surface microlayer, but little information exists on the microbial community composition of this ecosystem due to sampling complexities. In this work, we present an improved method to sample and compare the bacterial diversity of the sea surface microlayer with that of subsurface water at the same site. Bacterial samples were collected from the sea surface microlayer with a sampling method, which minimized sample contamination from the research platform and the subsurface water. Sampling was conducted using a polycarbonate membrane filter to obtain the bacterial community structure at open water and coastal water sites in the Straits of Florida. The microlayer sampling was planned to coincide with synthetic aperture radar satellite overpasses (COSMO SkyMed), which capture a range of fine-scale features on the sea surface. The presence of surfactants affect the synthetic aperture radar imaging process because surfactants in the sea surface microlayer suppress short gravity-capillary ocean surface waves, thereby decreasing the backscatter and allowing the radar to detect surfactant-covered areas. Although sources of surfactants vary, certain marine bacteria are known to produce and transform surfactants, which suggest that these surfactant-related marine bacteria have an important biological influence on fine-scale synthetic aperture radar satellite imagery. Therefore, the comparison between synthetic aperture radar satellite images and in situ field samples may be used for interpreting and studying fine-scale features on the sea surface. The surfactant-associated bacterial composition of the sampling sites was determined using high-throughput, 454 pyrosequencing methods. A total of 61,663 sequences were analyzed and the results indicated the presence of surfactant-associated bacteria such as *Moraxellaceae, Halomonadaceae, Enterobacteriaceae*, *Bacillaceae*, and *Nocardiaceae*. By establishing these bacterial groups that influence the presence of surfactants, remote sensing techniques which involve monitoring the microlayer are expected to be enhanced and may provide additional information on the state of the upper ocean ecosystem.

Keywords: sea surface, synthetic aperture radar, pyrosequencing, bacteria.

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1.0 Introduction

1.1 Background

The sea surface microlayer represents the boundary between the atmosphere and the ocean, with a total thickness between 1 and 1,000 μm (Wurl et al. 2011). Serving as both a source and a sink for materials in the atmosphere and the water column, the sea surface microlayer (SML) can be summarized as being a micro-habitat that covers about 70% of the Earth's surface (Murrell et al. 2007; Wurl et al. 2011). Being such a widely distributed microbial ecosystem, the SML is often termed neuston to distinguish the microorganisms associated with the air-water interface from the subsurface plankton (Naumann, 1917). Despite the thinness of the SML, this interface is distinct from the subsurface water below and may play a significant role in biological processes on a global scale, including microbial loops (Wurl et al. 2011).

The neuston was first studied as a new branch of marine biology in 1971 by Yuvenaly Zaitsev, where he emphasized the importance of the neuston in the reproductive cycles of marine organisms (Zaitsev, 1971). However, a greater appreciation of the microlayer's role in global-scale microbial processes is now emerging (Cunliffe et al. 2011). Consequently, there is renewed interest in the microbiological composition and how this may vary in space and time. The SML results from the accumulation of both discrete molecules and larger particles at the air-water interface to form a film (Cunliffe et al. 2011). Historically, the depth of the SML has not been well defined, having been determined by the prevailing sampling protocol. Nevertheless, early descriptions of the SML depict a distinct entity with a stratified structure comprising an upper lipid layer containing highly surface-active molecules overlying a protein-polysaccharide layer extending into subsurface waters (Fig. 1.1). The lipid layer components were typically considered to be of low solubility and contain hydrophobic ends extending into the air (Cunliffe et al. 2011). This was the first basic structure referred to as the SML (Hardy, 1982).

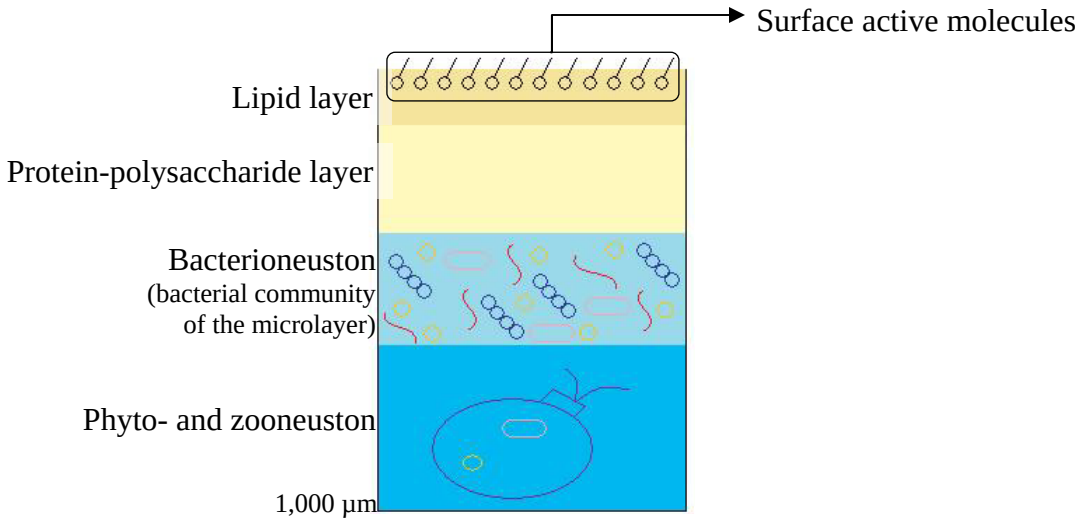

Figure 1.1: Classical Sea Surface Microlayer Model (Adapted from Cunliffe et al. 2011).

The early view of lipids as important surface microlayer components has been revised where lipids are no longer considered to be present in such sufficient concentrations (Sieburth, 1983). A more modern model of the SML consists of macromolecules that are produced from dissolved organic matter (Fig 1.2). An important component of this SML organic matter is transparent exopolymer particles (TEPs). These TEPs are sticky gel particles produced in the water column by phytoplankton, which expel TEPs in the ocean (Alldredge et al. 1993). The coagulation of dissolved organic matter readily allow TEPs to form aggregates in the water column with other particles such as detritus, and as recent evidence suggests, some of these TEPs then migrate up to the surface via rising bubbles and diffusion, and form a gelatinous film (Cunliffe et al. 2009). Consequently, as TEPs are colonized by microorganisms, a surface microlayer film is believed to be formed.

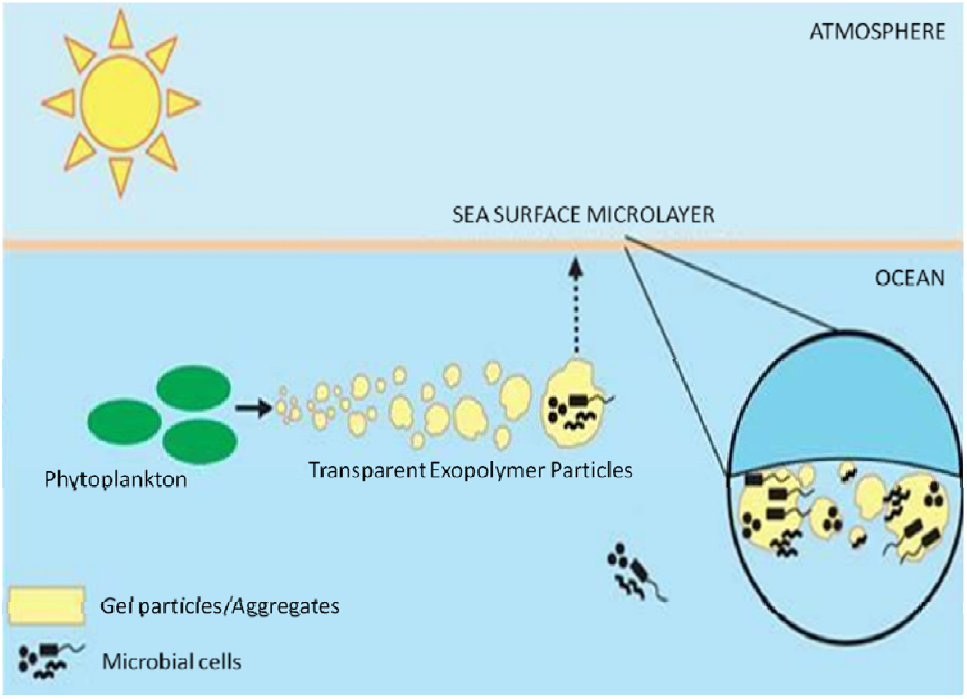

Figure 1.2: Modern Sea Surface Microlayer Model (Adapted from Cunliffe et al. 2009).

The SML is also known to concentrate, in varying degrees, surface active compounds or surfactants (Wurl et al. 2011). These surfactants, such as oleic acid and oleyl alcohol, reduce surface tension (Elraies et al. 2009) and are amphiphilic, meaning they can also increase the solubility, mobility, and subsequent biodegradation of organic compounds (Singh et al. 2007). A major source of surfactants is through the production by phytoplankton, which exude natural surfactants as metabolic by-products (Liss et al. 1997). Rising air bubbles coated with surface-active material have been recognized as a major transport vector of surfactants from subsurface water to the ocean surface (Liss, 1975). When the bubbles burst at the ocean's surface, a small fraction of the organic matter eject into the atmosphere and the remaining fraction is available for the formation of surfactant films (Liss, 1975).

1.2 Microbial Composition in the Microlayer

Microorganisms are vital to the function of all ecosystems, largely because they exist in enormous numbers and so have immense cumulative mass and activity (Whitman et al. 1998). They are also more diverse than any other organisms, so it is easy to see why the structure of microbial communities, that is, the different kinds of organisms and their

abundances, is so important to the way in which ecosystems function (Fuhrman, 2009). Even with modern tools however, it is not easy to determine microbial community structure and map its variations in space and time (Fuhrman, 2009). Changes in community structure in space and time are very informative because they show us what scales a particular sample represents and help us to understand factors that control communities (Fuhrman, 2009). This is crucial for extrapolating from individual samples to the world at large (Fuhrman, 2009). Understanding ecosystem function calls for much better knowledge than we have today about microbial processes and interactions (Fuhrman, 2009).

Comparison between studies on the SML composition is problematic because there is currently no consensus as to the most appropriate strategy for sampling. Different microlayer samplers yield varying defined depths (Cunliffe et al. 2011). Therefore, early studies that utilized molecular methodologies to study microbial ecology in the SML offered conflicting conclusions, as shown in Figure 1.3. For example, a study comparing bacterioneuston (the bacterial community of the surface microlayer) community structure with subsurface water bacterial community structure at two sample sites detected no consistent difference between the two communities at either site (Agogue, Casamayor, et al. 2005). Similarly, in a study done in the Blyth River estuary, the results indicated that the microbial community structures present in both the microlayer and the subsurface waters were relatively similar (Cunliffe et al. 2008). By contrast, surface microlayer samples collected off the UK North Sea coast contained a distinct bacterioneuston community compared to the subsurface water and was dominated by only two genera: *Vibrio* spp. and *Pseudoalteromonas* spp. (Franklin et al. 2005). Moreover, surface microlayers of marine and inland waters have reportedly greater biological activity than in the subsurface water, as seen in a study of SML samples taken from the Bay of Marseilles in France which exhibited higher chlorophyll *a* concentrations and bacterial counts when compared to those in underlying subsurface water, and as a whole contained a higher particulate organic fraction (Garabetian, 1991). Furthermore, when surface microlayer samples were collected for a study in the Mediterranean Sea and Atlantic Ocean, there were significantly higher rates of bacterial respiration in the SML than subsurface water (Reinthaler et al. 2008).

