Exploring the Unseen: From Microplastic pollution to the Microbial world of Cruise Ships.

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NSF Research Experience for Undergraduates
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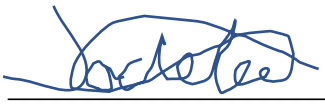
Summer 2018



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Exploring the Unseen: From Microplastic pollution to the Microbial world of Cruise Ships.

Abstract:

Cruise ships account for less than 1% of the world's merchant fleet but generate 25% of all its waste; disposing of over one billion gallons of treated and untreated sewage into the ocean each year. This large volume of waste can pose a major threat to human health and puts pressure on the environment due to its deleterious effect on marine biota, ecosystem structure and function. Because ship-generated waste disposal is of particular concern for public health at ports of call, our study focused on the impacts of cruise ships on water quality in Frenchman Bay. We specifically monitored for the 'unseen' effects of these vessels. We looked at the possible introduction of microplastics (MP) from paint of ship's hulls, and from blackwater and greywater discharges. In addition to wastewater discharges, cruise ships have been found to be vectors for invasive species by potentially introducing alien microbes into the harbor's waters *via* hull fouling. Consequently, we also investigated the microbiota around the ships by using metabarcoding analysis of extracted eDNA from water samples taken in the proximity of different cruise ships. The samples were run with one primer: targeting the V4 region of the 16S small subunit (SSU) rRNA to amplify microbial prokaryotes. Our data suggest that there was no major impact of cruise ships in Bar Harbor from microplastics or detectable presence of foreign species from ship hulls.

Introduction:

As cruise ship traffic continues to increase all over the world, so too does the industry's environmental impact. In 2005, the United Nations Environmental Programme (UNEP) identified tourist ships as one of the principal pollution sources of marine ecosystems (Caric et al. 2014). Cruise ships have been found to generate 25% of all global merchant waste; an average cruise ship carrying 2000 to 3000 passengers can produce about 1000 tonnes of waste per day, including 100000 to 115000 litres of sewage or blackwater (Ocean Conservancy 2002). This amount of waste, if intentionally or accidentally discharged, can have a major impact on the marine ecosystems. Indeed, wastewater usually contains chemical contaminants and large amounts of phosphorus and other fertilizing compounds, which can stimulate an overgrowth of algae and other aquatic plants (Slomp et al. 2004). The overgrowth of algae consumes oxygen

and can result the creation of anoxic zones, which makes it impossible for aquatic life to survive (Slomp et al. 2004). Furthermore, wastewater discharges can also negatively affect human health, mostly because of risks of outbreaks caused by pathogenic bacteria present in untreated sewage (Disney et al. 2015). Therefore, because of its proximity to populated areas, ship-generated waste disposal is of particular concern at ports of call.

Currently in Maine, cruise ships with over 250 passengers are prohibited from releasing any wastewater (grey or black) within the harbor, unless they possess a permit and the discharged waste is adequately treated (Disney et al. 2015). Furthermore, smaller commercial passenger vessels are encouraged to discharge wastewater while the ship is at least 4 nautical miles away from shore and proceeding at a speed not less than 6 knots (Disney et al. 2015). However, with a growing number of cruise ship visits in Bar Harbor (from 116 in 2016 to 180 in 2018) there is still citizen concern over any potential environmental impact visiting ships could have in the harbor.

Water quality parameters around cruise ships have been monitored in Bar Harbor fairly regularly since 2004 to assess whether cruise ships were complying with Maine's legislation on wastewater discharges. These parameters included Dissolved Oxygen (D.O.), Biochemical Oxygen Demand (B.O.D), pH, *Enterococcus* levels, phytoplankton, transparency, nutrients (Dissolved Inorganic Nitrogen and phosphorus levels), turbidity and chlorine (Disney et al. 2015). Our research project focused on parameters that could be indicative of water quality impacts from sources other than wastewater discharges. Specifically, we monitored for microplastics (polymer-based materials less than 5 mm in size) and for invasive or distinctive bacterial species that could be introduced in the harbor by visiting cruise ships.

