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## DNA Analysis of Surfactant-Associated Bacteria in a Natural Sea Slick Observed by TerraSAR-X and RADARSAT-2 Over the Gulf of Mexico

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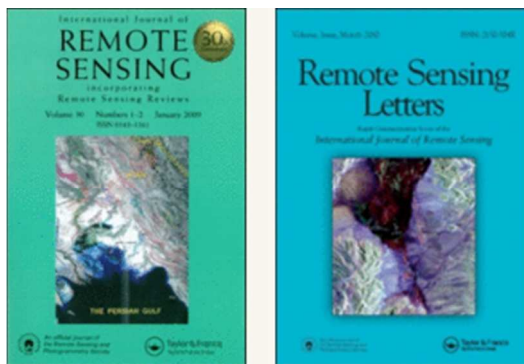
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**DNA analysis of surfactant-associated bacteria in a natural sea slick observed by TerraSAR-X and RADARSAT-2 over the Gulf of Mexico**

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## DNA analysis of surfactant-associated bacteria in a natural sea slick observed by TerraSAR-X and RADARSAT-2 over the Gulf of Mexico

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### Abstract

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29 The damping of short gravity-capillary waves (Bragg waves) due to surfactant accumulation  
30 under low wind speed conditions results in the formation of natural sea slicks. These slicks are  
31 detectable visually and in synthetic aperture radar (SAR) imagery. Surfactants are produced by  
32 natural life processes of many organisms, such as bacteria, phytoplankton, seaweed, and  
33 zooplankton. By using DNA analysis, we are able to determine the relative abundance of  
34 surfactant-associated bacteria in the sea surface microlayer and the subsurface water column.  
35 A method to reduce contamination of samples during collection, storage, and analysis (Kurata  
36 et al., 2016; Hamilton et al., 2015) has been implemented and advanced by increasing the  
37 number of successive samples and changing sample storage procedures. In this work,  
38 microlayer samples have been collected in the Gulf of Mexico during a research cruise (LASER)  
39 on the R/V *F.G. Walton Smith* during RADARSAT-2 and TerraSAR-X overpasses. We found that in  
40 slick areas surfactant-associated bacteria mostly reside in subsurface waters, producing  
41 surfactants, which move to the surface, accumulate on and enrich the sea surface microlayer.  
42 This is consistent with previous studies (Kurata et al., 2016; Hamilton et al., 2015) and with the  
43 experimental results of Cunliffe et al. (2010).  
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## 1. Introduction

### *Sea surface microlayer*

The sea surface microlayer (SML) covers approximately 70% of the Earth's surface and is the boundary between the atmosphere and ocean where many biogeochemical processes occur (Liss and Duce, 1997). It is considered an extreme environment due to high variability in fluxes of nutrients, salinity, temperature, radiation (solar and UV), heat, momentum, and gas.

The aforementioned processes include particle cycling and microbial loops due to the SML's input from and output to the subsurface water and atmosphere. Particles from the atmosphere, for instance aerosols and dust, are deposited into the SML from the air side of the air-sea interface. Organisms can either be permanent residents of the SML (bacteria, phytoplankton, zooplankton) or temporary inhabitants (fish eggs, invertebrate larvae). Sea slicks act as a physical barrier to gas exchange at the air-sea interface (Cunliffe et al., 2011).

There have been several attempts to define the exact structure of the SML. Hardy (1982) depicted the SML as having distinct, stratified layers in which surface-active agents (surfactants), lipids, and alcohols are fixed above a protein and carbohydrate layer. Current models show a lesser degree of organization with gel-like particles and bacterioneuston mixed heterogeneously in the upper portion of the microlayer (See Figure 1 in Cunliffe et al., 2010).

The physical structure of the SML consists of the viscous sublayer (~1500  $\mu\text{m}$  thick), thermal sublayer (~500  $\mu\text{m}$  thick), and salinity diffusion sublayer (~50  $\mu\text{m}$  thick). Under moderate wind speed conditions, these molecular sublayers are mainly controlled by microscale wave-breaking associated with capillary waves and have a great impact on the gas exchange between the ocean and atmosphere (Soloviev and Lukas, 2014).

There are several techniques to sample the microlayer, including hydrophilic and hydrophobic polycarbonate filters, glass plates, mesh screens (metal or nylon), and rotating drums. Each sampling technique defines the SML as a different thickness. For example, the hydrophilic polycarbonate filter used in this study has a maximum sampling depth of 42  $\mu\text{m}$ . Glass plates sample from 20-150  $\mu\text{m}$ , mesh screens from 150-400  $\mu\text{m}$ , and membrane filters

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3 from 6-42  $\mu\text{m}$ . Subsurface water sample depths can also vary from 0.1-20 m and the sampling  
4 devices include bottles, pumps and rosettes. This discrepancy in sampling depth with different  
5 methods makes comparing microlayer community composition very difficult. Franklin et al.  
6 (2005) proposed using membrane filters for bacterial studies. Kurata et al. (2016) and Hamilton  
7 et al. (2015) substantially advanced those techniques.