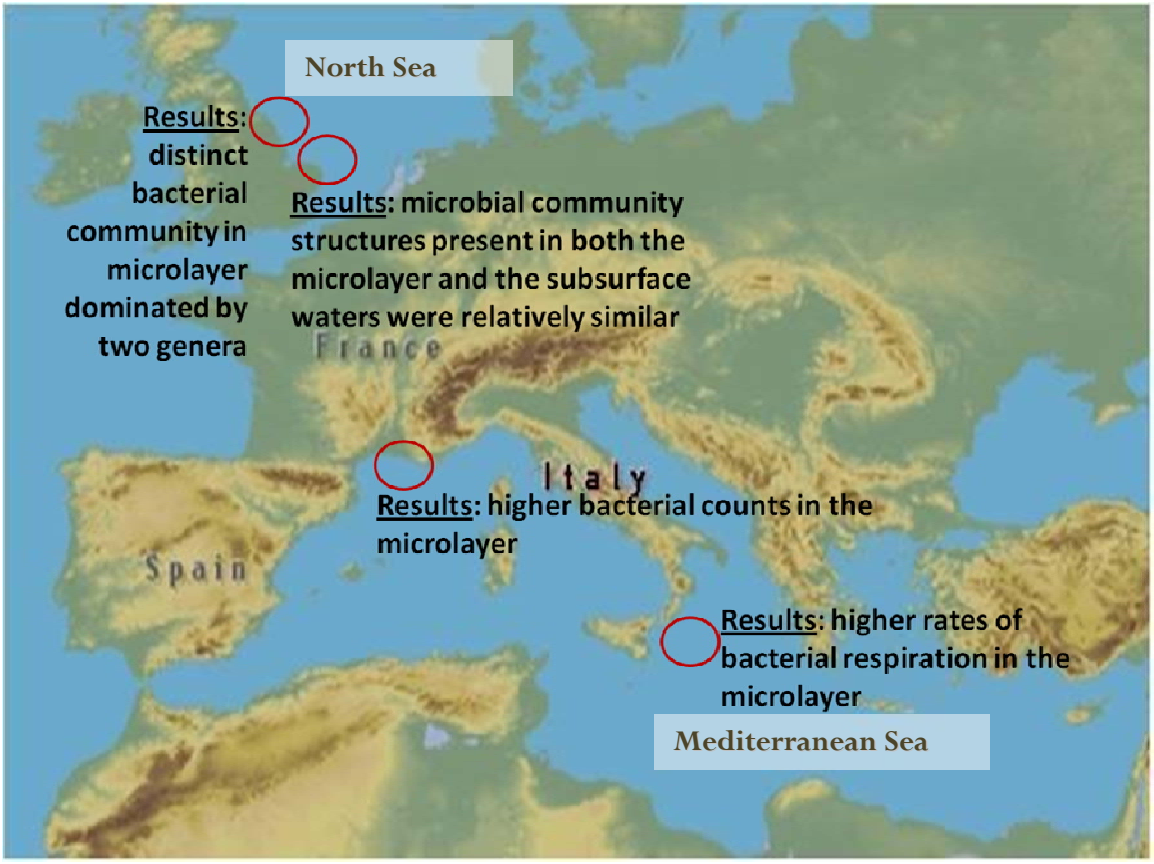

Figure 1.3: Map of previous studies done on the microbial ecology in the SML.

Given that these studies were carried out at different locations and during different seasons, the differences in results are possibly due to differences in the types of marine systems or seasonal effects. The variability in microbial diversity reported in the literature might also be related to natural ecological variability of the enrichment, in addition to different sampling devices used to collect the sea surface microlayer (Agogue et al. 2004). Despite different reports on the microlayer microbial community structure, bacterial communities thriving at the SML are poorly characterized (Agogue, Casamayor, et al. 2005). Further work is needed to address the importance of microbial communities in the sea surface microlayer at a broad range of local, regional, and global scales.

1.3 Microorganism's Role in Microlayer Surfactant Concentrations

A variety of microorganisms are able to produce surfactants and biosurfactants, which are surface-active compounds comprised of glycolipids, lipopeptides, phospholipids, esters groups and certain polysaccharide-protein complexes (Pogorzelski et al. 2006).

Surfactants are produced by microbes, secreted either extracellularly or attached to parts of cells, predominantly during growth on water-immiscible substrates (Desai et al. 1997). Their industrial applications include enhanced oil recovery and surfactant-aided bioremediation of water insoluble pollutants (Sullivan, 1998).

Biosurfactants have several advantages over chemical surfactants, such as lower toxicity, higher biodegradability, better environmental compatibility, and specific activity at extreme temperatures, pH, and salinity (Desai et al. 1997). Due to their structural diversity and environmental compatibility, their production makes them very attractive in their potential areas of use (Lang et al. 1999). A possible additional source of these biosurfactants may be from zooplankton.

While some microorganisms produce surfactants, there are strains of bacteria that have been shown to degrade surface-active substances. A community of strains from the genus *Pseudomonas*, for example, removes surface-active substances from waste waters (Klimenko et al. 2004). *Pseudomonas rathonis*, *Pseudomonas alcaligenes TR*, *Pseudomonas aureofaciens*, and *Pseudomonas mendocina* are all surfactant degraders, capable of degrading anionic and nonionic surface-active substances (Klimenko et al. 2004). Furthermore, microorganisms that are immobilized on the surface are often capable of oxidizing compounds that normally do not lend themselves to destruction (Klimenko et al. 2004).

Some considerations regarding which bacterial populations are responsible for biological degradation in the marine environment are that all species may not be able to be cultivated in the lab (Rusch et al. 2007). Even though marine microbes are the most abundant life form in the ocean, they remain elusive because only a small percentage can be grown and studied (Rusch et al. 2007). Often less than one percent of bacterial cells form colonies on standard culture media (Agogue, Casamayor, et al. 2005). Since such a small percentage of microbial taxa can currently be cultured from the environment, the ocean serves as a potential source of new marine organisms (Sfanos et al. 2005). Therefore, cultivation-independent methods need to be utilized in order to look at population dynamics (Murrell et al. 2007).

1.4 Food Webs and Horizontal Transport in the Microlayer

Food webs in aquatic environments are exemplified by the microbial loop (Fig 1.4), in which protists such as flagellates and ciliates prey on bacterial cells and are in turn preyed upon by relatively larger aquatic organisms (Pomeroy, 1974). Trophic interactions can influence the structure of microbial loop communities, for example, when protists selectively target a particular bacterial group and in turn profoundly affect their bacterial community structuring (Pernthaler, 2005). Certain amoeba and ciliates actively feed on bacterial cells at the SML interface and microscopic surveys of the SML have revealed the presence of flagellate and ciliate protists, indicating that complex protist communities are present (Joux et al. 2006). The protists grazing in the SML therefore contribute to the structure of the film, because ciliates in particular are able to produce significant amounts of surface-active organic compounds that are part of the surface microlayer film (Kujawinski et al. 2002).

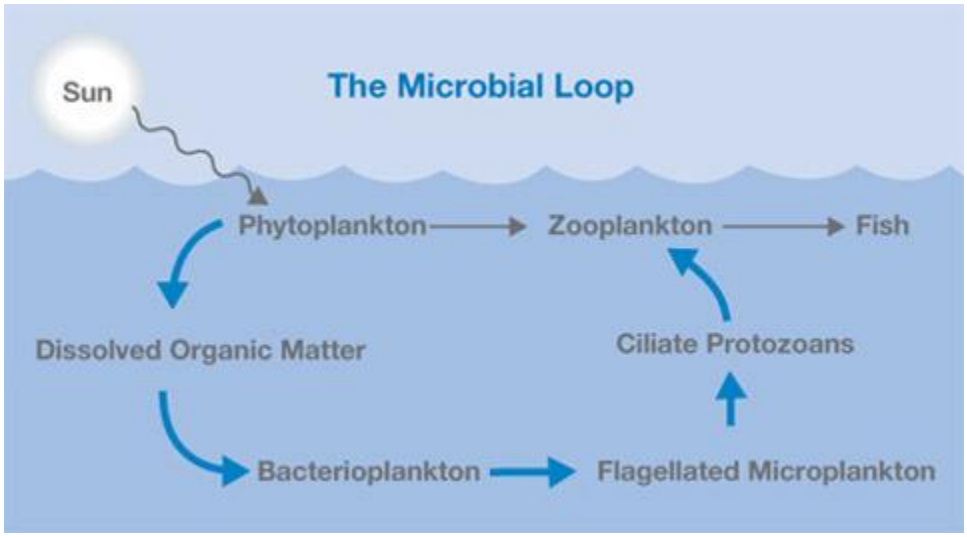

Figure 1.4: Energy flow of the microbial loop. (Adapted from Moen, 2005)

The location of surface microlayers also makes them a highly dynamic system (Cunliffe et al. 2011). Exchange with the atmosphere is strongly influenced by the microbiological nature of surface microlayers (Cunliffe et al. 2011). As the microlayer contains differing concentrations of bacterioneuston compared to underlying subsurface waters, horizontal transport in the sea surface microlayer may be of particular importance as a mechanism in bacterial community structuring (Hale et al. 1997). If sea surface microlayer transport

acts independently of subsurface water circulation, it may be difficult to accurately predict the fate of bacterioneuston from subsurface water circulation patterns (Hale et al. 1997). Therefore, rates of surfactant spreading and the extent to which the chemistry and biology of surface waters may be altered are unclear. Also, the composition of marine aerosols formed from bursting bubbles at the sea surface changes in response to the occurrence of dense microlayers (O'Dowd et al. 2004). These marine aerosols contain microorganisms, therefore bubble bursting and aerosol formation is an important transport mechanism for microlayer components (Kuznetsova et al. 2005; Russell et al. 2010).

1.5 Solar Radiation's Effect on the Microlayer

Unlike those in the underlying waters, organisms within the SML receive maximal solar radiation, in particular UV radiation, which has the potential to cause direct DNA damage or indirect damage via the formation of destructive intermediates (Cunliffe et al. 2011). Although microorganisms in the SML are exposed to high intensities of UV radiation, high concentrations of toxic organic substances and heavy metals, and unstable temperature and salinity conditions, the SML has been reported to have high abundances of microorganisms, suggesting that the bacterioneuston has developed strategies to survive in this extreme environment (Agogue, Joux et al. 2005). Bacteria within the SML studied under exposure to solar radiation and in the dark showed similar abundances and activity (Sintes et al. 2006). Furthermore, as concluded from a study done off coastal waters in the northwest Mediterranean Sea, pigmented bacteria were not more resistant to solar radiation than non-pigmented bacteria, indicating that resistance to radiation is well distributed among bacterial species present in the surface microlayer (Agogue, Joux et al. 2005).

There are, however, conflicting reports on the effects of UV radiation on the neuston. For example, a study done on surface microlayers off California showed neuston communities not to be measurably affected by either visible or UV radiation (Carlucci et al. 1985), while a similar study in Chesapeake Bay showed effects from both (Bailey et al. 1983). Moreover, photo-damage does occur to phytoplankton in the microlayer when under an excessive increase of UV-B radiation, which could affect their subsequent

production of surfactants (Falkowska et al. 2005). Photodegradation is an important UV effect in surface microlayers and it may be a primary transformation mechanism (Cunliffe et al. 2011). Further investigations are necessary to characterize the mechanisms involved in the resistance of marine bacteria to solar radiation. (Agogue, Joux et al. 2005).

1.6 Sea Surface Microlayer of Synthetic Aperture Radar Remote Sensing

Synthetic aperture radar (SAR) satellite imagery is used for a wide variety of environmental applications and is quite an effective tool for monitoring the sea surface (Wiley, 1985). SAR is implemented by mounting a single antenna on a moving platform, such as a satellite (Fig 1.5). Microwave pulses are transmitted by the antenna towards the ocean surface and the microwave energy scattered back to the satellite is measured (Liew, 2001). The radar forms an image by using the time delay of the backscattered signal and uses long-range propagation characteristics, which produce high resolution images and capture fine-scale features on the ocean surface (Angus, 2008). The SAR satellite images can also provide broad-area imaging during both night and day (Angus, 2008). Therefore, advantages of SAR capabilities include minimum constraints on time-of-day as well as atmospheric conditions.

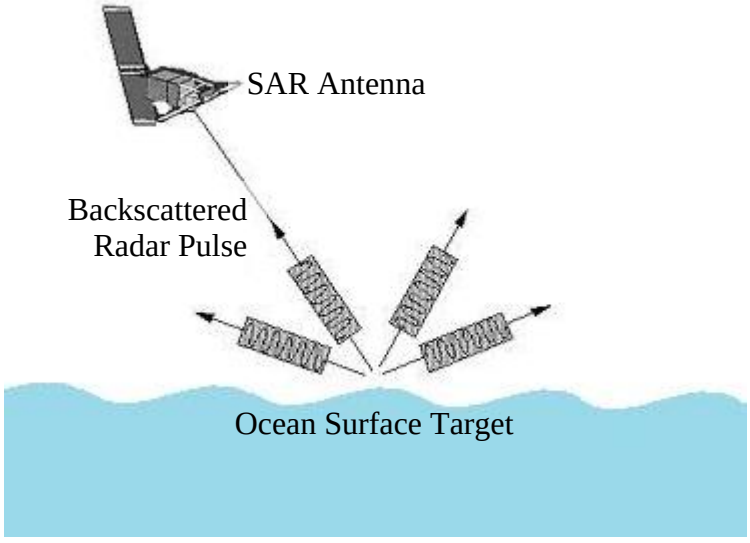

Figure 1.5: SAR radar pulse. (Adapted from Liew, 2001)

By establishing the bacterial groups that influence the presence of surfactants, remote sensing techniques which involve monitoring the surface of the ocean will be enhanced. This is because SAR images of the surface of the ocean are affected by the presence of surfactants, which change the backscatter characteristics of the ocean (Angus, 2008). Surfactants are detected by the radar because they dampen gravity-capillary ocean surface waves, thereby decreasing the backscatter (Alpers et al. 2008). As a result, surfactant-covered areas appear dark in SAR images relative to surfactant-free areas (Fig 1.6). Although sources of surfactants vary, certain marine bacteria are known to produce and degrade surfactants, which make them valuable ecological contributors (Satpute et al. 2010). Therefore, these surfactant-related marine bacteria may have an important biological influence on fine-scale SAR satellite imagery.