The number of microplastics has been increasing in oceans for over the last four decades and are of particular concern because of their ability to bioaccumulate in food chains, and adsorb Persistent Organic Pollutants (POPs) and other anthropogenic contaminants (Andrady 2011). They can originate from both primary or secondary sources; primary microplastics are intentionally produced for direct use, and secondary microplastics originate from the fragmentation of larger pieces of plastic (Lassen et al. 2015). From cruise ships, microplastics are likely to originate from abrasion and maintenance of hull paint or by the releases of self-polishing antifouling paints (Lassen et al . 2015). The Danish Environmental Protection Agency in 2015 reported that total releases of microplastics to aquatic environments from the use of

paints for large vessels is estimated to be about 16-190 tonnes/year. Wastewater is also an important source of microplastics, as certain cosmetic products can contain primary microplastics and end up into black and grey waters (Prata 2018). It is also estimated that 35% of microplastics in oceans are thought to be fibers from synthetic textiles, which may originate from the wash of polymer textile clothing (Prata 2018). Therefore, any accidental or intentional release from cruise ships may result in an increased concentration of microplastics in the harbor.

The other parameter that we monitored for was the presence of invasive or unique bacteria around cruise ships. Ships have been a source of invasive species since global travel first started, as they have the ability to transport organisms across distances they could not achieve by drifting on their own (Carlton 1999). The introduction of alien aquatic species usually poses major threat to the native biodiversity and may adversely affect human health. One way alien species can be introduced to new environments by cruise ships is through hull fouling. This occurs when microorganisms, plants, algae and animals accumulate on submerged surfaces, or ships' hulls (IMO 2018). The bacteria or organisms may then shed when they stop at ports of call. We therefore looked at the possibility that ships may have a 'microbial signature' that they drag from port to port.

Materials and Methods:

For microplastic monitoring, the samples were manually collected using a 1L wide-mouth brown plastic bottle around 8 different ships over the course of nine weeks, the bottle was rinsed three times before sampling. We collected samples from surface waters at the bow and at the stern of the ships, in addition samples were taken when no ship was present at bell buoy #7 (usual control site for the Cruise Ship Monitoring Program), at anchorage alpha and at the Town Pier (see **figure 1** in the appendix). Samples at the bow were used as control samples; water flows from bow to the stern; possibly shedding the ship's biofilm and associated microplastics. These samples were then filtered using a vacuum hand pump with a 0.45-micron pore sized filter paper. The filter papers were then analyzed using a dissecting microscope at the 32 magnification, by counting the number of microplastics on each filter paper. Statistics were done using R, a t-test was performed to compare microplastics from the cruise ship's stern and bow.

For bacterial monitoring, samples were collected by filtering 180 mL of water with a syringe through a Sterivex™ filter. The filters were stored at -80°C before they were sent for

analysis and were processed by Dr. Kelley Thomas at the University of New Hampshire. eDNA metabarcoding analysis involves the extraction of DNA, using PCR to amplify DNA (the samples were run with one primer: targeting the V4 region of the 16S small subunit (SSU) rRNA to amplify microbial prokaryotes), Next generation sequencing of purified DNA. The sequences were then processed by using qiime2, we used the following protocol (Thomas 2018).

```
sort_reads.py
```

nano file:

```
import glob
import os
import shutil
os.mkdir("reads")
for f in glob.glob("*/*.gz"):
    shutil.copy2(f, "reads/" + f.split("/")[1])
for f in glob.glob("reads/*R3*"):
    os.rename(f, f.replace("R3", "R2"))
qiime tools import\
      --type 'SampleData[PairedEndSequencesWithQuality]'\
      --input-path reads\
      --source-format CasavaOneEightSingleLanePerSampleDirFmt\
      --output-path demux.qza


qiime demux summarize\
      --i-data demux.qza\
      --o-visualization demux.qzv
```

The forward read was truncated at the 238 bp, and the reverse reads at the 200 bp.

```
qiime dada2 denoise-paired\
      --i-demultiplexed-seqs demux.qza\
      --p-trim-left-f 0 --p-trim-left-r 0\
      --p-trunc-len-f 238 --p-trunc-len-r 200\
      --p-n-threads 72\
      --o-representative-sequences rep-seqs --o-table table --o-denoising-stats dns


qiime feature-table summarize\
      --i-table table.qza\
      --m-sample-metadata-file mdat.tsv\
```

```
    --o-visualization table


qiime feature-table tabulate-seqs\
    --i-data rep-seqs.qza\
    --o-visualization rep-seqs
```