### 13 *Surfactants and bacteria*

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15 Surfactants are amphiphilic compounds composed of various phospholipids, glycolipids,  
16 lipopeptides, fatty acids, and other complex molecules. The amount and type of surfactant  
17 produced depends on many factors, including availability of nutrients, such as nitrogen,  
18 magnesium, and potassium, as well as physical factors of pH, temperature, salinity, etc.  
19 (Karanth et al., 1999).

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21 Many organisms, such as bacteria, phytoplankton, zooplankton, and seaweed, produce  
22 surface-active agents, or surfactants, during various life processes (Gade et al., 2013). For  
23 example, bacteria produce surfactants for food capture, motility, protection, and aggregation  
24 (Burch et al., 2010). Several bacteria genera that are well-known for producing, degrading, or  
25 having an unknown association with surfactants include *Pseudomonas*, *Bacillus*,  
26 *Corynebacterium*, *Enterobacter*, *Rhodococcus*, *Halomonas*, and *Acinetobacter* (Satpute et al.,  
27 2010). It is interesting to note that *Pseudomonas* and *Rhodococcus* are also oil-associated  
28 bacteria (Sekhon et al., 2012). Since bacteria are part of the microbial loop and other important  
29 ecosystem functions at the air-sea interface, it is important to know their diversity and  
30 abundance in the SML. Kurata et al. (2016) and Hamilton et al. (2015) found that in slick areas,  
31 surfactant-associated bacteria mostly reside in subsurface waters, producing surfactants which  
32 move to the surface and enrich the sea surface microlayer. This is consistent with the  
33 experimental results of Cunliffe et al. (2010).

### 34 *Natural sea slicks*

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36 Under low wind speed conditions, accumulation of surfactants forms natural sea slicks. These  
37 slicks cause dampening of the short ocean waves and can thus be detected visually and in  
38 optical and synthetic aperture radar (SAR) satellite imagery. The smoother surface reflects the  
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3 incoming light or radar beam and thus optically, the slicks are seen because of their glossy  
4 appearance; in SAR, they appear as dark areas. Natural slicks are believed to be highly variable  
5 in time and space. Increased wind speeds or wave breaking can easily disturb the slick and the  
6 associated microbial communities. We use satellite imagery to detect slicks and thus relate the  
7 findings of the microbiological campaign to their presence. There are many causes for natural  
8 sea slicks: build-up of organic material, terrestrial runoff (Wurl et al., 2011), oceanic features  
9 such as convergence zones or frontal interfaces (Gade et al., 2013), high biological productivity,  
10 and sediment upwelling/resuspension (Espedal et al., 1996).  
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### 13 *SAR Imaging of Slicks*

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Satellite SAR imagery is used in this study to visualize slick presence. SAR satellite microwaves can penetrate cloud cover and fog in both daytime and nighttime conditions. Recently, high resolution SARs, like TerraSAR-X, that are well suited to image highly variable coastal and oceanographic processes are available to the scientific community (<http://terrasar-x.dlr.de>). As the SAR images the properties of the scattering surface, this normalized calibrated radar backscatter can be used to measure the roughness of the sea surface, which has been related to wind speed (Lehner et al., 1998). The roughness of the surface is not just dependent on the wind speed though; surfactants or oil spills dampen the short gravity-capillary waves. This causes slicks to appear as a darker area in SAR imagery than the surrounding rougher sea surface, as the slick reflects the microwaves away from the receiving antenna. Other features besides biogenic slicks can cause dark patches in SAR imagery, such as oil spills, grease ice, wind shadowing/sheltering (Soloviev et al., 2010), rain, ship wakes, and internal waves (Velotto et al., 2011). Bright targets (speckles) in SAR images are caused by man-made features (oil rigs, ships, etc.). Surfactant-associated bacteria is believed to be essentially invisible to ocean color satellite sensors, but can potentially be identified in SAR by the presence of surface slicks (Kurata et al., 2016; Hamilton et al., 2015; Soloviev and Lukas, 2014).

This paper is organized as follows. Section 2 describes *in situ* and remote sensing methods. Section 3 presents the results of the experiment in the Gulf of Mexico. Discussion and conclusions are given in Section 4.



## 2. Methods

### *In Situ Bacterial Sampling*

Over 100 samples were collected during a Gulf of Mexico Research Initiative (GoMRI/CARTHE) research cruise, LAngrangian Submesoscale ExpeRiment (LASER), in February 2016 in the Gulf of Mexico (Table 1). Figure 1 shows sampling locations. All sampling was recorded on video using a GoPro camera to identify the sea state and possible instances of contamination during sampling.

