Relationship of total *Vibrio* spp. and *Vibrio vulnificus* to phytoplankton and water quality parameters in the Neuse River Estuary, North Carolina

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A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the Department of Environmental Sciences and Engineering, Gillings School of Global Public Health.

Chapel Hill
2010

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Abstract

Emma Susick: Relationship of total *Vibrio* spp. and *Vibrio vulnificus* to phytoplankton and water quality parameters in the Neuse River Estuary, North Carolina
(Under the direction of Dr. Rachel Noble and Dr. Gregory Characklis)

Vibrio bacteria are widely distributed in estuarine and coastal aquatic systems across the globe and not only play vital roles in nutrient cycling but are also important human pathogens. *V. vulnificus* is especially important in the United States as it is responsible for a majority of deaths seafood-related deaths. This study examined dynamics among total *Vibrio*, *V. vulnificus*, plankton populations and environment parameters in the Neuse River Estuary. Size fractionation was used to crudely partition zooplankton from phytoplankton. While there was substantial variation in total *Vibrio* concentrations, the ≥180μm fraction, containing primarily large phytoplankton and zooplankton, exhibited more rapid growth over the course of the experiment compared to fractions containing smaller organisms and control treatments. Responses of *V. vulnificus* were also tested, but results showed that dynamics are complex and highly variable. Further exploration of the species-specific nature of these relationships is necessary to improve understanding of *Vibrio* ecology.

Acknowledgements

This work has been financially supported by the National Science Foundation and National Institutes of Health Ecology of Infectious Disease Program Grant Number 0812913, Collaborative Research: Linkage of Bacterial Pathogens to Human Infectious Disease in an Estuary Subjected to Extreme Climatic Events. I would first like to thank my advisors, Dr. Rachel Noble and Dr. Greg Characklis who have given me endless support and guidance over the past two years. Also, I would like to thank the Paerl lab who collected the water on which my research depended, everyone in the Noble lab, especially Denene Blackwood, for their support and advice over the summer, and my committee member, Dr. Jill Stewart.

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Introduction:

Bacteria in the genus *Vibrio* are widely distributed in estuarine and coastal aquatic systems across the globe, and play vital roles in these ecosystems by processing and recycling nutrients through the degradation of chitin and other organic materials (Hunt et al., 2008, Li and Roseman, 2004). In addition to their role in the global nutrient cycle, certain *Vibrio* species also cause disease in aquatic organisms and humans making them important from an economic and public health perspective (Oliver et al., 2005, Todd, 1989).

There are three pathogenic *Vibrio* species of public health importance: *V. cholerae,* the causative agent of the disease cholera; *V. vulnificus*, which causes wound infections and primary septicemia; and *V. parahaemolyticus*, which causes gastroenteritis (West et al., 1989). Other *Vibrio* species, while posing less threat to human health, can cause illnesses in marine life as well (Austin and Zhang, 2006, Ben-Haim and Rosenberg, 2002).

In 2005, there were 131,943 reported cases of cholera worldwide resulting in 2,272 deaths, which may only represent 5-10% of actual disease incidence (WHO, 2006). In 2006, the CDC reported 730 confirmed cases of illness in the U.S. resulting in 36 deaths due to *Vibrio* bacteria (including *V. vulnificus, V. parahaemolyticus,* and *V. cholerae*) through the Other Vibrio Illness Surveillance System (CDC, 2006). Understanding how *Vibrio* interact with their environment and associate with other marine organisms is important in understanding their ecology. This information could

lead to better understanding and prediction of the timing and location of increased threats to public health and efforts to establish preventative measures.

Previous work has examined how environmental parameters and water quality in aquatic systems impacts *Vibrio* growth, as variability in *Vibrio* concentrations has been associated with temperature, salinity, nutrient concentrations, sediments and the presence of other aquatic organisms such as plankton. Temperature and salinity have been recognized as the major predictive factors in *Vibrio* abundance (Wetz et al., 2008, Hsieh et al., 2007, Randa et al., 2004). The highest concentrations of *Vibrio* are generally reported in the summer months when water temperature is warmer (Blackwell and Oliver, 2008, Hsieh et al 2007, Paz et al., 2007). The observed reduction in *Vibrio* isolated in winter months is likely due to their ability to enter a viable but non-culturable (VBNC) state during times of duress, a state from which they can still react and become infectious if ingested (Pruzzo et al., 2003). It has also been observed that *Vibrio* can reside in the sediment, which may provide a reservoir of *Vibrio* that can be resuspended into the water column during a storm event (Fries et al., 2007). In addition to warm water temperatures, *Vibrio* concentrations have been positively correlated with salinity measurements in the Neuse River Estuary (Fries et al., 2008, Hsieh et al., 2007, Lipp et al., 2001). However, the optimal salinity range may change depending on temperature, nutrient availability and the specific *Vibrio* species (Blackwell and Oliver, 2008, Kaspar and Tamplin, 1993).