For the taxonomy:

```
ime feature-classifier classify-consensus-blast\
    --i-query rep-seqs.qza\
    --i-reference-taxonomy
/data/share/databases/SILVA_databases/SILVA_132_QIIME_release/taxonomy/taxonomy_all/99/majority_taxonomy_
all_levels.qza\
    --i-reference-reads
/data/share/databases/SILVA_databases/SILVA_132_QIIME_release/rep_set/rep_set_all/99/silva132_99.qza\
    --o-classification taxonomy\
    --p-perc-identity 0.8\
    --p-maxaccepts 1

qiime metadata tabulate\
    --m-input-file taxonomy.qza\
    --o-visualization taxonomy.qzv

qiime taxa barplot\
    --i-table table.qza\
    --i-taxonomy taxonomy.qza\
    --m-metadata-file mdat.tsv\
    --o-visualization taxa-barplots.qzv

qiime feature-table heatmap\
    --i-table table.qza\
    --m-metadata-file mdat.tsv\
    --o-visualization heatmap.qzv\
    --m-metadata-column Ship
```

The samples were normalized and statistics were performed:

```
qiime diversity alpha-rarefaction\
    --i-table  table.qza\
    --p-max-depth 834\
```

```
    --p-min-depth 2\
    --p-steps 30\
    --o-visualization alpha-rarefaction
qiime diversity core-metrics-phylogenetic\
    --i-phylogeny rooted-tree.qza\
    --i-table table.qza\
    --p-sampling-depth 2\
    --m-metadata-file mdat.tsv\
    --output-dir core-metrics-results
qiime diversity alpha-group-significance\
    --i-alpha-diversity core-metrics-results/faith_pd_vector.qza\
    --m-metadata-file mdat.tsv\
    --o-visualization core-metrics-results/faith-pd-group-significance.qzv
qiime diversity alpha-correlation\
    --i-alpha-diversity core-metrics-results/faith_pd_vector.qza\
    --m-metadata-file mdat.tsv\
    --o-visualization core-metrics-results/faith-pd-correlation.qzv
```

Results:

Microplastic data:

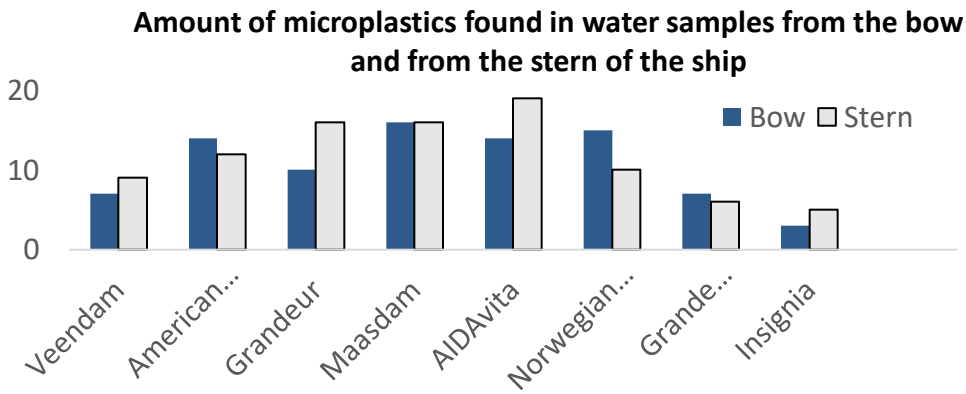


***Figure 2**: Microplastics found in the Bow and the stern of various ships.*

The sample with the most microplastics (17) was found at the stern of AIDAvita and the sample with the least amount of microplastics (1) at the bow of Insignia. Microplastics were found in all samples collected and all microplastics found were microfibers.