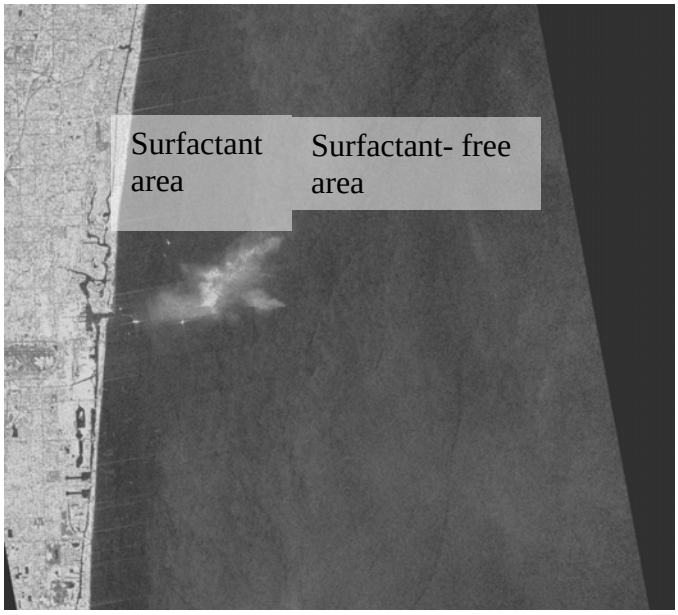

Figure 1.6: COSMO SkyMed satellite image showing surfactant and surfactant-free areas. The bright spot in the middle is a rain signature.

The Earth observation satellite system, constellation of small satellites for the Mediterranean basin observation (COSMO SkyMed), was used in this study. This observation satellite is funded by the Italian Ministry of Research and Ministry of Defense and is conducted by the Italian Space Agency, utilized by both military and civilians (Candela et al. 2003). The system includes four medium-sized satellites equipped with SAR sensors with global coverage of the planet (Fig 1.7). Observations of any area of interest can be repeated several times a day in all weather conditions (Italian

Space Agency, 2007). The imagery can then be applied to environmental monitoring, as well as mapping, hazard analysis, and defense assurance.

Figure 1.7: The four COSMO SkyMed satellites monitoring the Earth. (Photo credit: ASI)

2.0 Objectives

2.1 Significance

Surfactants suppress short gravity-capillary surface waves of the ocean. This effect is most pronounced under low and moderate wind speed conditions (Alpers et al. 2008). These surfactants may be of anthropogenic origin or naturally produced by marine organisms such as bacteria and phytoplankton, and possibly other organisms. The connection from surfactants to microorganisms is not clear, however. Surfactants might also be produced from the decay of organic material. Anecdotal evidence suggests a possible connection between surfactants and zooplankton, where zooplankton secrete surface active agents under stormy conditions, in a stress response (Hühnerfuss, private communication). Those surface active agents then act to reduce turbulence and waves, which would lower the stress on the zooplankton. Although this implies a potential link between zooplankton and surfactant production, the association between surfactants and marine bacteria is still one that needs to be better established.

This research is significant for understanding what types of marine bacteria dwell within the microlayer and subsurface water in coastal versus open water regions, in order to offer a better understanding of how these microbial populations effect surfactant production and transformation. The comparison between synthetic aperture radar satellite imagery and in situ field samples is also important, because these comparisons may be used for interpreting SAR satellite imagery in application to studying fine-scale features on the sea surface. Results from this study are expected to be useful for: environmental monitoring, applications in monitoring biological properties of the sea surface microlayer across the globe, as well as future studies that pose questions regarding the sea surface microlayer.

2.2 Hypotheses

The objectives of this research are to test the following hypotheses:

1. Higher surfactant-associated bacterial diversity is present in the sea surface microlayer compared to the subsurface waters in both coastal and open water regions.
2. Different surfactant-associated bacterial diversity is present in the coastal sea surface microlayer compared to the open water sea surface microlayer.
3. Sea surface features detected with satellite remote sensing techniques can be linked to the presence of surfactant-associated bacteria in the near surface layer of the ocean.

Hypothesis 1: As discussed in section 1.2, studies showed conflicting results on microlayer composition; however one study did find higher bacterial counts in the sea surface microlayer when compared to subsurface water (Garabetian, 1991). This seems more likely considering all the biological activity that occurs at the SML interface. Since surfactants are known to concentrate in the sea surface as well, a greater diversity of surfactant-associated bacteria is expected in the microlayer. Two sampling sites comparing microlayer and subsurface waters were tested to examine these results, using 454 pyrosequencing technology to determine bacterial groups present.

Hypothesis 2: Different surfactant-associated bacterial diversity is expected in the coastal microlayer as compared to the open water microlayer due to expected differences in oceanographic properties in coastal areas. In the sampling region from this study, there may be a coastal counter-current where water moves in the opposite direction than the Gulf Stream (Soloviev et al. 2012). This may produce different biophysical water properties in the coastal area compared to the Gulf Stream. To test this hypothesis, 454 pyrosequencing was utilized to determine bacterial groups present in two sampling sites comparing coastal and open water microlayers.

Hypothesis 3: In order to examine this hypothesis, sea surface microlayer sampling was conducted during COSMO SkyMed satellite overpasses. Additional samples were collected in slick and out of slick areas during the RADARSAT-2 satellite overpass by Naoko Kurata, which are discussed in the companion thesis (Kurata, 2012).

3.0 Materials and Methods

3.1 Study Areas

Samples have been collected at a coastal and open water site in the Straits of Florida on September 13, 2011. Four samples were used to pursue DNA analysis. The first sample set was comprised of a microlayer sample and corresponding subsurface water sample, collected from the open ocean approximately five miles offshore. The second sample set was comprised of a microlayer sample and a corresponding subsurface water sample, collected from coastal water approximately one mile from the shore. Two additional control samples were collected earlier on September 10, 2011. These controls were used to check for potential bias that could be introduced by bacteria in the air and/or from the sampler itself. The control samples also determine whether selective adsorption to the sample surface exists (Agogue et al. 2004). All samples are summarized in Table 3.1 with further references to provide details of the sampling conditions. The sampling areas represent four environments: coastal microlayer, coastal subsurface water, open water microlayer, and open water subsurface. Although there were numerous other samples taken on different days, the samples from these days were used for downstream analysis in this pilot project, because they were the cleanest in terms of no subsurface

contamination, they were collected during a satellite overpass, and were also properly recorded to ensure the new method was carefully followed. Even though there were other samples collected that met these criteria, we had to limit the number of samples to be analyzed in this project, due to the relatively high cost of DNA pyrosequencing.

Table 3.1: Details of all sampling conditions in the Straits of Florida.

Sample	Date Time (EST)	Coordinates (GPS)	# of Samples
SML OW	9/13/2011 8:41am	N26°06.858 W79°59.890	1
SSW OW	9/13/2011 8:51am	N26°06.999 W79°59.926	1
SML CW	9/13/2011 9:29am	N26°06.719 W80°04.248	1
SSW CW	9/13/2011 9:36am	N26°06.825 W80°04.299	1
Control Air	9/10/2011 10:55am	N26°09.948 W79°59.653	1
Control Empty	9/10/2011 10:59am	N/A	1

SML OW = sea surface microlayer, open water
SML CW = sea surface microlayer, coastal water
Control Air = sample exposed only to air
Open Water = ~5 miles offshore
SSW OW = subsurface water, open water
SSW CW = subsurface water, coastal water
Control Empty = sample not exposed to any elements
Coastal Water = ~1 mile from coastline

The sample sets were collected during a COSMO SkyMed satellite overpass at 7:21am (Fig 3.1). The comparisons between SAR satellite images taken of the sampling region with the in situ field samples can potentially aid in establishing the link between the presence of surfactant-associated bacteria in the microlayer and SAR imagery of the sea surface. Therefore, further applications in studying fine-scale features on the sea surface and remote sensing techniques used in monitoring the microlayer are expected to be enhanced.

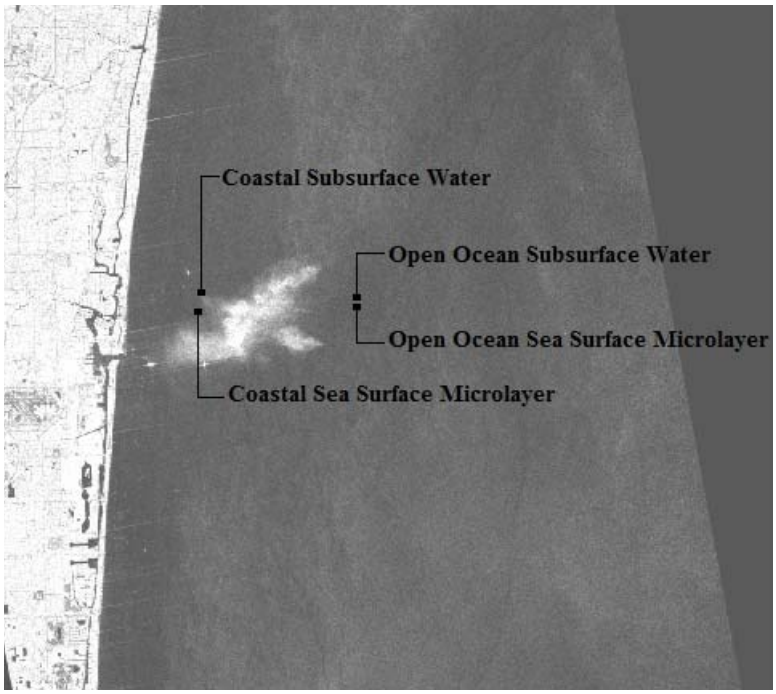

Figure 3.1: COSMO SkyMed satellite image showing sampling locations on 9/13/2011. The bright spot near the sampling locations is a rain signature.

3.2 Sea Surface Microlayer Sample Collection

Two SML samples were collected, one from a coastal region and another from an open water region, using polycarbonate membrane filters (47mm diameter, 0.2µm pore size). These filters retain particles or microorganisms larger than their pore size primarily by surface capture (Advantec MFS, Inc. 2005). A great deal of creativity was involved in developing a proper technique to sample the microlayer without subsurface contamination. Initially, a forcep method was attempted, which was replicated from a previously published study (Franklin et al. 2005). This method involved standing on the dive platform of the research vessel and very carefully placing a polycarbonate membrane filter on the surface of the ocean with sterile forceps. The filter was placed onto the surface of the water for 10 seconds (in calm conditions). However, after testing this sampling method and finding that is was inadequate for use in the high energy environment of the coastal and open ocean waters off South Florida, and was leading to a contamination of the SML samples with subsurface water, a new sampling method was developed.

A superior approach to sample the SML was first tested in the NSUOC boat basin, which provided a calm and controlled area where the technique could be developed and a preliminary sample set was more easily obtained. Once this sampling method was perfected, samples were then collected from the Straits of Florida. This new and improved method consisted of attaching a membrane filter to a fly-fishing nymph hook, which was pre-sterilized in ethanol. The fly-fishing nymph hook was then tied to sterilized fly-fishing line, creating a loop on the opposite end of the hook. The filter, hook, and line were all placed inside a sterile, plastic zip-lock bag until sampling commenced (Fig 3.2). Then when we arrived at the sampling location of interest, the loop created on the fly-fishing line was attached to a snap-swivel at the end of a fishing pole. The zip-lock bag containing the membrane filter was then opened, freeing the filter to cast out from the bow of the research vessel (Fig 3.2). By using the fishing pole, we were able to gain control in allowing the filter to only touch the sea surface, without submerging and this also provided more space between the sample and the research vessel, which eliminated potential contamination from the ship wake. After approximately ten seconds, the filter was removed from the surface and with the use of sterile forceps, removed from the fly-fishing nymph hook (Fig 3.2). The filter was then placed into a new and sterile, plastic zip-lock bag where it was immediately stored on dry ice. This process was repeated, per sampling location and all filters that submerged were rejected. The filters were later stored at -80°C until further DNA analysis was performed.

Figure 3.2: Sampling technique developed for the sea surface microlayer.