In addition to water quality parameters, previous work has examined how plankton populations can impact *Vibrio* concentrations. It has been shown that *Vibrio* can gain protection and nutrients from associations with plankton (Eiler et al., 2006,

Islam et al., 1994). *Vibrio* are through to benefit from their ability to degrade the chitinous exterior of zooplankton, as evidenced by studies that found *Vibrio* attached to zooplankton and benefiting from these associations (Hunt et al., 2008, Huq et al., 1983). With phytoplankton, *Vibrio* populations often increase after phytoplankton blooms, suggesting that they feed off the subsequent release of dissolved organic matter and decaying cells (Rehmstam-Holm et al., 2010, Mourino-Perez et al., 2003).

Previous studies have indicated that *Vibrio* can survive longer and grow faster in association with zooplankton, particularly copepods (Tamplin et al., 1990, Huq et al., 1983, Kogure et al., 1980). Turner et al. (2009) examined concentrations of free-floating *Vibrio* and *Vibrio* attached to various plankton classified by size (63-200μm, ≥200μm plankton). They found that for free-living *Vibrio*, temperature and salinity are the primary factors in predicting population levels. For attached *Vibrio*, it was determined that plankton species composition, especially the abundance and the life stage of copepods, was also important. Eiler et al. (2007) looked at *Vibrio* growth in response to increases in the concentration of cyanobacteria-derived organic matter and found it resulted in increased growth of *V. cholera* and *V. vulnificus*. One study by de Mageny et al. (2008) compared cholera cases to chlorophyll *a* measurements, an indirect measure of phytoplankton concentration, in coastal areas and found strong associations between increases in chlorophyll *a* measurements and subsequent increases in cholera cases inland. This study tied relationships between phytoplankton and *Vibrio* populations to reported cholera cases and public health risk. Thus, changes in plankton populations and composition will likely have an impact on *Vibrio* concentrations and provide a useful indicator of human health threats.

Climate change may be another important factor in evaluating public health risk associated with *Vibrio.* Current climate change models predict warming of waters and increased rainfall in coastal areas, potentially impacting the concentration and range of *Vibrio* populations (Houghton et al., 2001). As the water gets warmer and stays warmer longer, there may be an increase in *Vibrio* levels and changes in the composition of plankton populations, which has also been shown to influence *Vibrio* concentrations (Paerl et al., 2007, Lipp et al., 2002). Climate change could also impact associations between *Vibrio* and plankton or larger macrobiota, such as oysters. Clinical strains of *V. vulnificus* were found at higher concentrations in oysters during warmer months (Han et al. 2009, Warner and Oliver, 2008). This is particularly alarming as *V. vulnificus* have been found at higher concentrations in oysters than surrounding water (Wright et al., 1996). In addition to temperature, Fernandez-Delgado et al. (2009) found that increased rainfall corresponded to increased isolation of *V. cholera*, a trend likely linked to decreases in salinity.

While previous work has identified relationships between zooplankton and *Vibrio*, potential relationships between phytoplankton and *Vibrio* concentrations are not nearly as well characterized. The goal of this research is to examine how associations with zoo- and phytoplankton influence the survival of Vibrio and to compare total *Vibrio* concentrations to those of *V. vulnificus*. These results provide insights useful in assessing human health risks and could act as inputs for predictive models of water quality. In addition to determining total *Vibrio* concentrations through culture methods, *V. vulnificus* was identified using quantitative polymerase chain reaction (QPCR). These results are important for understanding how *V. vulnificus*

concentrations, measured using molecular methods, change relative to total *Vibrio* concentrations, measured through culture-based methods. It is important to note that culture-based methods for *Vibrio* are not as selective and specific as DNA-based methods, as culture-based methods could lead to overestimation due to false-positives and/or underestimation due to competition with other organisms, particulate matter or inhibitory compounds in the media (Harwood et al., 2004, Wright et al., 1993, Lotz et al., 1983). The use of a time series method to examine how *Vibrio* populations, especially *V. vulnificus*, change with the concentration of various plankton groups was designed to add to our current understanding of *Vibrio* ecology.