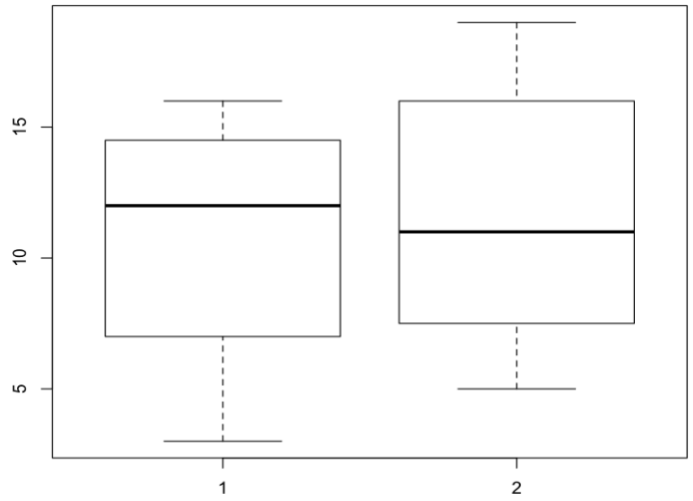


***Figure 3**: Boxplot from R of microplastics in the Stern and Bow of the different ships, with 1 being microplastics found at the stern and 2 microplastics found at the bow.*

The average number of microplastics found at the bow of the cruise ships sampled was 10.75 compared to 11.625 at the stern. A paired t test performed with R, to see if the samples were statistically different, gives a p value of 0.5187.

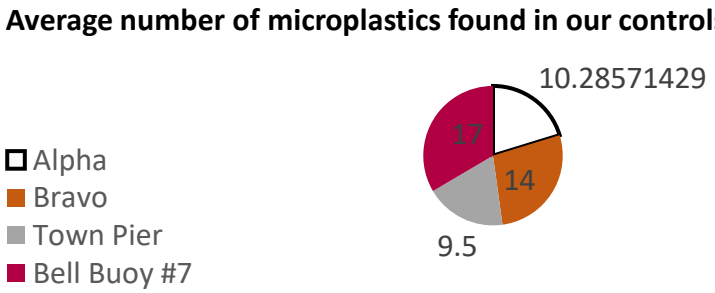
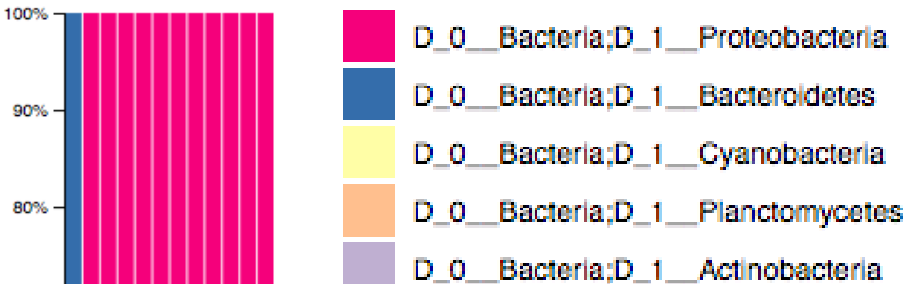


***Figure 4**: Comparison of the average number of microplastics from samples taken with ships and with no ships from the sites: Bell Buoy #7, Alpha, Bravo and Town Pier. There was only one sample taken from Bell Buoy #7 and Bravo.*

The site where most microplastics were found was site Bell Buoy #7 and the lowest amount of microplastics were found at the Town Pier.

Microbiome data:



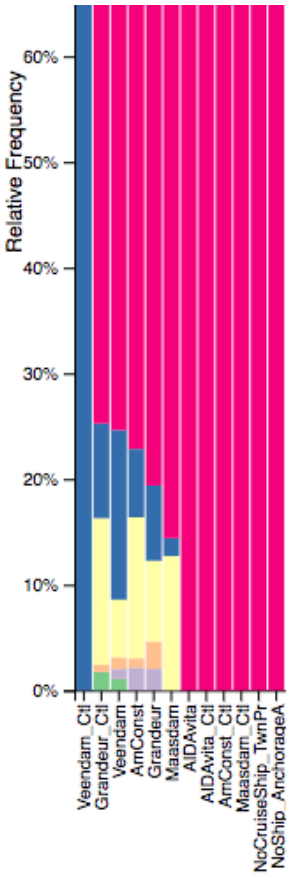


Figure 5*: Bar plot showing bacterial relative abundance at the second taxonomic level. Taxonomy was assigned with QIIME2 (v.6) using the SILVA database, based on DNA sequences from the v4 region of the 16S gene (Yilmaz et al. 2014).*

Samples from Anchorage Alpha and the Town Pier sampled when no ships were present, as well as samples at the bow of AIDAvita, Maasdam, American Constitution and Veendam only contained one bacterial species. The most abundant bacterial species found in all but one sample, was an uncultured gram negative Pelagibacterium (SAR11) from the class Alphaproteobacteria and phylum Proteobacteria.