*Table 1. Sample collection information during the 2016 GoMRI LASER research cruise.*

Site	Date	Slick Present	Wind Speed (m s <sup>-1</sup> )	CTD Casts	Sampling Platform
1	2/6/16	No	4-5	0	Small Boat
2	2/6/16	No	7-8	1	Small Boat
3	2/6/16	No	5-7	1	Small Boat
4	2/10/16	No	5-7	1	R/V <i>Walton Smith</i>
5	2/10/16	No	7-8	1	R/V <i>Walton Smith</i>
6	2/12/16	Intermittent	2-3	2	R/V <i>Walton Smith</i>
7	2/12/16	Yes	2-3	2	R/V <i>Walton Smith</i>

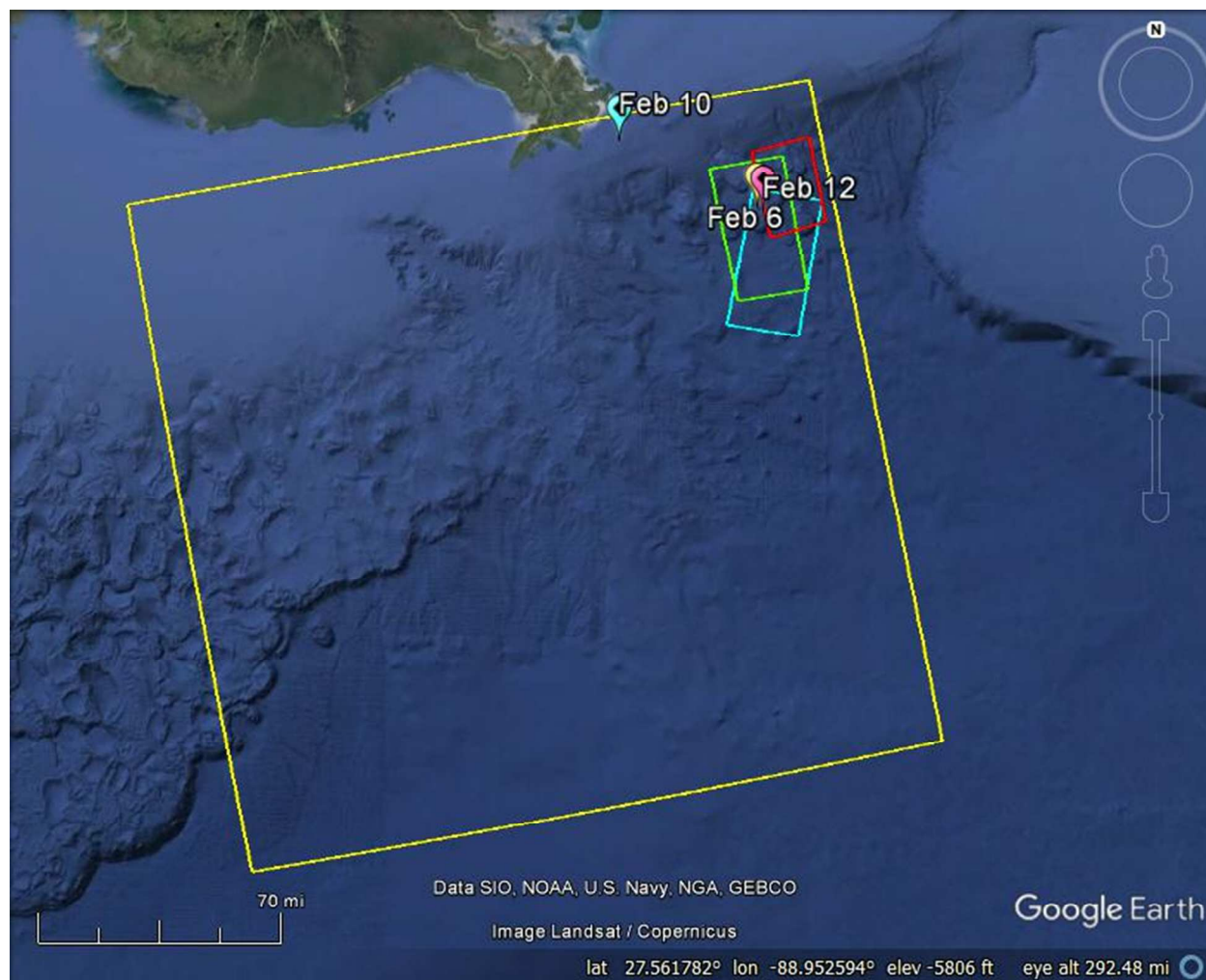


Figure 1. In situ sampling sites in the Gulf of Mexico during the 2016 GoMRI LASER research cruise and footprints of SAR satellite images. The TerraSAR-X footprints are in blue (February 10) and green (February 11). The RADARSAT-2 footprints are in red (February 10) and yellow (February 13).