Methods:

Sampling. The Neuse River Estuary (NRE) is an important resource for fishing and recreation in eastern North Carolina and hosts a natural population of *Vibrio* bacteria. It is typically a partially-mixed, shallow, drowned river valley estuary. Six large volume NRE samples were collected from June to August of 2009. Water was obtained from station 120, which is approximately 17 miles downstream from New Bern (Figure 1). Station 120 is located at an elbow-shaped bend in the estuary, which regularly has a salinity gradient and chlorophyll *a* concentration that is favorable for *Vibrio* populations (Wetz et al., 2009). During the sampling period, station 120 temperatures ranged from 26-30°C, salinity from 12-27ppt, and chlorophyll *a* from 3-19μg/L. Samples were collected from 0.2 to 0.5 meters below the surface in acid-rinsed 5 or 10 L Nalgene containers, placed in coolers and transported within four hours to the

laboratory for immediate analysis. In situ salinity, temperature, chlorophyll *a*, dissolved oxygen, turbidity and pH were measured using a YSI multiprobe Sonde (Yellow Springs Instruments).

Laboratory Analysis.

Experimental Design. Particulate matter from NRE water was separated into different size fractions of ≥180µm, and <180µm using a Nitex mesh. A raw, unmanipulated control was also included for each sample. Samples were stored at room temperature, and not in direct sunlight. The containers were mixed at each time point, and subsamples were taken every 4-12 hours. At each time point aliquots were taken to analyze total *Vibrio* concentrations using Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) and *V. vulnificus* concentrations using quantitative polymerase chain reaction (QPCR). Chlorophyll *a* analysis and phytoplankton microscopy was also performed (Table 1). All analyses were conducted in duplicate.

Size Fractionation. To examine the different effects zooplankton and phytoplankton have on *Vibrio* populations size fractions representing each group were created. The method of using size fractionation to examine relationships between *Vibrio* and plankton has been used previously by Turner et al. (2009) and Montanari et al. (1999). Size fractionation was accomplished using 47mm 180µm Nitex filters (Millipore), and included a ≥180µm size fraction representing zooplankton (typically larger than 200µm) and larger estuarine plankton, as well as a <180µm size fraction representing

phytoplankton and smaller estuarine plankton. There were two different methods used during the summer, discussed below (Figure 2).

In June and July, 1 L sample volumes were used to create the size fractions and control. The ≥180μm size fraction was filtered through an 180μm nylon net filter and the material left on the filter was rinsed into an equal volume of NRE water that had been filtered through a 0.4μm filter. An equal volume of NRE water was also passed through a 5μm filter and the filtrate was added into the ≥180μm size fraction. This water represents an additional inoculation of bacteria from the original water samples, and was added with the intention of increasing the speed and magnitude of *Vibrio* response to plankton. The <180μm size fraction was created by combining equal volumes of filtrate that passed through the 180μm nylon filter (the <180μm fraction) and a 5μm nylon filter (the bacterial inoculation). A control was also established with raw, unmanipulated NRE water. Additional size fractions of <100μm and <20μm were created in June and July but were not continued in August and not included in analyses as they yielded similar results to the <180μm (See Appendix A).

In August, the volumes studied were increased to 5 L to run additional assays, reduce the likelihood of "bottle effects" and maintain potential ecological relationships. The NRE water was filtered through an 180μm filter and the material on top of the filter was rinsed into an equal volume of water that was filtered through a 20μm filter. This change was made due to time limitations and with the intention of looking at zooplankton, which would likely not be found in the 20μm filtrate. The <180μm size fraction was created from the filtrate that passed through the 180μm nylon filter. No 5μm filtrate was added to these size fractions. This was due in part to time limitations,

but also to determine how *Vibrio* concentrations would react in a more natural setting without supplementation. A control was also established with raw, unmanipulated NRE water.

Membrane Filtration. At each time point aliquots were taken out of each size fraction and filtered through a 47mm 0.45μm grid GN-6 Merticel MCE membrane filters (Pall) in duplicate. Aliquot volume was determined by using the framework described by Hsieh et al. (2007) to guide dilutions for *Vibrio* analyses using initial measures of NRE temperature and salinity. The filter was then placed onto TCBS agar and placed in a 37°C incubator for 24 hours. TCBS media has been shown to be selective for *Vibrio* (West et al., 1982). All yellow and green colonies were counted as *Vibrio.*

QPCR. Aliquots of 100ml or 50ml, depending on the time point, were subsampled from each bottle, filtered through 47mm 0.40μm polycarbonate filters (Millipore) and stored at -80°C for later analysis. DNA extraction and analysis was completed following a protocol established by Wetz et al. (2008). DNA was extracted using 0.1mm silica/zirconium beads (BioSpec Products), 490μl extraction buffer AE (Qiagen), 10μl of 10μg/mL salmon sperm DNA (Sigma), and a mini bead beater for each sample. Salmon sperm DNA (from *Oncorhynchus keta* [sketa]), was added into the extraction buffer as a control to measure extraction effectiveness and inhibition (Haugland et al., 2005). The beads, extraction mixture and polycarbonate filters were placed in the bead beater for 1 minute and then centrifuged for 1 minute at 12,000 x g. The supernatant was extracted, leaving the pellet intact and added into a clean 1.7μm microcentrifuge tube and

centrifuged for 5 minutes at 12,000 x g. The supernatant was removed and added into a clean 1.7µm microcentrifuge tube, leaving the pellet in place. This final supernatant was the extracted DNA.