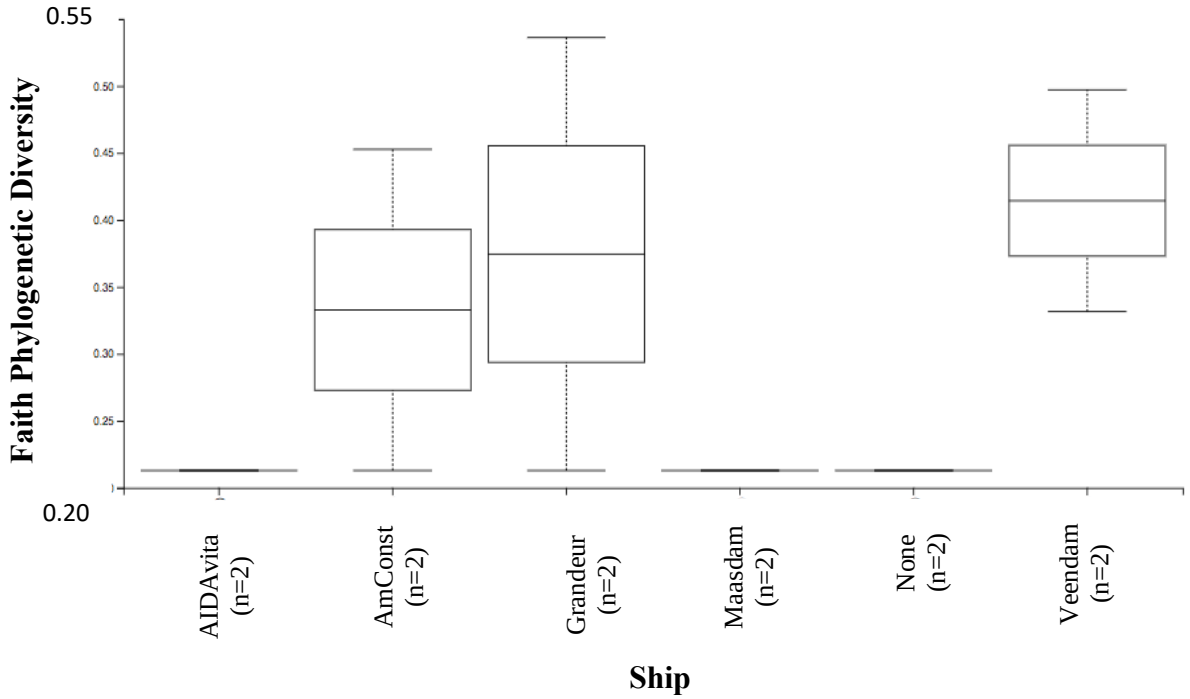


***Figure 6**: Phylogenetic alpha diversity based on Faith's PD between ship samples. p =0.2992 for all groups (Kruskal-Wallis method). Comparison of ship to ship gave no p values below 0.05. Therefore, there was no statistical difference between ships.*

The most genetically diverse samples were taken from Veendam and the samples containing only one bacterial species were the least genetically diverse samples (American Constitution, No Ship and AIDAvita).