The method of Franklin (2005) was expanded upon by Kurata et al. (2016) and implemented in this study in order to decrease contamination of the sample by the ship wake, boat, and researcher. A hydrophilic polycarbonate membrane filter was attached to a sterile hook and line, which was then stored in a sterile bag until its deployment in the field. A fly-fishing technique using a ten-foot fishing pole was used to reach an area outside the ship's wake to lay the filter on the ocean surface for three to five seconds. Using the fishing pole, the filter was lifted off the surface and caught using sterile forceps. This study enhanced contamination safeguards and sample collection/storage methods in comparison to Kurata et al. (2016), for example the filter was directly placed in a labeled MoBio bead tube, which is later

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3 used for DNA extraction. This ensured there was no loss of sample, which is vital since only a  
4 small amount is collected on the filters. The 47 mm hydrophilic polycarbonate filter used in this  
5 study defined the sampling depth of the microlayer, which ideally was on the order of 40  $\mu\text{m}$ .  
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7 Samples were stored on ice in the field and transferred to a  $-80^{\circ}\text{C}$  freezer prior to extraction.  
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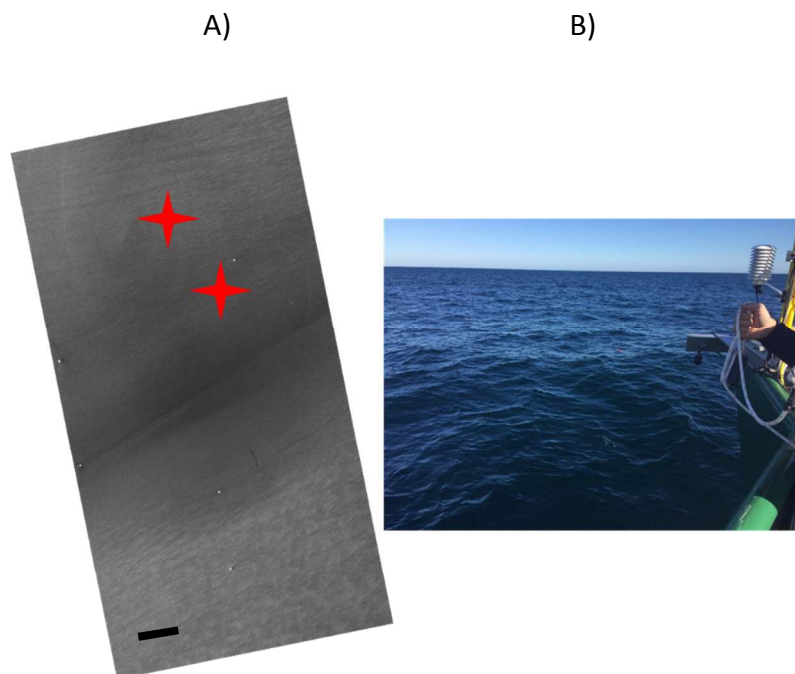
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11 The SSW was sampled at 0.2 m, using a peristaltic pump with tubing sterilized with 90%  
12 isopropanol and then rinsed with SSW. After approximately 45 s of SSW flowing through the  
13 tube, the water sample was collected in a sterile bag. A filter was dipped in the bag, swirled  
14 around, and then placed in a labeled MoBio bead tube for DNA extraction. Samples were stored  
15 on ice during collection and then placed in a  $-80^{\circ}\text{C}$  freezer prior to extraction.  
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19 Control filters were collected as a baseline for DNA analysis and in addition analyzed for  
20 possible contamination. Air-control filters were exposed to the air at the sampling site for  
21 approximately 30 s. Non-exposed control filters were never removed from the lab space, which  
22 would provide insight into laboratory contamination.  
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### 32 *Remote Sensing Data*

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34 For this study, we selected RADARSAT-2 satellite Wide Scan and TerraSAR-X in Stripmap mode.  
35 The SAR data help identify slicks. The reduced roughness of the sea surface in the slick results in  
36 reduced radar backscatter, which shows up as a dark area on the image. The presence of slicks  
37 is dependent mainly on wind speed, and other environmental conditions (internal waves,  
38 fronts, etc.).  
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45 Slicks were observed during sampling on February 12. The sampling conducted on  
46 February 12 occurred several hours after a TerraSAR-X satellite overpass, during rather low  
47 wind speed conditions of  $2\text{ m s}^{-1}$  to  $3\text{ m s}^{-1}$ . The TerraSAR-X Stripmap intensity image shows an  
48 area 30 km wide by 50 km long acquired in VV polarization (Fig. 2).  
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Figure 2. A) The TerraSAR-X image acquired in VV on February 11, 2016 at 23:49:10 UTC with sampling Sites 6 and 7 superimposed. Scale bar represents 5 km. B) Photograph of the intermittent slick on February 12.