Inhibition was measured using QPCR to quantify the remaining portion of the known amount of salmon sperm DNA that was originally spiked onto the filter. QPCR reactions were run in duplicate using the Cepheid SmartCyclerII with the primers, Sketa probe and Omnimix. For each reaction, 14.75µl of nuclease-free water, 2.5µl of 10µm reverse sketa primer, 2.5µl of 10µm forward sketa primer, 0.25µl of 10µm sketa probe, 0.5 Omnimix beads and 5µl of sample were used (nuclease free water: OmniPu from VWR EM-9610; reverse sketa primer: MWG Biotech Inc. 5' CCG AGC CGT CCT GGT CTA 3'; forward sketa primer: MWG Biotech Inc. 5'GGT TTC CGC AGC TGG G 3'; sketa probe: MWG Biotech Inc., 5'-6FAM-AGTCGCAGGCGGCCACCGT-TAMRA; Omnimix, TaKaRa Bio Inc., Omnimix HS lyophilized PCR master mix containing 3U TaKaRa hot start Taq Polymerase, 200µM dNTP, 4mM $MgCl_2$ in 25µM HEPES buffer, pH 8.0 +- 0.1) (Haugland et al., 2005). The QPCR assay started at 94°C for 120 seconds, then 45 cycles at 94°C for 15 seconds followed by 60°C for 30 seconds. Samples were considered inhibited if samples were measured at 1.5 cycle time (Ct) values away from the standard. If samples were inhibited the sample was diluted accordingly until no inhibition was observed. If inhibition could not be removed through dilution, the DNA was further purified using an additional DNA extraction method, DNA-EZ RW02 Extraction (GeneRite) following the K102-02C Extraction Protocol.

The *V. vulnicifus* QPCR primers and probe were designed to target the hemolysin gene vvh, unique to *V. vulnificus* (Wetz et al., 2008, Harwood et al., 2004). QPCR

reactions were run in duplicate using the Cepheid SmartCyclerII with the vvh primers, probes and Omnimix HS. For each reaction, 14.75µl of nuclease-free water, 2.5µl of 10µm reverse primer, 2.5µl of 10µm forward primer, 0.25µl of 10µm probe, 0.5 Omnimix HS beads, and 5µl of sample were used (nuclease free water: OmniPur from VWR EM-9610; reverse vvh 1973 primer: MWG Biotech Inc. 5' TCG ACT GTG AGC GTT TTG TC 3'; forward vvh 1795 primer: MWG Biotech Inc. 5' TGC CTR GAT GTT TAT GGT GAG ACC 3'; vvh 1914 FAM probe: MWG Biotech Inc., 5' TAG CCG AGT RGC ATC CGA TCG TTG TT 3'; Omnimix, TaKaRa Bio Inc., Omnimix HS lyophilized PCR master mix containing 3U TaKaRa hot start Taq Polymerase, 200µM dNTP, 4mM $MgCl_2$ in 25µM HEPES buffer, pH 8.0 +- 0.1).

In addition to the samples, negative extraction controls, negative controls and positive standards were run to confirm technique and create a standard curve. The standard curve was constructed of four serial dilutions of a known about of *V. vulnificus*. Standards were created by growing up a *Vibrio vulnificus* culture (ATCC 27562) and making serial dilutions in 1X Phosphate Buffered Saline (PBS) according to Wetz et al. (2008). Concentrations were determined under fluorescent microscopy using SYBR Green I following Noble and Fuhrman (1998). The culture was then diluted to a final concentration of 100,000 cells per 100ml. The 100ml of the *V. vulnificus* dilution was then filtered through 47mm 0.4µm PC filters and stored at -80°C for later analysis.

Four serial dilutions of the *V. vulnificus* standard were made to create the standard curve. The SmartCyclerII Software uses a linear regression of known samples to create the standard curve to compare known amounts of DNA to the unknown

samples. Negative extraction controls included an unused PC filter. Negative controls used nuclease free water instead of sample.