3.3 Subsurface Water Sample Collection

Two subsurface water samples were collected from the corresponding microlayer sample sites by pumping water from approximately twenty centimeters of depth below the

surface. The pumping was performed by a portable peristaltic pump (Fig 3.3). This pumping system allows fluid to travel through just the interior of the tubing without touching other pump components. There is no need for tubing connectors, but instead spring loaded clamps grip the exterior portion of the peristaltic tubing and secure it during operation, which reduces the risk of contamination because the tubing and water sample were never in direct contact with the pump mechanics. The tubing was sterilized prior to sampling by pumping ethanol through. Subsurface water was then pumped from the stern of the ship into a sterile, plastic zip-lock bag, filling the bag approximately halfway with water. A polycarbonate membrane filter was then dipped into the pumped water, by use of sterile forceps (Fig 3.3). After dipping the membrane filter for approximately ten seconds, the filter was transferred into a new and sterile, plastic zip-lock bag. This process was repeated at each sampling location. Both the bags containing the pumped water and the membrane filters were immediately stored on dry ice. Later the water and filters were stored at -80°C until processing in the lab.

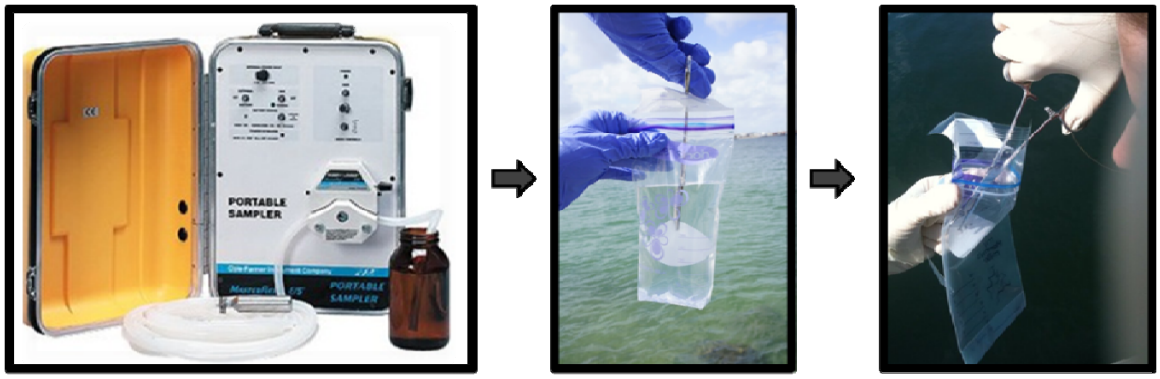

Figure 3.3: Sampling technique developed for the subsurface water.

3.4 Control Sample Collection

Control samples were collected on the same polycarbonate membrane filters. One control was exposed solely to air (referred to as 'control air') for approximately 10 seconds and then placed in a sterile, plastic zip-lock bag. This control was used to check for potential bacterial contamination introduced in the air. The other control sample (referred to 'control empty') was not exposed to any elements, but instead was immediately transferred from its original container straight into a sterile, plastic zip-lock bag. This control was used to check for potential bacterial contamination introduced

from the sampler itself. Both of these control samples were stored at -80°C until processing in the lab.

3.5 DNA Lab Analysis

3.5.1 DNA Extraction and Purification

Direct extraction of total DNA was initially taken from cells on the polycarbonate membrane filter samples taken in the NSUOC boat basin by use of a RapidWater DNA Isolation Kit. This kit is a tool for isolation of genomic DNA from a variety of filtered water samples (MO-BIO Laboratories, Inc. 2010). The kit can isolate high quality DNA from common filter membrane types and is designed for low DNA concentration samples. However, after processing the preliminary samples and not achieving positive results, a different kit was used.

A QIAamp DNA Investigator Kit was instead used to extract the DNA from the polycarbonate membrane filters (following protocol: isolation of total DNA from paper and similar materials). This kit provided fast and efficient purification of genomic DNA from the samples. The main principle of this kit that makes it so efficient is it required the polycarbonate membrane filters to be cut into small pieces before extraction began. Cutting the filters into pieces successfully yielded better results, most likely because there was more surface area available for DNA material to be extracted from. A total volume of 40µl of DNA was extracted from the polycarbonate membrane filters of each sample.

DNA cleanup was then performed on that 40µl of extracted DNA, using the same QIAamp kit (following appendix B: cleanup of DNA). This DNA cleanup offered high DNA purity and concentration. The resulting purified DNA totaled 20µl for each sample, and performed well in downstream analyses.

3.5.2 Polymerase Chain Reaction

Once the DNA was extracted and purified from the filters, polymerase chain reaction (PCR) was then performed. PCR amplifies the desired DNA sequence, which for this case was the bacterial 16S rRNA gene. This gene is highly conserved between different species of bacteria and contains hyper-variable regions which can provide species-

specific signature sequences useful for bacterial identification (Coenye et al. 2003; Rusch et al. 2007). The relationships between 16S genes reflect evolutionary relationships between organisms and therefore a comparison of 16S gene sequence similarities is usually used as the 'gold standard' for taxonomic identification at the species level (Armougom et al. 2009). Universal primers 27F and 1492R (detailed in Table 3.2) were used to target and amplify the 16s rRNA genes of different species of bacteria.

Table 3.2: Universal primers used in polymerase chain reaction.

Primer	Primer Sequence 5' to 3'	Target Group	Reference
27F	AGAGTTTGATCMTGG	Universal	Lane, 1991
1492R	TACCTTGTTACGACTT	Universal	Lane, 1991

The following PCR reaction was setup for each sample, which consisted of:
1.0 µl of DNA (as extracted earlier)
1.0 µl of 27F primer
1.0 µl of 1492R primer
2.5 µl of buffer
0.5 µl of dNTPs
0.5 µl of Qiagen Taq polymerase
18.5 µl of molecular grade H_2O
25.0 µl total per sample reaction

A master mix containing all of the above components was prepared to facilitate pipetting, and then divided according to the number of samples/reactions (6 for this study). This master mix was prepared on ice, with the molecular grade water and buffer added first, and the Taq polymerase added last. Then PCR was facilitated in a thermocycler. The specific PCR cycle conditions are summarized in Table 3.3, to provide further details of the settings administered.

Table 3.3: PCR cycle conditions.

Step	Cycles	Temperature (°C)	Duration
1. Initial Denaturation	1	95	2 minutes
2. Denaturation	1	95	30 seconds
3. Annealing	1	50	30 seconds
4. Elongation	1	72	1 minute
5. Repeat #2 - 4	30		
6. Final Elongation	1	72	5 minutes
7. Cooling	1	4	forever

Normally after PCR cycles have completed, an agarose gel is prepared in order to visualize the PCR products. However in this case, and in order to conserve as much PCR product for downstream analysis, no gel was prepared but instead nested PCR was immediately performed.

3.5.3 Amplicon Library Construction (Nested PCR)

Often following the first PCR amplification of the 16S rRNA gene, non-specific binding in products occurs due to the amplification of unexpected primer binding sites. Therefore, a second PCR reaction (referred to as nested PCR) is a modification that reduces that non-specific binding. In this study, nest PCR was utilized to prepare amplicon libraries for downstream amplicon sequencing. Amplicon library construction involves two fusion primers, intended to amplify a secondary target within the first run PCR product (Roche Applied Science, 2007). When preparing DNA samples for amplicon libraries, the fusion primers must be designed according to the particular requirements of the experiment.

Specific fusion primers were designed in constructing amplicon libraries for each sample of this study (Roche Applied Science, manual version 001-2009). Each forward fusion primer contained a directional Primer-A sequence at the 5-prime end of the oligonucleotide (Fig 3.4; Table 3.4). This sequence binds to the DNA capture beads and anneals the amplification and sequencing primers of emPCR kits (Roche Applied Science, 2007). The Primer-A sequences end with a four-base sequencing key "TCAG", used in downstream software for base calling and to recognize legitimate library reads (shown in red in Fig 3.4). A unique multiplex identifier (MID, also referred to as a 'barcode') sequence was then attached after the sequencing key, which allows for independent samples to be pooled together for sequencing (Roche Applied Science, 2009). Each sample was assigned with its own, 10-nucleotide barcode (Table 3.5), which also allows for automated software identification of samples after sequencing and subsequent bioinformatic segregation (Parameswaran et al. 2007). The reverse primer designs (Primer-B) did not require a MID because the amplicon libraries were unidirectionally sequenced, meaning the amplicons were only sequenced from fusion Primer-A. Finally, following the MID was a template-specific primer (shown in purple

in Fig 3.4), or for the reverse primers the template-specific primer followed the sequencing key. The template-specific primers (357F, 805R) were designed to anneal to either side of the target to be sequenced (detailed in Table 3.4). This end also served as the PCR amplification primer during library preparation (454 Life Sciences, 2006).

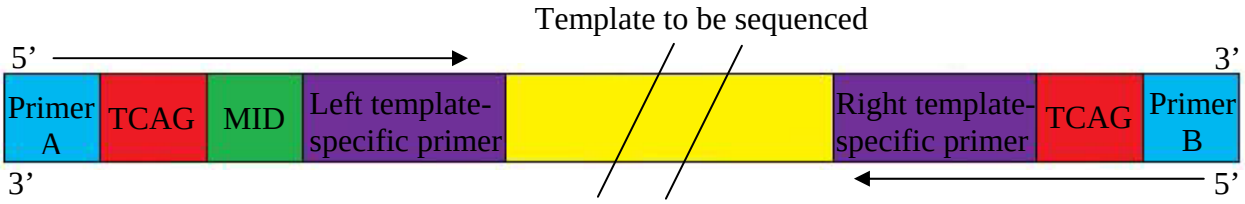

Figure 3.4: Schematic representation of an Amplicon library product (Adapted from Roche Applied Science, 2007).

Table 3.4: Directional and template-specific primer sequences used to construct all amplicon libraries.

Primer	Primer Sequence 5' to 3'	Reference
Primer-A (forward)	CCATCTCATCCCTGCGTGTCTCCGAC	Roche Applied Science, 2007
357F	TACGGGAGGCAGCAG	Lane, 1991
Primer-B (reverse)	CCTATCCCCTGTGTGCCTTGGCAGTC	Roche Applied Science, 2007
805R	GACTACCAGGGTATCTAATC	Sfanos et al. 2005

Table 3.5: Unique Multiplex Identifier (MID) sequences assigned to individual samples in constructing amplicon libraries.

Sample	MID ID#	MID Sequence 5' to 3'	Reference
SML OW	MID9	TAGTATCAGC	Roche Applied Science, 2007
SSW OW	MID10	TCTCTATGCG	Roche Applied Science, 2007
SML CW	MID11	TGATACGTCT	Roche Applied Science, 2007
SSW CW	MID12	TACTGAGCTA	Roche Applied Science, 2007
Control Air	MID5	ATCAGACACG	Roche Applied Science, 2007
Control Empty	MID8	CTCGCGTGTC	Roche Applied Science, 2007

SML OW = sea surface microlayer, open water
SML CW = sea surface microlayer, coastal water
Control Air = sample exposed only to air
Open Water = ~5 miles offshore
SSW OW = subsurface water, open water
SSW CW = subsurface water, coastal water
Control Empty = sample not exposed to any elements
Coastal Water = ~1 mile from coastline

The following reaction was setup for each sample using a FastStart High Fidelity PCR System kit (Roche Applied Science, version 6.0), which consisted of:
5.0 µl of DNA (PCR product of first reaction)
1.0 µl of Fusion forward primer
1.0 µl of Fusion reverse primer
5.0 µl of FastStart High Fidelity buffer
1.0 µl of dNTPs
1.0 µl of FastStart High Fidelity Taq polymerase
41.0 µl of molecular grade H_2O
55.0 µl total per sample reaction

Again, the above reaction was prepared on ice, with the molecular grade water and FastStart High Fidelity buffer added first, and the FastStart High Fidelity Taq polymerase added last. The same PCR cycle conditions summarized in Table 3.3 were again administered. Then, after the PCR cycles completed, an agarose gel was prepared in order to visualize the nested PCR products (Fig 3.5).

The following 1% agarose gel was prepared, which consisted of:
50.0 ml of TAE buffer
500.0 mg of agarose powder
5.0 µl of Sybrsafe

The agarose powder was mixed in the buffer and microwaved until the agarose was dissolved. The solution was then cooled until warm to the touch and the sybrsafe was added. The gel was mixed well, poured into a boat, and cooled for another 15 minutes (to solidify). The first lane of the gel was loaded with 6.0 µl of 100bp DNA ladder (BioLabs, Inc. N3231S). The subsequent lanes were loaded with 1.0 µl of loading dye and 5.0 µl of the nested PCR product. The gel ran for one hour at 120 volts.