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There was a well-defined convergence zone in the sampling area on February 12. Convergence zones associated with downwelling are known for the accumulation of organic matter and microbial life (Espedal and Johannessen, 1996). The dark elongated area and surrounding dark areas in the middle of the SAR image shows the slick. The lighter area at the bottom of the image is rougher water, and indicates the presence of atmospheric convective cells due to warmer temperature on the southern side of the front. Oil rigs appear in this image as bright spots.

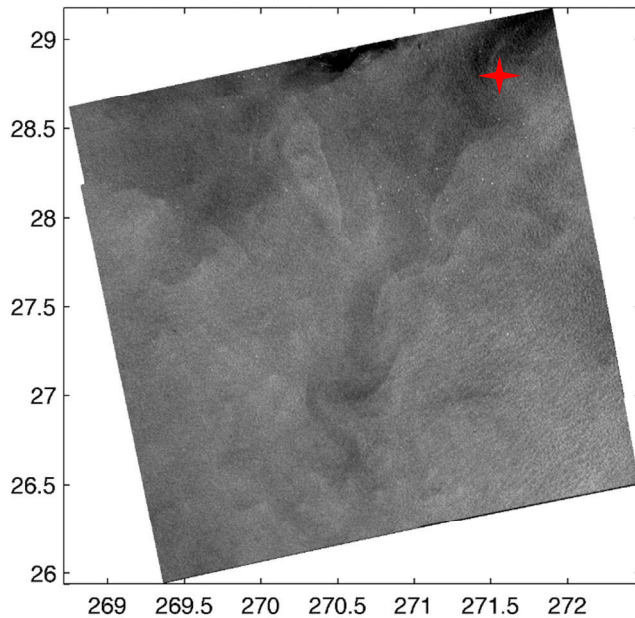
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A RADARSAT-2 ScanSAR mode image passed over the area of *in situ* sampling site at 23:57 UTC on February 13, 2016, one day after the *in situ* sampling. The wide coverage of ScanSAR image (500km by 500km) shows a dark pattern during the observation time. The low wind speed of  $2 \text{ m s}^{-1}$  was measured from the research vessel one day before.

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The SAR images on February 10, 2016 (Figs 4 and 5) were collected under moderate wind speed conditions. During the experiment on February 10, no slicks were detected visually due to wind speeds above  $5 \text{ m s}^{-1}$  (Table 1). Neither TerraSAR-X nor RADARSAT-2 images showed slick presence (Figs 4 and 5). However, these images were collected not exactly over

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3 the sampling site on this day. The cellular structure in images in Figures 4 and 5 is due to the  
4 strong atmospheric convection. Note that this was wintertime and water was warmer than the  
5 air, while the cold, northern wind came from the land.  
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36 *Figure 3. The RADARSAT-2 (C-band) image acquired in VV on February 13, 2016 at 23:57 UTC, corresponding to the yellow box in*  
37 *Fig 1. Red star indicates sampling location on February 12, 2016.*  
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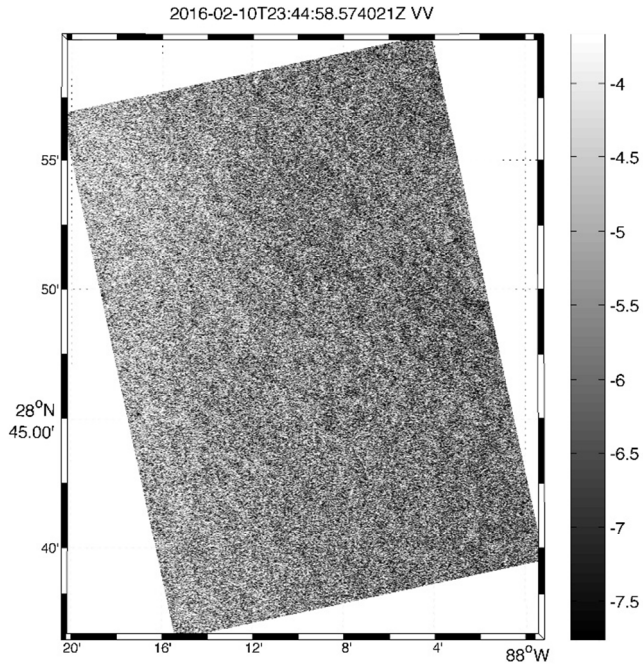


Figure 4. The RADARSAT-2 (C-band) image acquired in VV on February 10, 2016 at 23:44 UTC, corresponding to the red box in Fig 1.