Chlorophyll a. 50ml of sample water was filtered through a 25mm Glass Fiber Filter (GFF), wrapped in aluminum foil to prevent exposure to light and stored at -20°C for later analysis. Chlorophyll *a* was analyzed following the Modified Fluorometric Technique in EPA Method 445. The chlorophyll *a* extraction was completed by placing the filters into 15ml tubes with 10ml of 90% acetone. The tubes were then placed in a sonication bath with water and ice, covered with aluminum foil and sonicated for 10 minutes. Tubes were then placed in a -20°C freezer for 20 hours. Samples were removed and the liquid was filtered through a 25mm GFF filter to remove any particulates and debris. The filtrate was placed in a fluorometer (Turner, TD-700) and the fluorescence was translated into chlorophyll *a* concentrations.

In addition to measuring chlorophyll *a* concentrations, 50ml of sample was transferred to brown bottles and Lugol's solution (5g iodine, 10g potassium iodide, 85ml distilled water) was added at a 1% total concentration to preserve and stain the phytoplankton. Aliquots of 18ml were then added to settling chambers, and left for 24 hours. Phylogenic groups of phytoplankton (diatoms, cyanobacteria, dinoflagellates, chlorophytes/cryptophytes) were enumerated using microscopy.

Statistical Analysis. Total *Vibrio* and *V. vulnificus* concentrations were log transformed for analysis. For additional analyses, log growth was also calculated for total *Vibrio* by dividing the concentration at time X by the initial concentration and taking the log of

that value. Next, data was tested for normality by plotting data points against their modified z-distribution and looking for a linear relationship. All data, except for the log transformed total *Vibrio* concentrations for June/July, were normally distributed. For normally distributed data, parametric tests were used, including independent, two-tailed, two sample t-tests, and correlation analysis with Pearson correlation coefficients. Non-parametric analyses were used for data that was not normally distributed, including two-tailed Wilcoxon-Mann-Whitney tests and correlation analysis with Spearman correlation coefficients. The t-test and Wilcoxon-Mann-Whitney test compare the means of two populations and the correlation coefficients examine variation shared by two populations. Statistical analyses were run separately for the June/July samples and the August samples due to the difference in methodology. Analyses were conducted using Excel (Microsoft, 2008) and SAS Statistical Software (Cary, NC, USA). Relationships were deemed significant at $p \leq 0.05$.

Results

Environmental Parameters. Over the sampling period, water temperatures ranged from 26-30°C, salinity from 12-27ppt, and chlorophyll *a* from 3-19µg/L. There were also several storm events, defined as greater than or equal to 0.5 inches of daily rainfall (Figure 3). A storm event occurred within two days before both the August 4th and 17th sample dates.

Plankton and Chlorophyll a. The phytoplankton population was estimated using two methods, chlorophyll *a* detection and direct microscopic enumeration. Previous work

has examined zooplankton in the NRE and similar estuarine waters and found summer concentrations to range from 10-200 zooplankton/L (Mallin, 1991). For this study's sample dates, NRE zooplankton concentrations ranged from 5.3 to 36.4 organisms/L, with copepods comprising 34-67% of the total zooplankton population (J. Leonard, unpublished data). Volumes used in these experiments were 1 and 5 L. While zooplankton were not directly counted due to time and sample limitations, it was assumed based on previous work that zooplankton were present in the ≥180μm fraction.

For June/July samples, the ≥180μm fraction had significantly lower chlorophyll *a* concentrations than <180μm (p=0.028) (Table. 2, Figure 4a). It also appears that the ≥180μm fraction has lower chlorophyll *a* concentrations than the control, however, this could not be confirmed through statistics due to low sample size. The control and the <180μm size fractions do not appear to differ significantly from one another (Figure 4a). This suggests that the ≥180μm fraction contained fewer phytoplankton than the <180μm fraction and control, which is expected as phytoplankton should be filtered out of the ≥180μm fraction but remain in the <180μm and control. For August samples, none of the size fractions differed significantly in chlorophyll *a* concentration (Table 2, Figure 4b). This is not unexpected as the August ≥180μm fraction combined material on top of the 180μm filter with the filtrate from a <20μm filter, which contains significant concentrations of phytoplankton (confirmed by microscopy).

Over all size fractions, chlorophyll *a* concentration was not found to vary significantly over time for either the June/July or August samples (p=0.415, p=0.119, respectively) (Table 6). However, it does appear that chlorophyll *a* did increase over

the course of the experiment when looking at individual sample dates. The control from July 15th and the <180µm fraction from July 20th do increase over time (Appendix C). This variation could be the result of a particular group of plankton that found the experimental conditions favorable for growth, or a lack of zooplankton grazers (Paerl et al., 2007). This may explain why the <180µm fraction, containing more phytoplankton than the ≥180µm fraction, exhibited slow, but significant, growth over 48 hours ($p=0.05$) (Table 6).