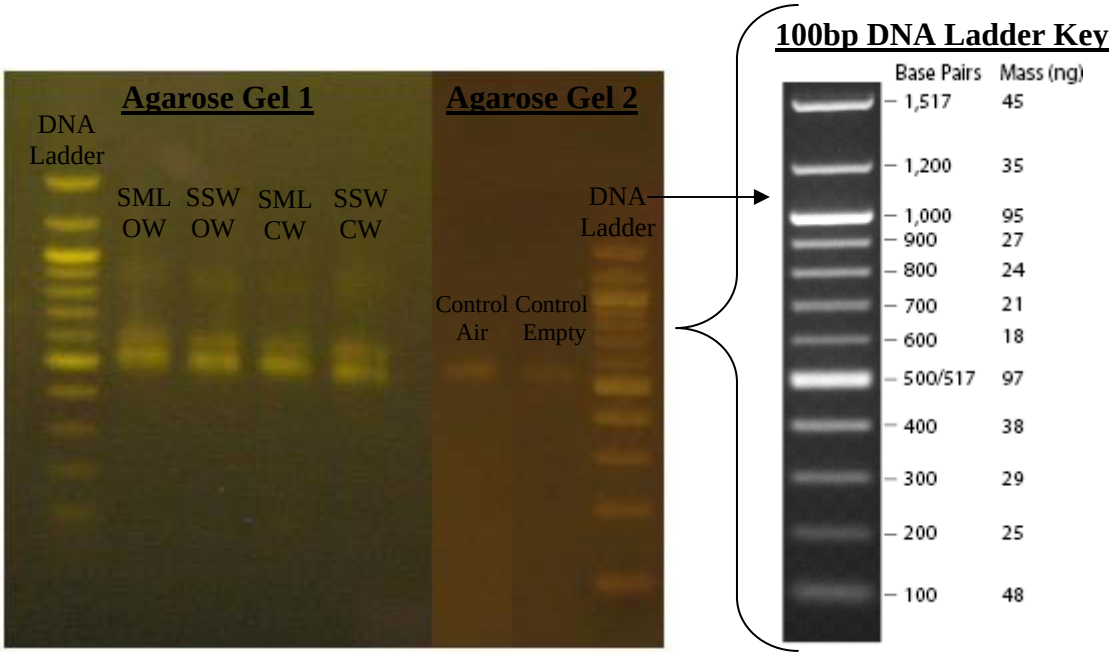

Figure 3.5: Agarose gel pictures of the nested PCR products from the 4 samples collected on 9/13/2011 (Agarose Gel 1) and the two control samples collected on 9/10/2011 (Agarose Gel 2). On the far right is the 100bp DNA ladder key, showing standard marker sizes (BioLabs, Inc. N3231S).

As seen on the agarose gel pictures in Figure 3.5, the bands of all the samples were near the 500 base pair marker of the DNA ladder. This was an important requirement in designing the amplicon libraries. The amplicon could not be any longer than 500 base pairs, from end to end (including the fusion primers) because templates longer than this do not amplify well in downstream emulsion-based clonal amplification. Therefore, an insert size of 448 base pairs was accomplished by using the template-specific primers (357F, 805R), which was still sufficient to cover two of the hyper-variable regions of the 16S rRNA gene.

However, the bands in agarose gel 1 showed smearing and contained multiple bands that were not targeted. A method to eradicate the smearing and multiple bands would be to perform an agarose gel extraction. However in an effort to conserve time, no gel extraction was administered but instead the amplicon libraries were purified before undergoing emulsion-based clonal amplification in the next step (by the University of Florida).

After the amplicon libraries were prepared, they were placed on dry ice and shipped overnight to the University of Florida (UF), ICBR Genomics Division, where emulsion-

based clonal amplification and 454 pyrosequencing were administered. The UF lab preferred to have at least 300ng of each target amplicon library. Meaning, the microlayer and subsurface samples all had approximately 10ng/µl, so then 30µl (or 300ng) of each sample was needed to send to UF. To ensure that UF had enough product to work with, 40µl (or 400ng) of each sample were sent to their lab. The control samples, however, both had more mass and therefore a smaller volume was required to send to the UF lab. The details of each amplicon library sent to UF are summarized in Table 3.6.

Table 3.6: The mass and appropriate volumes of each amplicon library sent to the University of Florida.

Sample	SML OW	SSW OW	SML CW	SSW CW	Control Air	Control Empty
ng/µl	~10	~10	~10	~10	~40	~20
Volume (µl)	40	40	40	40	15	15
DNA Size	~500bp	~500bp	~500bp	~500bp	~500bp	~500bp

SML OW = sea surface microlayer, open water
SSW OW = subsurface water, open water
SML CW = sea surface microlayer, coastal water
SSW CW = subsurface water, coastal water
Control Air = sample exposed only to air
Control Empty = sample not exposed to any elements
Open Water = ~5 miles offshore
Coastal Water = ~1 mile from coastline

3.5.4 Emulsion-Based Clonal Amplification

Once the amplicon libraries arrived at the University of Florida's ICBR Genomics Division lab, sample processing began with Quality Control, which evaluated the amplicon libraries for size and quantity to ensure the samples were pure (no primers). As suspected, the data showed the presence of smaller fragments, so the samples underwent cleanup to remove these smaller fragments. Equal amounts from each sample were then pooled based on their concentrations and the pooled DNA was quantitated again before being subjected to emulsion-based clonal amplification (emPCR).

The emPCR amplification process was performed on the whole amplicon library, using a GS FLX Titanium emPCR Kit (Roche Applied Science, manual version FLX.Ti.00 – USM-00056.B). In emPCR, the DNA fragments were physically separated in an emulsion. This allowed for bias-free amplification of the DNA molecules by entrapping them in lipid microreactors, which eliminated any competition from multiple templates (Schuster, 2008). Meaning that during the PCR, each DNA fragment was independently confined into a droplet of oil and water containing the PCR reagents (Armougom et al.

2009). This emPCR is a unique process that eradicated the need for cloning the target sequences because the templates were handled in bulk within their respective emulsions (Margulies et al. 2005). The entire amplification process consisted of seven main steps and took a few hours, where the final product was a sequencing-ready library of clonally amplified, single-stranded DNA fragments. The DNA library was then loaded onto a picotiter plate (PTP) device, where the clonally amplified fragments were distributed evenly and were then ready for sequencing.

3.5.5 454 Pyrosequencing

The amplified DNA fragments that were previously loaded onto the PTP device were inserted into and sequenced on a Genome Sequencer FLX Instrument (Roche Applied Science, instrument version GS FLX – 2.0.01). This instrument automatically performed and monitored the sequencing reactions in all the wells of the PTP device simultaneously, providing a unique technology that efficiently sequenced the single DNA molecules and enabled a comprehensive view into the diversity of the environmental samples of this study.

The Genome Sequencer process is referred to as 454 pyrosequencing because the sequencing technology is based on the detection of pyrophosphates released during DNA synthesis (Parameswaran et al. 2007). Therefore, this DNA sequencing method follows a 'sequencing-by-synthesis' principle, which relies on efficient detection of the sequential incorporation of natural nucleotides during the synthesis of DNA (Ronaghi et al. 1998). Pyrosequencing is thus a technique built on a 4-enzyme real-time monitoring of DNA synthesis by bioluminescence (Ahmadian et al. 2006). The pyrosequencing technique includes four enzymes that are involved in a cascade reaction system (Fig 3.6). When a nucleotide is introduced in the DNA-strand, a detectable light signal is produced (Ahmadian et al. 2006). Therefore, a light signal is only detected if a base pair is formed with the DNA template, and the signal strength is proportional to the number of nucleotides incorporated in a single nucleotide flow (Armougom et al. 2009).

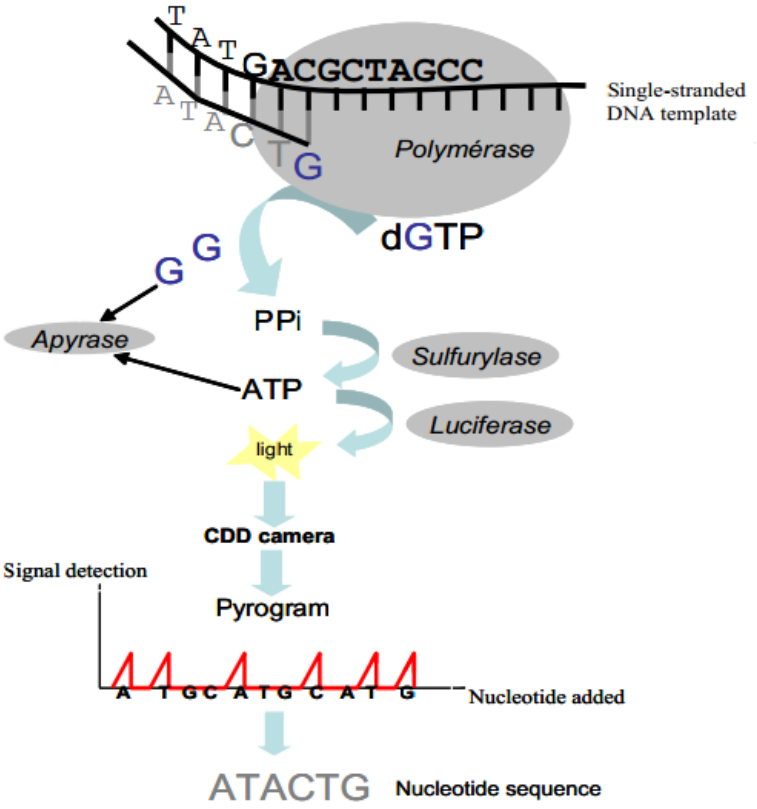

Figure 3.6: Principle of pyrosequencing technology.
A single-stranded DNA template is incubated with the enzymes, DNA polymerase, ATP sulfurylase, luciferase and apyrase. The incorporation of a nucleotide is accompanied by release of pyrophosphate (PPi). The ATP sulfurylase converts PPi to ATP. The signal light produced by the luciferase-catalyzed reaction in presence of ATP is detected by a charge coupled device (CCD) camera and integrated as a peak in a Pyrogram. The process continues with addition of the next dNTP and the nucleotide sequence of the complementary DNA strand is inferred from the signal peaks of the pyrogram. (Armougom et al. 2009).

The output of the sequencing run contained raw DNA sequencing data that was further analyzed using software, according to the objectives of this study. However, analyzing such massive nucleotide sequence collections can overwhelm existing computational resources and analytic methods (Cai et al. 2011). Therefore, sophisticated software that can handle this massive dataset was required.

3.6 Data Analysis - Bioinformatics

In order to take the sequencing data from raw sequences to interpretation, the quantitative insights into microbial ecology (QIIME) software was utilized. QIIME is an open-source

software pipeline built using the PyCogent toolkit and supports a wide range of microbial community analysis and visualizations (Caporaso et al. 2010). This software thus provided a robust platform for combining the experimental datasets and for rapidly obtaining new insights about various microbial communities targeted in this study.

The following analyses were performed using QIIME (software version 1.5.0) and are further addressed in the proceeding results:

- The DNA sequence reads were filtered for quality and multiplexed reads were assigned to samples by nucleotide barcode (parameter: barcode type = 10).
- Operational taxonomic units were picked based on sequence similarity within the reads, and a representative sequence from each was chosen.
- The operational taxonomic units were assigned to a taxonomic identity using reference databases.
- Communities were then summarized according to their taxonomic compositions.
- Diversity metrics were calculated for each sample to compare the types of communities, using the taxonomic assignments.

4.0 Results

4.1 Preliminary Study

In the preliminary analysis of the samples collected from the NSUOC boat basin, DNA was cloned following the initial PCR. In order to identify what bacterial populations were present in our samples, individual DNA fragments needed to be separated, which was facilitated by DNA cloning. DNA sequencing was then administered to determine the order of nucleotide bases of targeted DNA. From the sequenced data, information regarding the taxonomic origins of the samples was obtained through the basic local alignment search tool (BLAST) program. This program allows for the comparison of the sequenced data with a library or database of sequences, and then identifies library sequences that resemble the original sequence.

The preliminary sequencing analysis suggested the presence of bacteria related to a strain of uncultured gammaproteobacteria, previously isolated from coastal sediment along a hydrocarbon contamination gradient (BLAST accession: FR670377.1; EC-value: 97%).

Many genera of the gammaproteobacteria are known to be involved in surfactant production and degradation, which is discussed later.

This preliminary study indicated that the bacterial composition of the sea surface microlayer could be effectively determined using the proposed sampling method. Therefore, further DNA analysis was warranted. Instead of cloning DNA fragments, the subsequent samples were processed using next generation 454 pyrosequencing technology (as discussed in section 3.5.5) and then analyzed in QIIME (section 3.6) to compensate for the small sample size and to achieve more comprehensive results.