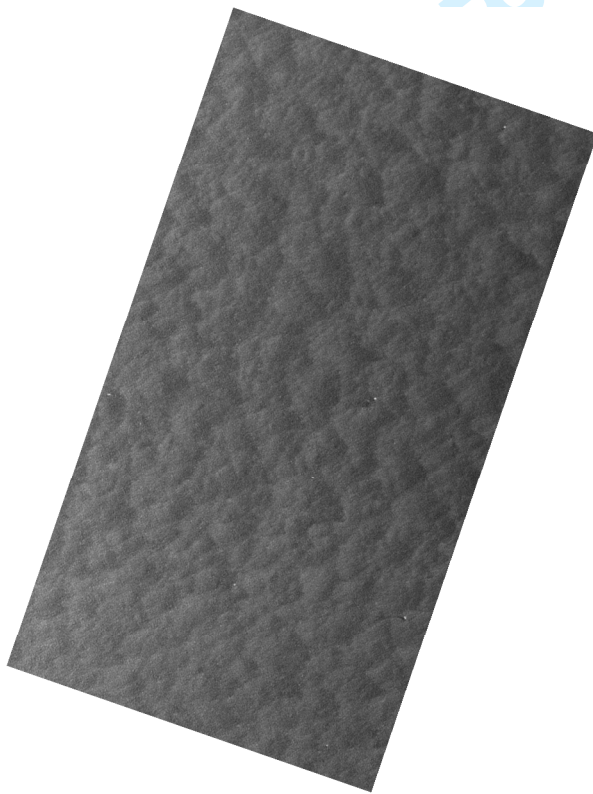


Figure 5. The TerraSAR-X image acquired in VV on February 10, 2016 at UTC, corresponding to light blue box in Fig 1.

### DNA Analysis

Bacterial DNA was extracted from SML and SSW samples using a MoBio PowerWater DNA Isolation Kit and the associated protocol was followed (MoBio Laboratories, Inc., Carlsbad, CA). Quantitative polymerase chain reaction (qPCR) is real-time monitoring of the amplification of a target gene, and was performed on the extracted DNA. All samples were processed in duplicate.

The 16S ribosomal RNA genes were targeted using *Bacillus*-specific primers (Bac265F and Bac525R) in order to amplify a 260-basepair gene sequence. *Bacillus cereus*, provided by the Microbiology Lab at Nova Southeastern University, served as the positive control for this study. *Bacillus* is a well-known surfactant-associated bacteria that was not found on the control filters from Kurata et al (2016) and Hamilton et al. (2015b). It was chosen as a positive control due to the probability it would not be found on the future control filters or in the lab setting. A FastStart Essential DNA Green Master Kit and LightCycler were used to follow the qPCR procedure by Hamilton et al. (2015), except without the nested PCR prior to qPCR analysis. The LightCycler software sets an automatic threshold and the sooner a sample crosses that threshold, the more of the targeted gene sequence is in the sample. Figure 6 is an example qPCR plot generated by the LightCycler software, without the threshold shown. The bright blue line is the positive *Bacillus cereus* control and the red line is the non-template (PCR-grade water) control.

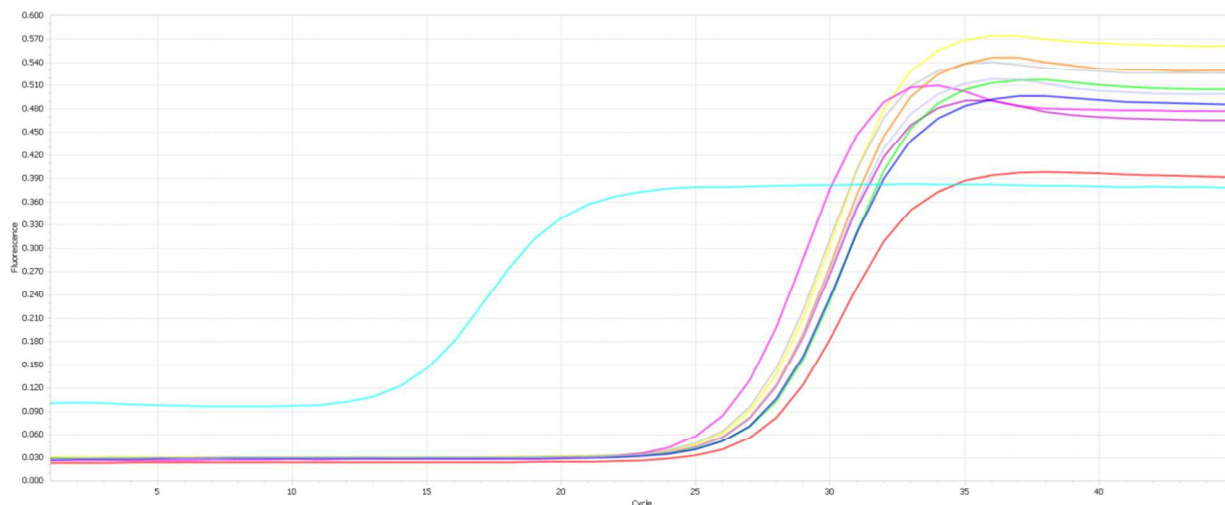


Figure 6. Example of a qPCR plot. Cycle number is on the x-axis and fluorescence is on the y-axis. The automatically set threshold is not shown.