Microscopic phytoplankton counts were also conducted to estimate phytoplankton populations and percent composition of specific phytoplankton groups (cyanobacteria, diatoms, dinoflagellates, chlorophytes/cryptophytes). These counts further confirmed the chlorophyll *a* observations in June/July samples, which showed that the ≥180µm fraction contained, on average, half the phytoplankton in the <100µm and control fraction (Table 3). The <100µm fraction served as an estimate of the <180µm fraction for phytoplankton for this assay, as the microscopic counts only identified organisms under 100µm. These results suggest that the 180µm filter was successful in removing a significant portion of the phytoplankton. However, for August samples, phytoplankton counts were similar across both size fractions and the control (Table 3). Again, this agrees with the chlorophyll *a* observations, that phytoplankton concentrations were similar across size fractions in August.

Analysis of total Vibrio. Total *Vibrio* concentrations were quantified through membrane filtration and plating on TCBS agar to count colonies. The two sets of experiments reveal different trends that are likely due to the bacterial inoculation and

community composition differences in zoo- and phytoplankton in the June/July samples from those in August. One established difference between the two sets of experiments is the increased number of phytoplankton in the ≥180μm fraction in the August samples compared to those in June/July (Figure 4).

While there is substantial variability among the experiments conducted, some trends did emerge. In the June/July samples, initial total *Vibrio* concentrations for ≥180μm fraction was significantly lower than the <180μm and control ($p=0.0001$, $p=0.037$ respectively) (Table 4, Figure 5). Examining log growth, each size fraction differed significantly from one another for the June/July samples after 48 hours, with the ≥180μm fraction significantly higher than the <180μm and control ($p<0.0001$, $p=0.004$ respectively) (Table 5, Figure 6). This agrees with previous work that found *Vibrio* growth positively correlated with zooplankton concentrations, especially copepods, which are likely contained in the ≥180μm fraction (Huq et al., 1983, Turner et al., 2009). Another possibility is that grazers are being filtered out of the ≥180μm and therefore there is less negative selective pressure. Longnecker et al. (2010) found *Gammaproteobacteria*, of which *Vibrio* are a member, exhibited higher bacterial production after reduction of grazers and were less affected by virus reduction than other bacteria groups. This underscores the importance of examining specific relationships between *Vibrio* and its surrounding environment, as *Vibrio* may react differently than other well studied bacteria. *Vibrio* may even react differently depending on their specific environment, as seen with their association with salinity (Kasper and Tamplin, 1993). Additionally, a small, but significant correlation was found with total *Vibrio* concentrations and time ($r = 0.524$, $p<0.0001$) (Table 6).

In general, size fractions in August did not vary significantly from one other in total *Vibrio* concentration, both initially and over time. This may be due in part to significantly higher phytoplankton concentrations in the ≥180μm fraction for August compared to June/July, a difference that can be seen in the plankton analysis (Figure 4, Table C-5). This implies that the first method used in June/July represents a more meaningful separation, which may have led to increased differentiation of *Vibrio* concentrations between the treatments. Also, there was a small but significant correlation between time and total *Vibrio* concentration ($r=0.477$, $p<0.0001$), indicating there was some growth over the course of the experiment (Table 6).

Comparing total *Vibrio* concentrations across the two sets of experiments, the June/July <180μm fraction were initially larger than the ≥180μm fraction. This trend was not seen in the August samples (Figure 7). This is likely due to differences in methodology. The June/July ≥180μm fractions showed increased growth compared to those in August (Figure 8). This trend was not as pronounced in the <180μm fractions. This is not surprising as the <180μm fraction had similar phytoplankton populations across all sample dates, whereas the ≥180μm fraction differed between the June/July and August samples. The increased growth seen in the ≥180μm fraction compared to the <180μm fraction could potentially suggest that the plankton smaller than 180μm, including phytoplankton, are less beneficial in aiding *Vibrio* growth than zooplankton, which are typically larger than 180μm.

The bacterial inoculant added in the June/July samples could also have increased the likelihood for bacterial-plankton interactions. While *Vibrio* concentrations have been found to increase during phytoplankton blooms, there are also negative selective

pressures, such as grazers and viruses that could limit growth (Worden et al., 2006). These interactions with other bacteria, protozoan grazers and viruses, as well as their surrounding environment, are complex and not yet well understood. While this study did not closely examine the plankton community composition, this could potentially play a large role in *Vibrio* population dynamics.