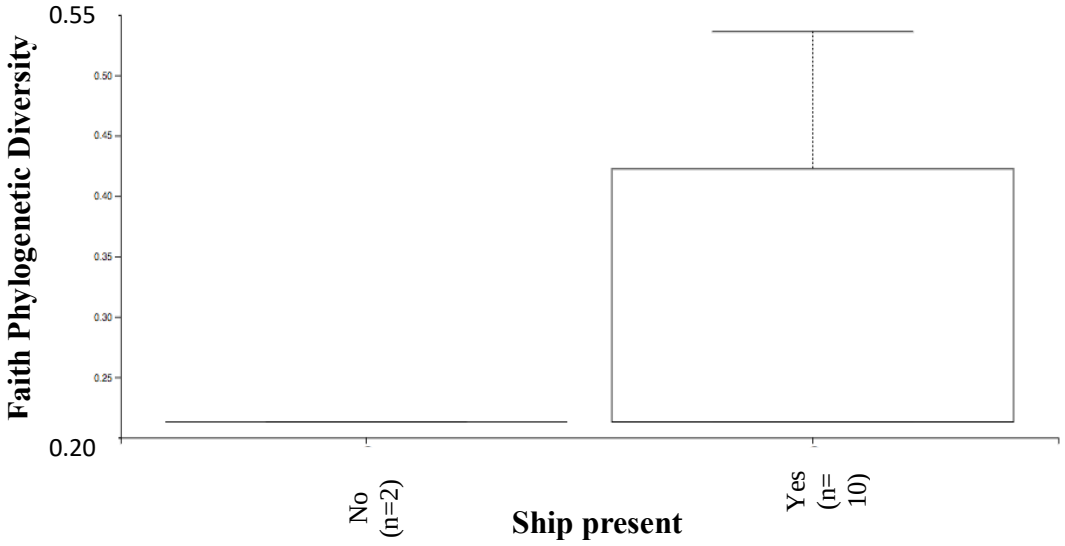


***Figure 7**: Phylogenetic alpha diversity based on Faith's PD between ship samples and samples when no ships were present. p =0.3065 (Kruskal-Wallis method).*

Discussion:

We found no significant difference between microplastic data at the bow and at the stern of visiting cruise ships: our paired t test gave a p value of 0.5187 > 0.05. Furthermore, microplastics were found at an average of 9.5 at the Town Pier with no ship present and 17 at the Bell Buoy #7. These results suggest that microplastics are always present in Bar Harbor. However, some factors may have influenced our data analysis, such as the deposition of microplastics from other sources. Indeed, Dris et al. found that indoor microplastic concentration ranged from 1.0 to 60.0 fibers/m^3. These fibers may have fallen down on the filters during analyses and therefore resulted in errors in the true microplastic counts. A more accurate measure of microplastics, than just visual sorting, would have been by using FPA-based icro-FTIR spectroscopy. This method would eliminate human error and biases, mostly from possibly missing or miscounting any microplastic fragments. Moreover, the Shaw institute has been monitoring microplastics in Maine coastal waters and has found on average 17 plastic fragments per liter of water in Blue Hill and Penobscot Bay, which suggests that these pollutants are more widespread than previously thought in the "pristine" Maine waters. And so, it is very possible that Frenchman Bay also contains a certain amount of microplastics. The 2018 Cruise Ship monitoring project showed no signs of cruise ship discharge from the ships sampled so far and we found no significant difference between samples from the bow and the stern of cruise ships. We can therefore assume that a majority of the microplastic fragments found are likely to have originated from secondary sources (in-situ litter in Bar Harbor, mostly from fishing nets) and from primary sources (hygiene products, introduced via effluent) other than cruise ship wastewater discharges.

The most abundant bacterial species found in all but one sample, was an uncultured gram negative Pelagibacterium (SAR11) from the class Alphaproteobacteria and phylum Proteobacteria. This species of bacterium is ubiquitous in global oceans and so, it is not surprising that we detected it in the majority of our samples (Sunagawa et al. 2015). More surprisingly, we found archaeal DNA in two of our samples, specifically, Archaea from Marine Group II of the phylum Euryarchaeota. However, this group resides in the photic zones of oceans and so it is not unusual to find its DNA in surface seawaters (Zhang et al. 2015). All of the other bacterial species identified were mostly marine organisms, normally found in ocean waters.