Each sampling day was analyzed in a separate qPCR run and the numerical values of each run cannot be compared due to possible pipetting differences in reagent and sample amounts. Only the relative abundances within each sampling day can be compared.

Relative abundance ( $A$ ) of *Bacillus spp.* was calculated as follows:

$$A = 2^{(28-ct)} \quad (1)$$

where  $ct$  is the cycle number generated by the LightCycler software (Hamilton et al., 2015b). The cycle number threshold is set based on the linear phase of amplification for each sample. The 95% confidence intervals are then calculated using Student's distribution. The mean relative abundance per site for SML and SSW was calculated using the average relative abundance of all samples per water type per location (rather than averaging the cycle number, which would not account for conversion from the log scale).

### 3. Results

Our results elucidate the difference of abundance of *Bacillus spp.* between the SML and SSW. Sites 3, 6, and 7 show a statistically significant difference in the relative abundance between the SML and SSW, while Sites 2, 4, and 5 do not (Fig. 7). A 95% confidence interval was calculated using a Student's t-distribution test. Site 3, which had a wind speed of 5-7 m/s and no visible slicks, showed higher relative abundance of *Bacillus spp.* in the SML compared to



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3 SSW. Sites 6 and 7 with wind speeds of 2-3 m s<sup>-1</sup> had an intermittent and better-defined slicks,  
4 respectively, had a higher relative abundance of *Bacillus spp.* in the SSW compared to the SML.  
5 Note, Site 1 has been removed from analysis since only SML samples were taken at that  
6 location so there is no SSW for relative abundance comparison. There is significant variation of  
7 *Bacillus* abundance in both the SML and SSW, which is consistent with previous work of  
8 Hamilton et al. (2015).  
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15 A slick was observed during sampling on February 12, which was confirmed by  
16 TerraSAR-X imagery (Fig. 2). Site 6 samples were collected in an intermittent slick area, while  
17 Site 7 samples were collected in a better defined slick. In both cases, the SSW contained more  
18 *Bacillus spp.* than the associated SML.  
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24 February 10 was sampled under moderate wind speed conditions. No slicks were  
25 observed in SAR imagery. There were no statistically significant differences between SML and  
26 SSW in Sites 4 and 5 on February 10.  
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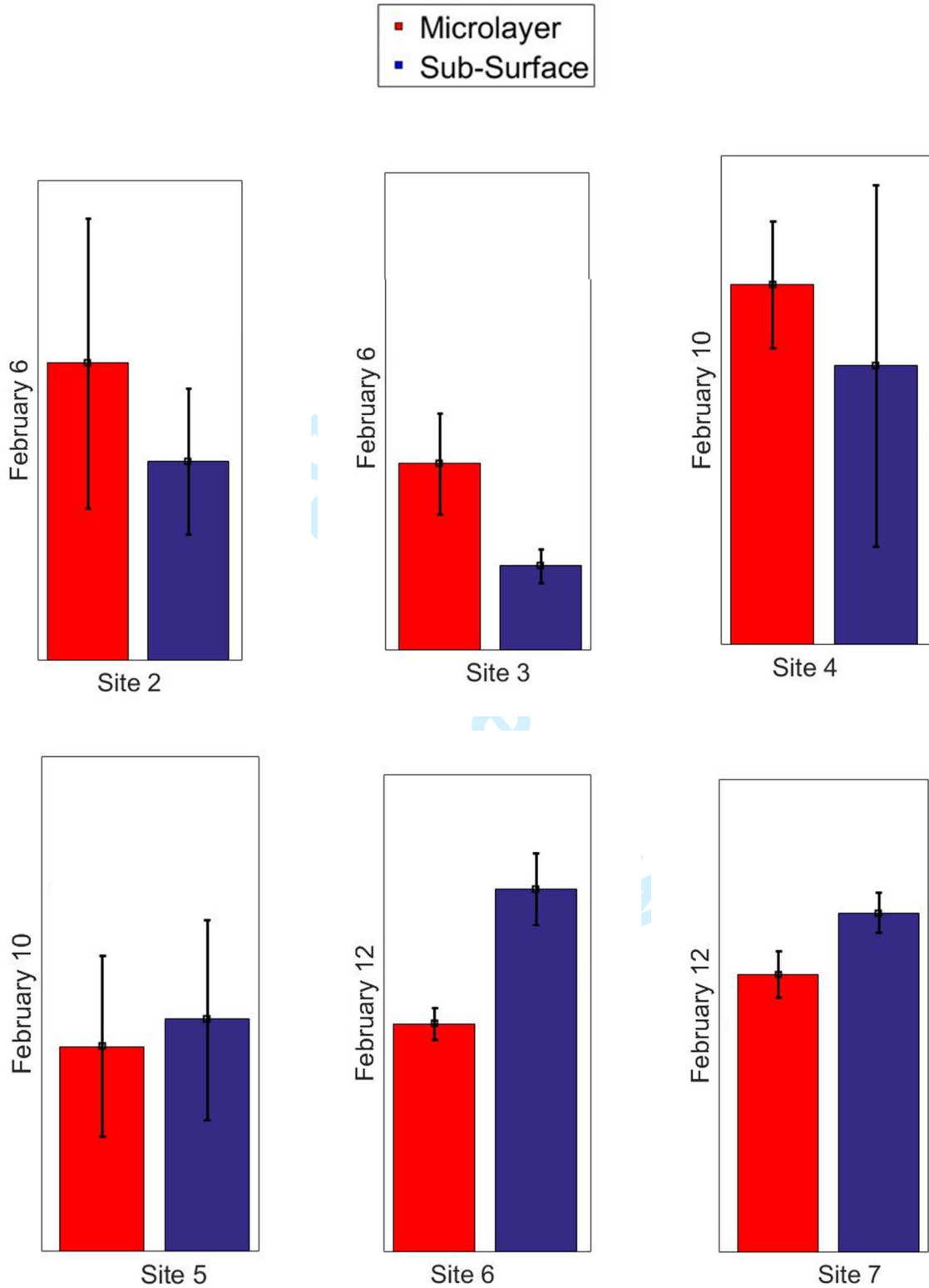