In addition to comparing *Vibrio* concentrations among size fractions and sample dates, *Vibrio* concentrations were also compared to corresponding chlorophyll *a* concentrations. While previous studies have found a positive correlation between bacterial production (μgC $L^{-1}h^{-1}$) and chlorophyll *a* (Apple et al., 2008), this research did not find total *Vibrio* concentrations to be significantly correlated with chlorophyll *a*. However, log *Vibrio* growth in August exhibited a small, but significant, correlation with chlorophyll *a* (r=0.363, p=0.002) (Table 6). After 36 hours, there is some trend between *Vibrio* concentration and initial salinity measurements. This could suggest that salinity influences the growth rate of *Vibrio* (Figure 9). Salinity has previously been associated with *Vibrio* concentration in estuarine and coastal environments, but its relationship to growth has not been directly examined in a time scale experiment (Blackwell and Oliver, 2008, Hsieh et al. 2008). These results provide additional information that further demonstrates the relationship between salinity and *Vibrio* concentrations.

Analysis of Vibrio vulnificus. QPCR was used to quantify *V. vulnificus* in samples from July 15th, July 20th, August 4th and August 17th. Each sample was extracted, tested for inhibition and then QPCR was conducted to determine the *V. vulnificus* concentration.

The August 17th samples were the only samples that showed substantial inhibition of PCR. It is possible that there were *V. vulnificus* concentrations above the detection limit of the assay (approximately 100 cells/100 mL), but that they could not be quantified. *V. vulnificus* was quantified on July 15th and August 4th, with some concentrations 10-100 times higher than corresponding total *Vibrio* concentrations as determined by membrane filtration with TCBS. On July 15th *V. vulnificus* was quantified at time 0, then again at 24 hours and remained relatively stable until 48 hours. On August 4th *V. vulnificus* was detected at 48 hours (Figure 10). Statistical differences in *V. vulnificus* across treatments could not be tested due to small sample size.

V. vulnificus is a species in the *Vibrio* genus and therefore concentrations of *V. vulnificus* should not exceed total *Vibrio* concentrations (Figure 10). However, quantification of *V. vulnificus* was based on the assumption of a single copy of the vvh gene, whereas total *Vibrio* was quantified based on colony formation using a membrane filtration approach. While TCBS is a popular and widely accepted media for culturing *Vibrio*, it lacks sensitivity and specificity (Harwood et al., 2004, Choopun et al., 2002, Massad and Oliver, 1987). Additionally, it has been documented that not all *Vibrio* species grow well on TCBS (such as *V. vulnificus*), leading to underestimations, and that non-*Vibrio* species can grow on TCBS (such as *Aeromonas* and *Pseudomonas*), leading to overestimation (Lotz et al., 1983, Wright et al., 1993, Pfeffer and Oliver, 2003). This difference in quantification using molecular versus culture methods underscores the need for standardized methods to quickly and easily identify and compare total *Vibrio* and species-specific forms of *Vibrio* in estuarine and coastal waters. With *V. vulnificus* concentrations often higher in oysters than in the surrounding waters, underestimating

concentrations could be dangerous to current public health risk models (Wright et al., 1996). Additional explanations for higher *V. vulnificus* concentrations compared to total *Vibrio* include possible viral infection, which could lyse cells and release genomic material. This DNA would be quantified during QPCR but not with membrane filtration.

A weak, but positive, correlation between total *Vibrio* and *V. vulnificus* was found in the July 15th sample ($r=0.777$, $p=0.020$). No correlation was found for the August 4th ($r=-0.200$, $p=0.747$); however, this could be due to the very small number of *V. vulnificus* observations (Table 6).

Conclusion

The aim of this study was to assess the dynamics of *Vibrio*, and specifically the potentially pathogenic *V. vulnificus*, in experiments that manipulated zoo- and phytoplankton communities. Previous work has shown positive relationships between *Vibrio* and copepods (zooplankton), but relationships are still unclear with respect to phytoplankton. As phytoplankton are vital and plentiful components of aquatic ecosystems, exploring possible relationships could provide important information about *Vibrio* ecology and potential public health impacts.

In this work, there was significant variability among experiments and size fractions within experiments. However, some trends emerged that seem significant and worth further exploration. In the June/July samples, which likely had better separation of zooplankton and phytoplankton due to methodology, total *Vibrio* concentrations in the ≥180μm fraction were lower initially but grew faster over time compared to the <180μm fraction and control. This agrees with previous work that found copepods

beneficial for *Vibrio* growth. However, the control should have the same zooplankton populations as the ≥180μm, yet exhibited less growth. This suggests that something smaller than 180μm, and present in the <180μm fraction and control group, is not as beneficial for *Vibrio* growth. Possible explanations include bacterial grazers that were mostly filtered out using 180μm Nitex filters or out-competition by other bacteria and phytoplankton for nutrients. Obtaining a more detailed picture of *Vibrio* ecology is important in understanding how these bacteria influence nutrient cycles and impact public health.