4.2 Assigning Samples to Multiplex Reads

The output from the 454 pyrosequencing run produced a total 61,663 raw sequences. The sequences were not evenly distributed among all the samples, but in fact the subsurface coastal water contained the largest number (Table 4.1). Also, the number of sequences was remarkably lower in both the controls, as compared to the rest of the samples, signifying a lower number of bacterial populations present within the control samples.

Table 4.1: The distribution of raw sequences among each sample.

Sample	SML OW	SSW OW	SML CW	SSW CW	Control Air	Control Empty	**Total**
# of Sequences	15,814	10,963	7,560	19,925	3,514	3,887	**61,663**

SML OW = sea surface microlayer, open water
SSW OW = subsurface water, open water
SML CW = sea surface microlayer, coastal water
SSW CW = subsurface water, coastal water
Control Air = sample exposed only to air
Control Empty = sample not exposed to any elements
Open Water = ~5 miles offshore
Coastal Water = ~1 mile from coastline

Assigning multiplexed reads to the samples according to their nucleotide barcode in QIIME allowed for quality filtering of the above sequences, based on the characteristics of each sequence. This task removed any low quality or ambiguous reads, which allowed for proper subsequent operational taxonomic unit (OTU) picking and taxonomic assignment.

4.3 Picking Operational Taxonomic Units

Using QIIME software, all the sequences from all of the samples were clustered into OTUs, based on their level of sequence similarity. In other words, OTUs are clusters of sequences, frequently intended to represent some degree of taxonomic relatedness (Sun et

al. 2010). For example, when sequences are clustered at 97% sequence similarity, each resulting cluster is typically thought of as representing a species (Crawford et al. 2009). Although the current techniques for picking OTUs are known to be imperfect, determining exactly how OTUs should be defined, and what they represent, is an active area of research (Crawford et al. 2009).

A total of 695 OTUs were picked from the raw sequence data. The distribution of those OTUs among each sample is outlined in Table 4.2. Since each OTU may be made up of many related sequences, a representative sequence from each OTU was picked for downstream analysis. This representative sequence was then used for taxonomic identification of the OTU.

Table 4.2: The distribution of operational taxonomic units among each sample.

Sample	SML OW	SSW OW	SML CW	SSW CW	Control Air	Control Empty	**Total**
# of OTUs	136	67	201	112	70	109	**695**

SML OW = sea surface microlayer, open water
SML CW = sea surface microlayer, coastal water
Control Air = sample exposed only to air
Open Water = ~5 miles offshore
SSW OW = subsurface water, open water
SSW CW = subsurface water, coastal water
Control Empty = sample not exposed to any elements
Coastal Water = ~1 mile from coastline

As shown in Table 4.2, there were more OTUs in the microlayer samples from both open and coastal water as compared to their corresponding subsurface samples. This indicated higher microbial diversity present in the SML samples, however that does not necessarily mean those OTUs were surfactant related. Further analyses of the targeted surfactant-associated bacterial populations are addressed later.

4.4 Taxonomic Identity of Operational Taxonomic Units

Each of the representative sequences mentioned in the previous section were assigned to taxonomic identities using the established database, Ribosomal Database Project (RDP) classifier, version 2.2 (Wang et al. 2007). This database provided information on the microbial lineages found within the samples. For each OTU considered, there was an RDP taxonomy assignment with a numerical confidence of that assignment. Then using those taxonomic assignments, an OTU heatmap (Fig 4.1) was assembled, which displays the OTU abundance in each sample and the taxonomic assignment for each OTU. The

counts on the heatmap are colored based on the contribution of each OTU to the total OTU count present in the sample (i.e. blue: contributes low percentage of OTUs to sample; red: contributes high percentage of OTUs). This provided a convenient way to look for organisms (and their lineages) of interest in this study.

Consensus Lineage					Samples						
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**SML OW**	**SSW OW**	**SML CW**	**SSW CW**	**Control Air**	**Control Empty**	**#OTU ID**
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	424		7		1	1	58
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		3	397				79
Bacteria	Cyanobacteria	Chloroplast	Stramenopiles	N/A	4	1	31				107
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	174		350		256	144	165
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae						327	470
Bacteria	Cyanobacteria	Chloroplast	Stramenopiles	N/A				312			515
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae		1	298				744
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	345			323			842
Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Litincolaceae				298			844
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae		3	672			1	1033
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	251						1144
Bacteria	Bacteroidetes	Flavobacteria	N/A	N/A	373		1				1145
Bacteria	Bacteroidetes	Flavobacteria	N/A	N/A				1278			1171
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	305	6	528				1297
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae			4		222	97	1374
Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	N/A	3	312					1377
Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	N/A	104	513	5	493			1416
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	1	1666	1				1430
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	121	879					1579
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	2355	1	3	3			1693
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	2	6	95	2			1801
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	985		231	30			1804
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	201					175	1892
Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	N/A				221			2075
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	837	2106	405	581	216	453	2171
Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	449			1			2195
Bacteria	Cyanobacteria	Synechococcophycideae	Synechococcales	Synechococcaceae	93	257	26	113			2256
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	319	2	293				2274
Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	92	798	1		68		2455
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	1385			3			2491
Bacteria	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	119		1	427			2598
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	92		22	410			2687
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	527		3				2770
Bacteria	Firmicutes	Bacilli	Bacillales	N/A	12	2		2		3	2876
Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	N/A	119		65	671			2899
Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	101			420			2902
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	698	1987	861	2140	292	591	3028
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae						341	3084
Bacteria	Cyanobacteria	Synechococcophycideae	Synechococcales	Synechococcaceae	15	244	116	255	24		3195
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae			1	537			3508
Bacteria	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	1	1	55	1	599	222	3527
Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	121		1	215			3560
Bacteria	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae			1	634			3738
Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae		350	133	833			3752
Bacteria	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae			2	366			3765
Bacteria	Bacteroidetes	Sphingobacteria	Sphingobacteriales	N/A	454	765		1			3769
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae		627	6				3840
Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	198		369				3906
Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	N/A		624					3966
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae			4				3986
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	2		22		1098		4010
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	802		440	3239			4044

Figure 4.1: Operational Taxonomic Unit Heatmap.

SML OW = sea surface microlayer, open water
SSW OW = subsurface water, open water
SML CW = sea surface microlayer, coastal water
SSW CW = subsurface water, coastal water
Control Air = sample exposed only to air
Control Empty = sample not exposed to any elements
Open Water = ~5 miles offshore
Coastal Water = ~1 mile from coastline

4.5 Community Summary by Bacterial Taxonomic Composition

Each OTU was further grouped into categories based on their different taxonomic levels, or the rank-based classification of bacteria. In biology, the scientific classification system establishes a hierarchy of rank in which each organism is assigned to (Linnaeus, 1758). In the currently accepted hierarchy of biological classification there are nine major taxonomic levels which include: Life, Domain, Kingdom, Phylum, Class, Order, Family, Genus and Species (Fig 4.2). With Life being the highest rank, there are then three domains that branch from Life: Archaea, Bacteria, and Eukaryotes. These domains have several different conventions between them and between their subdivisions in terms of taxonomy, and as such are studied by different disciplines (Woese et al. 1990). Following the bacteria domain is Kingdom, which includes six groups (animalia, archaea, bacteria, fungi, plantae, and protista). Next is Phylum, the taxonomic rank below kingdom, which is further analyzed in the following section.

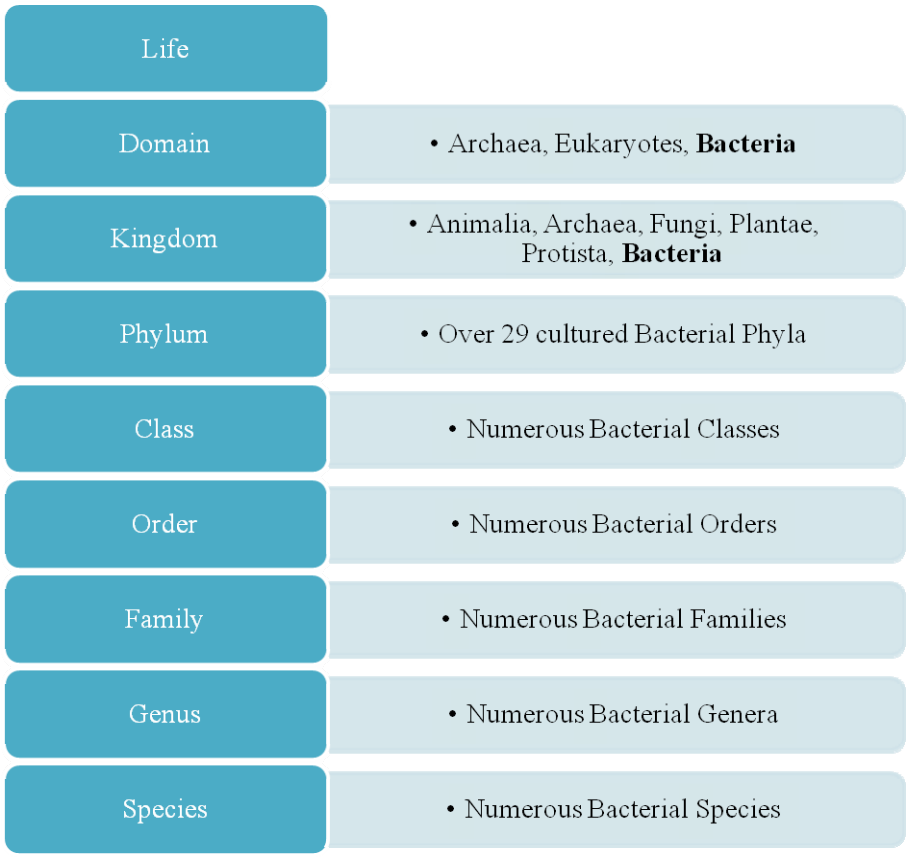

Figure 4.2: The hierarchy of biological classification, containing nine major taxonomic levels.

4.5.1 Phylum Taxonomic Classification

The phylum rank can be defined as grouping organisms based on a certain degree of morphological or developmental similarity, or with a certain degree of evolutionary relatedness (Valentine, 2004). In the classification system, there are over 29 bacterial phyla divisions that have been cultured and many others that cannot currently be cultured (Madigan et al. 2009). The uncultured groups are known solely by metagenomics (methods to analyze environmental DNA) and if included, the number of bacterial phyla would reach 52 or higher (Rappe et al. 2003). The distribution and relative abundance of the bacterial populations on the phylum level, found within each sample of this study are illustrated in Figure 4.3 and Table 4.3 respectively, and are useful for providing a broad overview of the important bacterial groups present.

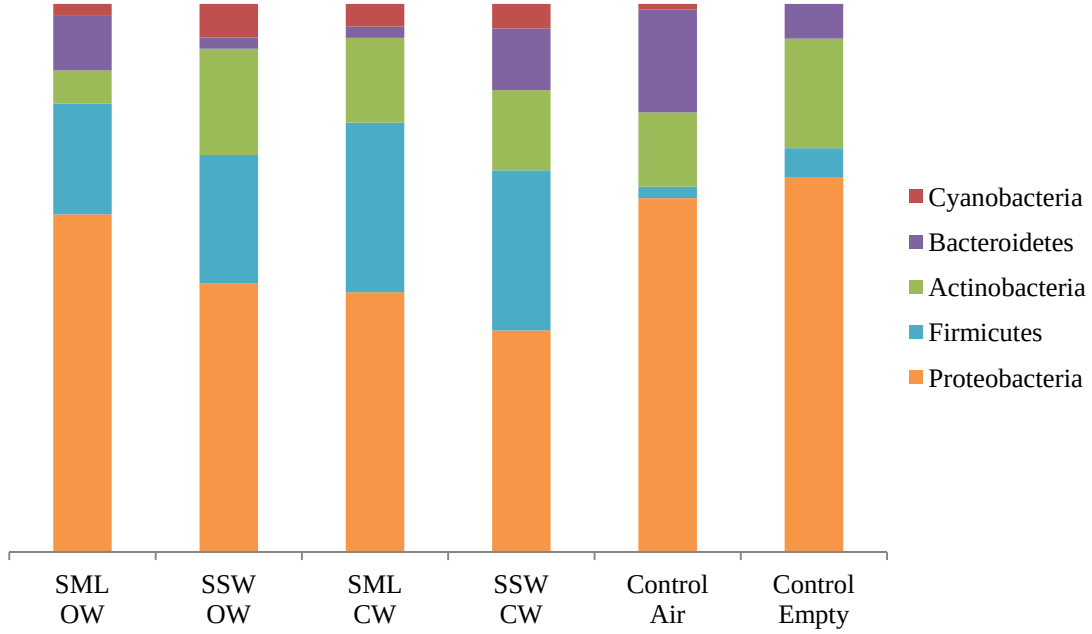

Figure 4.3: Phylum taxonomic assignment distribution in each sample.