It is unusual that so many of our samples contained only one bacterial species (seven in total). But there was also no statistical difference found in the genetic diversity between our different ship samples ($p = 0.2992 > 0.05$). And there was no genetic difference in samples taken when ships were present and when they were not ($p = 0.3065 > 0.05$). Consequently, we cannot say that the visiting cruise ships brought with them a significantly different microbiota than the surrounding waters in Bar Harbor. However, only two samples were taken when no ship was present, so more samples should be taken to better statistically compare samples and create baseline data of the microbes normally found in the harbor. Sampling methods could have also contributed to error in our results, as no gloves were worn during the collection of our samples. The boat we used for collection may also have not been close enough to the cruise ships in order to detect their microbiota and our distance away from the ships was not always the same. Finally, there is doubt as to how long it would take for bacteria to be detected from the hull after the visiting cruise ship is anchored in port.

Acknowledgements:

I am very grateful to Dr. Jane Disney for mentoring me and having me in the community lab this summer. I would like to acknowledge Anna Farrell for all of her help with this project, I couldn't have done it without her. I am grateful to Ashley Taylor for her mapping skills and welcoming me in the community lab. I would also like to thank Amber Wolf and Charlie Phippen the harbormaster for their assistance in sampling. Finally, I would like to acknowledge Dr. Kelley Thomas for being the initiator for the microbiome part of this project and Devin Thomas for DNA sequencing and his assistance in bioinformatics analysis. This research was funded by the Scott Murphy M.D. Fund.

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Appendix:

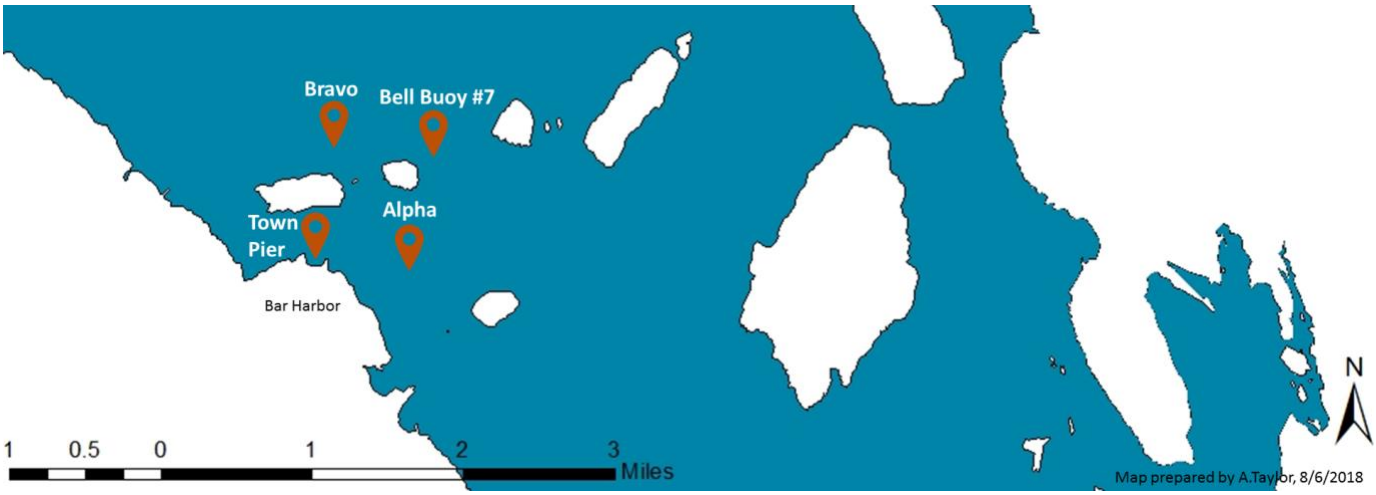

Figure 1: *Map of the different areas sampled, including the cruise ship anchorages Alpha, Bravo and the Town Pier (Taylor A. 2018)*

Field Sheet:

Microplastics and the microbiome around cruise ships		
Site name:		
Collected by:		
Date:	**Time:**	
Volume of water for microplastics:	**Volume of water for microbiome:**	
Weather Clear Fog Partly Cloudy Overcast Drizzle Downpour Snow Rain	**48 Hr. Rain** None Light Medium Heavy	**Tide Stage** Low Mid High
Inches of Rain:		

<table>
<tr><td>Water Temperature:</td><td>Air Temperature:</td><td>Cooler temp.:</td></tr>
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South
Southeast
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North
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All metadata for this project is on Anecdata®: Exploring the Marine Microbiome:

https://www.anecdata.org/projects/view/318/about