Figure 7. Relative abundance of *Bacillus* spp. from samples collected in the Gulf of Mexico during the 2016 LASER research cruise.

#### 4. Discussion and Conclusion

Our experimental results can be summarized as follows. Sites 6 and 7, sampled under low wind speed conditions, and Site 3, sampled under moderate wind speed conditions, showed statistically significant differences in relative abundance of surfactant-associated bacteria in the SML compared to the SSW. Sites 2, 4, and 5, sampled under moderate wind speed conditions, however, did not produce statistically significant differences between the SML and SSW. (Note that abundance of surfactant-associated bacteria was, in general, smaller under moderate rather than low wind speed conditions.) In this study, the number of successive SML samples was increased from a few in Kurata et al. (2016) and Hamilton et al. (2015) to as many as ten, which resulted in better confidence intervals. A further increase of the number of successive samples above ten is not feasible because the ship drifts and often leaves the slick area before completing the sampling set. One way to improve statistics is to increase the number of SSW samples. Note that the number of SSW samples was nine in Sites 3, 6, and 7 compared to three or six samples in Sites 2, 4, and 5. This is the probable explanation for the statistical significance of the results in Sites 3, 6, and 7 and the lack of statistical significance for Sites 2, 4, and 5.

Our results suggest that under calm weather conditions, more surfactant-associated bacteria are present in the SSW compared to the SML. This is consistent with observations by Kurata et al. (2016) and Hamilton et al. (2015). This indicates that surfactants are produced in SSW and transported to the SML via physical processes such as advection, bubble scavenging, and convection, accumulating on and enriching the sea surface microlayer, which is consistent with Cunliffe et al. (2010).

During the field campaign, we collected SAR images from TerraSAR-X and RADARSAT-2 satellites. It is very difficult to obtain *in situ* samples from a research vessel at exactly the same time and location as the satellite images because high-resolution SAR has a very limited footprint. In this work, the time of *in situ* sampling and SAR overpasses were reasonably close in time. (It should be noted that most SAR images collected in the World Ocean are never corroborated with field measurements.)

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Surfactant-associated bacteria are in general transparent and not visible in ocean color satellite imagery. SAR technology can help to visualize the slick areas often related to surfactant-associated bacteria, which are involved in processing organic material in the water column and production of surfactants. SAR technology can thus be implemented to track organic material, such as dissolved oil and other pollution in the water column, by the presence of surface slicks (Kurata et al. 2016).

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3 Contributions

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5 Contributed to conception and design: CWD, AVS, JK

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8 Contributed to acquisition of data: CWD, AVS, SL, WP

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10 Contributed to analysis and interpretation of data: CWD, AVS, AT, MS, SL

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40 Competing Interests

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42 The authors have declared that no competing interests exist.

47 Data accessibility statement (required for research articles)

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