Table 4.3: Relative abundance of phylum level bacterial populations present within each sample.

Sample	SML OW	SSW OW	SML CW	SSW CW	Control Air	Control Empty
Date Time (EST)	9/13/2011 8:41 am	9/13/2011 8:51 am	9/13/2011 9:29 am	9/13/2011 9:36 am	9/10/2011 10:55am	9/10/2011 10:59am
Proteobacteria Phylum	61%	48%	46%	36%	62%	65%
Firmicutes Phylum	20%	23%	30%	26%	2%	5%
Actinobacteria Phylum	6%	19%	15%	13%	13%	19%
Bacteroidetes Phylum	10%	2%	2%	10%	18%	6%
Cyanobacteria Phylum	2%	6%	4%	4%	1%	0%

SML OW = sea surface microlayer, open water
SSW OW = subsurface water, open water
SML CW = sea surface microlayer, coastal water
SSW CW = subsurface water, coastal water
Control Air = sample exposed only to air
Control Empty = sample not exposed to any elements
Open Water = ~5 miles offshore
Coastal Water = ~1 mile from coastline

4.5.1.1 Proteobacteria Phylum

Phylum: <u>Proteobacteria</u>
Class: Gammaproteobacteria
Order: Pseudomonadales
Family: Pseudomonadaceae
Genus: Pseudomonas - degrade aromatic hydrocarbons

Phylum: <u>Proteobacteria</u>
Class: Gammaproteobacteria
Order: Pseudomonadales
Family: Moraxellaceae
Genus: Acinetobacter – produce biosurfactants

Phylum: <u>Proteobacteria</u>
Class: Gammaproteobacteria
Order: Oceanospirillales
Family: Halomonadaceae
Genus: Halomonas – produce biosurfactants

Phylum: <u>Proteobacteria</u>
Class: Gammaproteobacteria
Order: Enterobacteriales
Family: Enterobacteriaceae
Genus: Enterobacter – produce biosurfactants

The results show that Proteobacteria were the most abundant bacterial group present throughout all the samples in the phylum level. This bacterial phylum contains many genera (listed above) of bacteria that are able to degrade aromatic hydrocarbons (Zocca et al. 2004) or that have been reported to produce biosurfactants (Satpute et al. 2010). This bacterial group is also responsible for nitrogen fixation as well as converting energy from light through photosynthesis (Stackebrandt et al. 1988). Although this group was present

in all samples, the highest percentage was found in the sea surface microlayer sample taken from the open water. Considering the SML is where exposure to the sun is most extreme, and this phylum is known for photosynthesis, their large presence in the microlayer would enable high productivity for photosynthesis. Although the abundance of Proteobacteria was slightly less in the SML sample taken from coastal water as compared to the open water, there was still a greater percentage present in the microlayer samples than in their corresponding subsurface water samples. This indicates that more of this bacterial group dwells in the microlayer.

Moreover, because this phylum contains numerous genera that are known to either produce surfactants or degrade aromatic hydrocarbons, their potential influence on surfactant production and transformation is of high interest. Considering this phylum group is widely dispersed in microlayer and subsurface waters, they may play a role in surfactant production and transformation throughout the whole water column.

It is also important to take into consideration the high percentages in both the control samples for this phylum. In view of the 'empty' control sample showing 65% proteobacteria present, there may be considerable contamination regarding this bacterial phylum.

4.5.1.2 Firmicutes Phylum

Phylum: <u>Firmicutes</u>
Class: Bacilli
Order: Bacillales
Family: Bacillaceae
Genus: Bacillus – produce biosurfactants

Firmicutes were the next largest phylum of bacteria present in all samples. Firmicutes contain the bacterial genus, Bacillus, which is known to produce biosurfactants (Satpute et al. 2010). They are also found in various environments, can survive extreme conditions, and produce energy through photosynthesis (Wolf et al. 2004). The highest abundance of this bacterial group was present in the coastal microlayer. The other samples had similar percentages, with only slight variations. Most importantly, this

group was present in very low numbers in the control groups, indicating little contamination in water samples.

Considering that this phylum contains the surfactant producer, Bacillus and was most abundant in the coastal microlayer, perhaps this group plays a significant role in surfactant concentration within the sea surface.

4.5.1.3 Actinobacteria Phylum

Phylum: <u>Actinobacteria</u>
Class: Actinobacteria
Order: Actinomycetales
Family: Nocardiaceae
Genus: Rhodococcus – produce biosurfactants

Actinobacteria are a phylum of bacteria that include some of the most common marine and freshwater life, playing an important role in decomposition of organic materials and thereby playing a vital part in organic matter turnover and carbon cycling (Ventura et al. 2007). This phylum also contains Rhodococcus, a genus of bacteria that produce biosurfactants (Satpute et al. 2010). The subsurface of the open water sample contained the highest abundance of this group, with the corresponding microlayer sample having considerably less of a percentage. Conversely, in the coastal water samples, there was a higher percentage in the microlayer than in the subsurface waters but not as much of a stark difference between the two. This data reveals some insight into the possible role that Actinobacteria may play in the production of surfactants throughout the water column. Furthermore, it seems their proposed influence on surfactant concentration is greatest in the coastal subsurface waters.

Bear in mind that the control samples did show a substantial percentage of this group, therefore there may be significant contamination in the other samples in regards to this bacterial phylum.

4.5.1.4 Bacteroidetes Phylum

The next phylum of bacteria present in all the samples was Bacteroidetes. Although there are no known genera from this phylum that are directly related to surfactant production or

transformation, Bacteroidetes are known to be widely distributed in the environment, including seawater and could therefore be potentially surfactant related (Gupta et al. 2007). Interestingly, the results from this phylum show inverse abundances for open water versus coastal water. Meaning, there was a greater percentage present in the open water microlayer than the corresponding subsurface, but equal and opposite abundances in the coastal waters. Because this group is known to be widely distributed in seawater, this could explain for the varying abundance results.

The control samples here also contained a significant percentage of this phylum, so there may be considerable contamination in the water samples.

4.5.1.5 Cyanobacteria Phylum

Cyanobacteria are the last bacterial phylum that was present in all water samples. Again, this phylum does not contain any known genera that are directly related to surfactant production or transformation, however, they can be found in oceans and freshwater, forming biofilms in marine environments (Flores, 2008). Aquatic cyanobacteria are best known for the highly visible blooms that can form in the marine environment that have a blue-green appearance. These blooms are toxic and frequently lead to closure of recreational waters. Cyanobacteria also produce exopolysaccharides, which are carbohydrate polymers that form a layer surrounding the cells that help them to withstand or resist adverse and extreme environmental conditions (Satpute et al. 2010). Despite there being no known genera from this phylum directly related to surfactants, there are many marine microbes that have yet to be identified. Therefore, cyanobacteria could potentially have a surfactant influence.

Cyanobacteria were more abundant in the open water subsurface than the corresponding microlayer, but present in equal percentages from coastal microlayer and subsurface waters. It is important to also note that the results show no cyanobacteria present in the control ‘empty’ sample, meaning there was no contamination introduced in the samples from this phylum.

4.5.2 Family Taxonomic Classification

Although Class and Order are the sequential taxonomic ranks that follow Phylum, the Family rank is the most specific classification the data analysis produced in this study and therefore contains the most valuable information regarding which surfactant-associated bacterial groups were present in the samples. Family is a stable taxonomic level for evolutionary studies, containing groups of organisms with a large degree of evolutionary relatedness (Sahney et al. 2010). Furthermore, the family taxonomic level is often used in biodiversity studies because genera and species cannot always be confidently identified (Sahney et al. 2010). The following results reveal the surfactant-associated bacterial populations found in the samples on the family level of classification (illustrated in Figure 4.4). Bacterial diversity was calculated in Table 4.4 for each sample based on the parameter, V_F, which is defined as the ratio of the number of surfactant-associated bacterial groups in an individual sample to the total number of identified surfactant-associated bacterial groups in all samples (9 families in our analysis).

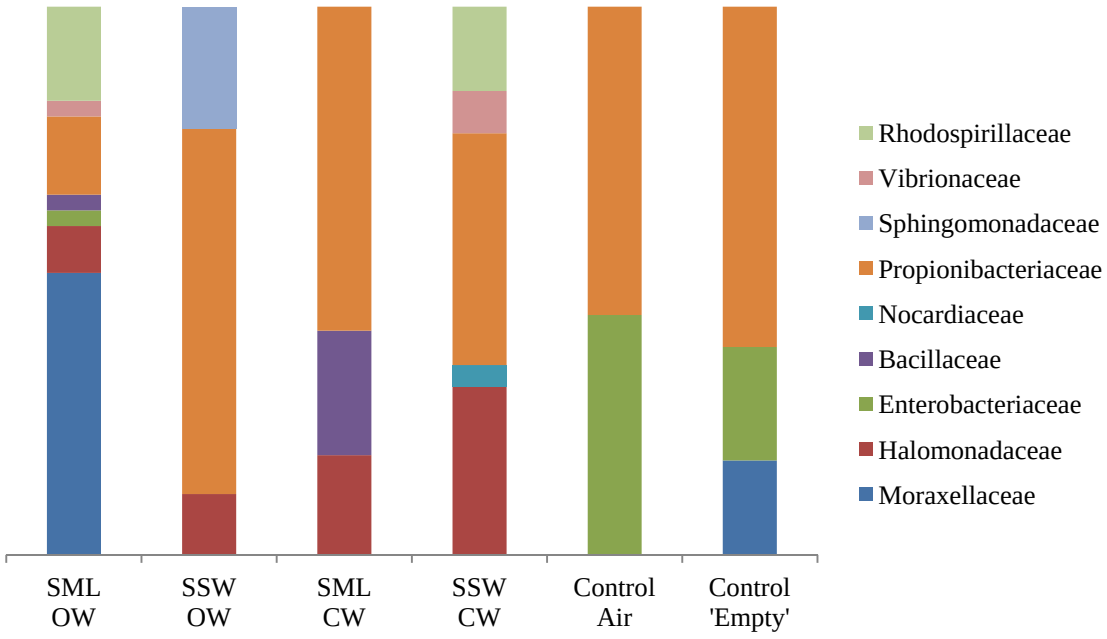

Figure 4.4: Distribution of the family taxonomic surfactant-associated bacterial populations found in each sample.

Table 4.4: Diversity and relative abundance of family level surfactant-associated bacterial populations present within each sample.

	Sample	SML OW	SSW OW	SML CW	SSW CW	Control Air	Control Empty
	Date Time (EST)	9/13/2011 8:41 am	9/13/2011 8:51 am	9/13/2011 9:29 am	9/13/2011 9:36 am	9/10/2011 10:55am	9/10/2011 10:59am
Phylum	**Family**						
Proteobacteria	**Moraxellaceae**	18%	0%	0%	0%	0%	5%
Proteobacteria	**Halomonadaceae**	3%	3%	4%	8%	0%	0%
Proteobacteria	**Enterobacteriaceae**	1%	0%	0%	0%	7%	6%
Firmicutes	**Bacillaceae**	1%	0%	5%	0%	0%	0%
Actinobacteria	**Nocardiaceae**	0%	0%	0%	1%	0%	0%
Actinobacteria	**Propionibacteriaceae**	5%	18%	13%	11%	9%	18%
Proteobacteria	**Sphingomonadaceae**	0%	6%	0%	0%	0%	0%
Proteobacteria	**Vibrionaceae**	1%	0%	0%	2%	0%	0%
Proteobacteria	**Rhodospirillaceae**	6%	0%	0%	4%	0%	0%
	Diversity among surfactant-associated bacteria, V_F	0.78	0.33	0.33	0.56	0.22	0.33

SML OW = sea surface microlayer, open water
SSW OW = subsurface water, open water
SML CW = sea surface microlayer, coastal water
SSW CW = subsurface water, coastal water
Control Air = sample exposed only to air
Control Empty = sample not exposed to any elements
Open Water = ~5 miles offshore
Coastal Water = ~1 mile from coastline
V_F = number of surfactant-associated bacterial families present in an individual sample ÷ the total number of identified surfactant-associated bacterial families in all samples

4.5.2.1 Moraxellaceae Family

Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Pseudomonadales
Family: <u>Moraxellaceae</u>
Genus: Acinetobacter – produce biosurfactants

The Moraxellaceae family are a part of the Proteobacteria phylum and are an important group of bacteria that were found in this study because Moraxellaceae are known to occur in water or soil and contain the genus, Acinetobacter, which are reported to produce biosurfactants (Rossau et al. 1991). The results show that Moraxellaceae were only present in the open water microlayer sample and were also the family with the largest

abundance for that sample. This data indicates that the Moraxellaceae family might play an important role in surfactant production in the sea surface microlayer.

However, the 'empty' control sample did contain a noteworthy percentage of this group. Therefore, there may be contamination regarding this bacterial family.

4.5.2.2 Halomonadaceae Family

Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Oceanospirillales
Family: <u>Halomonadaceae</u>
Genus: Halomonas – produce biosurfactants

Halomonadaceae are another significant family from the Proteobacteria phylum that include Halomonas, a genus of bacteria that are known to produce biosurfactants (Satpute et al. 2010). In the open water, this group was present in equal numbers for both the microlayer and subsurface samples. However, in coastal waters, the abundance of Halomonadaceae was twice as high in the subsurface water than the corresponding microlayer. This might imply that Halomonadaceae are influential in surfactant production in the coastal water column. Most importantly, this group was not present in the control samples, verifying no contamination.

4.5.2.3 Enterobacteriaceae Family

Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Enterobacteriales
Family: <u>Enterobacteriaceae</u>
Genus: Enterobacter – produce biosurfactants

A third family from the Proteobacteria phylum that was found in this study were the Enterobacteriaceae. This family contains the bacterial genus Enterobacter, which are recognized as biosurfactant producers as well (Satpute et al. 2010). Enterobacteriaceae were only found in the open water microlayer sample, however in a very low percentage. From this data it could appear that Enterobacteriaceae have a possible influence in surfactant production within the sea surface, although there were considerable abundances present in the control samples, signifying contamination from this family.

4.5.2.4 Bacillaceae Family

Phylum: Firmicutes
Class: Bacilli
Order: Bacillales
Family: <u>Bacillaceae</u>
Genus: Bacillus – produce biosurfactants

Bacillaceae are a family from the Firmicutes phylum. They are significant to this study because they contain the genus, Bacillus, which is documented as a surfactant producer (Satpute et al. 2010). Bacillaceae were present only in the microlayer samples from both coastal and open waters. Because this group contains a bacterial genus that is known to produce biosurfactants, their presence solely in the microlayer reveals a potential influence in surfactant concentration within the sea surface. Moreover, this family was also not present in the control samples, confirming no contamination.

4.5.2.5 Nocardiaceae Family

Phylum: Actinobacteria
Class: Actinobacteria
Order: Actinomycetales
Family: <u>Nocardiaceae</u>
Genus: Rhodococcus – produce biosurfactants

The family Nocardiaceae are part of the Actinobacteria phylum and are commonly found in water and soil (Stackebrandt et al. 1997). They also contain Rhodococcus, a genus of bacteria that are reported to produce biosurfactants (Satpute et al. 2010). Additionally, Nocardiaceae can degrade hydrocarbons and have been proposed as bioremediation agents for environmental spills (Aislabie el al. 1998). This family was only found in the subsurface from coastal waters, therefore having a potential role in surfactant transformation in the coastal water column. However, this bacterial group was present in a very low percentage. The control samples did not have any presence of this family, ensuring no contamination.

4.5.2.6 Propionibacteriaceae Family

Another family from the Actinobacteria phylum, Propionibacteriaceae, were present in significant numbers for all samples in this study. Much like their phylum abundance results, the subsurface of the open water sample contained the highest percentage of this

group, with the corresponding microlayer sample having considerably less of a percentage. Conversely, in the coastal water samples, there was a higher percentage in the microlayer than in the subsurface waters but not as much of a stark difference between the two. Although there are no known genera from this family that are directly related to surfactant production or transformation, because these bacteria include some of the most common marine and freshwater life and play an important role in the decomposition of organic materials, it is important to note their continual presence (Ventura et al. 2007). Additionally, considering Propionibacteriaceae are reported to play an important role in the decomposition of organic materials, they could also contribute to surfactant transformation in the water column.

However, the control samples did contain a significant percentage of this group, suggesting contamination from this bacterial family.

4.5.2.7 Sphingomonadaceae Family

Another group that was found at the family level was Sphingomonadaceae. There are no known genera from this family that are directly related to surfactant production or transformation, however, this group is known by their ability to degrade some aromatic compounds, which makes them of interest to environmental remediation (Balkwill et al. 2006). These bacteria were only found in the open water subsurface sample, revealing a possible influence in surfactant transformation in the water column. Additionally, this family was not present in the control samples, confirming no contamination.

4.5.2.8 Vibrionaceae and Rhodospirillaceae Families

Vibrionaceae and Rhodospirillaceae are the last noteworthy family groups of bacteria present in the samples of this study. Although neither is directly linked to having genera that produce or transform surfactants, Vibrionaceae inhabit fresh or salt water and most bioluminescent bacteria belong to this family (Madigan et al. 2005). They are also typically found as symbionts of deep-sea animals and members of this family can synthesize an ancient and powerful marine neurotoxin that protects some fish (Madigan et al. 2005). Rhodospirillaceae are mainly comprised of purple non-sulfur bacteria, which produce energy through photosynthesis (Dworkin et al. 2005). They are often found in anaerobic aquatic environments, such as mud and stagnant water (Garrity et al.

2005). Both of these families were found in the open water microlayer and the coastal subsurface water, but not at all in their corresponding sampling locations. Additionally, these groups were not present in the control samples, meaning there was no contamination introduced from these families.

5.0 Discussion

The 454 pyrosequencing platform utilized in this study was able to generate sufficient coverage for assembling the bacterial groups present in the sea surface microlayer and subsurface waters, which were otherwise inaccessible with lower-throughput sequencing methods because pyrosequencing analyzed hundreds of communities simultaneously, integrating information from all samples. The results revealed known and unknown microbes, further stressing the importance of taxonomy independent analysis, such as QIIME (Oh et al. 2006). And because many environmental microbes have not been formally described yet, this taxonomic independent analysis allowed for ecological estimations to characterize the microbial communities present. The pyrosequencing technology also eliminated the need of laboratory isolation and cultivation of individual species, and thereby opened the hidden world of microbial communities in the environment that have previously been poorly characterized (Sun et al. 2010).

5.1 Major Findings

The results of this study emphasize:
(1) Table 4.4 shows the diversity parameter, V_F, for surfactant-associated marine bacterial families, as identified in this study. The open water sea surface microlayer had larger diversity of surfactant-associated bacterial families (V_F = 0.78) than its corresponding subsurface water (V_F =0.33). However, the microlayer of the coastal water had less diversity of surfactant-associated bacterial families (V_F = 0.33) than its corresponding subsurface sample (V_F = 0.56). These findings support the first hypothesis in the open water, but not in the coastal water.

(2) Of the two microlayer regions evaluated in this study, the coastal microlayer had lower diversity of surfactant-associated bacterial families (V_F = 0.33) as compared to the open water microlayer (V_F = 0.78), which supports the second hypothesis. This may be

associated with biophysical properties of coastal and open ocean water masses in the sampling area.

Note that the improved method of the SML sampling developed in this work has been applied only to a limited number of samples. Future work implementing this method will be required to make conclusions based on more substantial statistics.

(3) Since surfactant-associated bacteria are linked to presence of surfactants on the sea surface, this may have implications for detecting surfactant-associated marine bacteria from space. This is due to the effect of surfactants on short gravity-capillary waves affecting SAR imagery, which has been widely reported in the literature (e.g., Alpers et al. 1989) but not in relation to marine bacteria. In our study, there was precipitation in the area of sampling during experimentation (seen as rain signature on the COSMO SkyMed satellite image, Fig 3.1) therefore we have not been able to definitively show that the presence of surfactants on the sea surface can be positively identified on SAR imagery. A companion thesis (Kurata, 2012) further explored this question by taking samples inside and outside slick areas and found greater diversity in the SML slick compared to non-slick SML, which was consistent with the corresponding SAR satellite image. This is in support of the third hypothesis.

This study examined the bacterial composition of the SML by capturing a snapshot of biogeographic patterns, which is often the case when sampling at single stations (Hewson et al. 2006). However, because ocean surface waters are dynamic and vertically mixed over relatively short time scales, composition of bacterial assemblages on very small scales has been demonstrated to vary remarkably (Long et al. 2001). This was reflected in the results of this study, which found varying results in microlayer and subsurface water, at coastal and open waters.

Additionally, marine bacteria that are rare in one season can be abundant in another. For example, in a four-year time-series study, a variety of taxa were undetectable in some months, but then made up several percent of the community in other months (Brown et al. 2005). This could be attributed by the fact that bacterial taxonomic composition in the SML is believed to result from selective environmental factors, such as resource

availability, the physical environment, and physical disturbances (Torsvik et al. 2002). All of the aforementioned factors can vary remarkably from season to season.

6.0 Conclusions

This pilot project introduces a new approach to sampling the sea surface microlayer and the importance of bacteria groups that effect the concentration of surfactants within the microlayer and subsurface of coastal and open waters in the Straits of Florida. The primary goal of this work was to understand the bacterial groups that are surfactant related in the microlayer in order to apply this knowledge to remote sensing techniques.

6.1 Limitations

The marine environment is vast, but there have only been a few microbiological studies done on the sea surface microlayer using molecular biology techniques. These, in total, have only covered less than one km^2 of the ocean. Efforts must therefore focus on a wider range of environments, using a universal sampling strategy in order to gain a more comprehensive understanding of this vast ecosystem. A continuing challenge is to better understand the links between microbial diversity and ecosystem function. For surface microlayer research to make progress in the future, multidisciplinary studies are essential.

Characterizations of bacterial groups could significantly improve by comparing larger sample sets at testing sites. This work took a small set of six samples because the focus was on formulating and perfecting an accurate sampling method. Therefore, future work implementing this method will require taking a larger sampling set and a larger area under consideration to provide more statistically significant results and to further validate that this new sampling approach can be replicated.

Additionally, the bioinformatic analysis was able to produce bacterial family taxonomic classification. Although several potential surfactant-associated bacterial families were found, this study cannot conclude that all play a direct role in surfactant concentration in the SML and subsurface waters. However, further analysis into the genus and/or species level of classification would ensure proper identification of surfactant-related bacterial groups present within the samples.

Moreover, because microbial communities, as part of natural ecosystems, are inherently complex, a more holistic approach can yield complementary data to help determine how particular organisms in a system occur together and vary with environmental parameters (Fuhrman, 2009). The traditional tools of microbiology tend to provide a narrow view, studying each organism in isolation. However, microbial communities include many interactions with protists and viruses, therefore all of these organisms should ultimately be included in the analysis; otherwise important controlling factors will be missed (Kirchman, 2008). This allows us to examine the potential interactions between organisms and aspects of the niches of microorganisms within extremely complex and dynamic natural communities.

6.2 Implications for Future Research (Future Envisions)

The limited data set obtained in this pilot project is not sufficient to definitively assess the microbial effects on the production and transformation of surfactants within the microlayer on a global scale. Instead, this research provides a baseline of data that demonstrates how well the proposed methods work and allow for other studies to be done that pose questions regarding the sea surface microlayer. This unique approach shows promise and further studies are needed with more robust sampling sets.

Future experiments on this research should first create a protocol for ensuring proper sterilization of the filters used for sampling. Although the polycarbonate membrane filters that were used in this study are commonly used for filtering water and for sampling without prior sterilization, there was significant contamination in several of the 'empty' filter samples that theoretically should not have had any presence of bacteria (Table 4.4). Contamination was diligently avoided by staying out of the ship wake and by using sterile instruments when sampling, but there were still strong numbers present. Therefore trying other, more sterile filter types or a metal mesh that can be sterilized is necessary to gather better results.

Further research in quantifying the absolute number of bacterial populations that are present in the sea surface microlayer and subsurface water is also important. This study offers the relative abundance of bacteria that have a potential effect on surfactant

concentrations in the microlayer and subsurface waters, however, real-time PCR could be utilized to quantify absolute abundance, by amplifying targeted bacteria. Real-time PCR, also called quantitative real time PCR (qPCR) is a technique that enables both detection and quantification of one or more specific sequences of a DNA sample (Kubista et al. 2006). This technique produces an absolute number of target DNA molecules by comparison with DNA standards (Dhanasekaran et al. 2010).

Not that long ago it seemed almost hopeless to sort out the identities and interrelationships among the trillions of microorganisms in a cubic meter of sea water, let alone a few hectares of ocean (Kirchman, 2008). But the new sampling method that we developed here, in addition to the high-throughput DNA sequencing technique used in this study can greatly advance the analysis of marine microbial community structures, especially for measurements spread over space and time. This will allow scientists to continue to follow, model and eventually predict the distributions of microorganisms and their activities, which is a critical aspect for understanding cycles in our oceans.

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