In addition to ecological relationships, this study also highlights the differences in quantification of potentially pathogenic *Vibrio* using molecular methods compared to the "gold standard" approach of quantifying total *Vibrio* concentrations using membrane filtration with TCBS. There are currently no QPCR assays known by the author to quantify the entire *Vibrio* genus due to taxonomic diversity. Clearly there are drawbacks to using current culture-based techniques, as found in this study and others, and underestimation of *Vibrio* bacteria using current culture methods may not allow for proper public health risk assessments. Future studies on ecological relationships of specific species of *Vibrio*, like *V. vulnificus*, with specific species of phytoplankton may allow for the development of more relevant predictive models to protect public health. Undoubtedly, the ecological dynamics of *Vibrio* and *V. vulnificus* are complex and warrant further study.

Tables

Table 1. Experimental summary.

Date	Location	Treatments	Time Points (hours)	Assays
June 22nd	Station 70/120	≥180μm (filter and 0.4μm) + 5μm filtrate <180μm + 5μm filtrate	0 14 36	TCBS (total *Vibrio*) Phytoplankton Microscopy
July 7th	Station 70/120	≥180μm (filter and 0.4μm) + 5μm filtrate <180μm + 5μm filtrate Control = Raw NRE	0 4 8 12 30	TCBS (total *Vibrio*) Phytoplankton Microscopy
July 15th	Station 120	≥180μm (filter and 0.4μm) + 5μm filtrate <180μm + 5μm filtrate Control = Raw NRE	0 8 24 36 48	TCBS(total *Vibrio*) Phytoplankton Microscopy Chl a qPCR (*V. vulnificus*)
July 20th	Station 120	≥180μm (filter and 0.4μm) + 5μm filtrate <180μm + 5μm filtrate	0 12 24 36	TCBS(total *Vibrio*) Phytoplankton Microscopy Chl a qPCR (*V. vulnificus*)
August 4th	Station 120	≥180μm (filter and 20μm) <180μm Control = raw NRE	0 6 16 24 36 48	TCBS(total *Vibrio*) Phytoplankton Microscopy Chl a qPCR (*V. vulnificus*)
August 17th	Station 120	≥180μm (filter and 20μm) <180μm Control = raw NRE	0 6 16 24 36 48	TCBS(total *Vibrio*) Phytoplankton Microscopy Chl a qPCR (*V. vulnificus*)

Table 2. Independent t-test results for chlorophyll *a* concentrations.

	July (n = 8)			**August** (n = 8)		
	≥180μm to <180 μm	≥180μm to Control	<180 to Control	≥180μm to <180 μm	≥180μm to Control	<180μm to Control
Time (hr)	t (p)	t (p)	t (p)	t (p)	t (p)	t (p)
T = 0	**-3.96 (0.028)**	--	--	0.06 (0.569)	-0.78 (0.464)	-2.18 (0.072)
T=24	**-3.74 (0.010)**	--	--	-1.04 (0.339)	-0.78 (0.467)	0.11 (0.916)
T = 48	-2.43[a] (0.093)	--	--	0.37 (0.721)	-0.37 (0.725)	-1.07 (0.324)

[a]n=6

Table 3. Microscopic phytoplankton counts (cells/L).

Size Fraction	**July 7th**	**July 15th**	**July 20th**	**August 4th**	**August 17th**
≥180μm	4.06e5	5.68e5	3.65e5	7.54e5	8.27e5
<180μm[a]	8.27e5	1.34e6	6.49e5	6.85e5	6.76e5
Control	--	--	--	6.57e5	7.10e5

[a]<100μm for July samples.

Table 4. Wilcoxon-Mann-Whitney and independent t-test results for total *Vibrio* using log (CFU/100mL).

	July			August		
	≥180µm to <180 µm	≥180µm to Control	<180µm to Control	≥180µm to <180 µm	≥180µm to Control	<180µm to Control
	Z	Z	Z	t	t	t
Time (hr)	(p)	(p)	(p)	(p)	(p)	(p)
T = 0	**-4.348**[a]	**2.084**[b]	-0.266[c]	-0.65	-1.65	-0.82
	(0.0001)	**(0.037)**	(0.790)	(0.522)	(0.117)	(0.422)
T = 12 (July)	**2.178**[d]	-1.088[e]	N/A	-1.12	-1.43	-0.13
T = 16 (Aug)	**(0.029)**	(0.277)		(0.270)	(0.162)	(0.901)
T = 24	-0.053[g]	**-1.958**[h]	-1.436[h]	0.48	0.07	-0.35[j]
	(0.958)	**(0.050)**	(0.151)	(0.633)	(0.947)	(0.732)
T = 36	0.734[h]	-1.718[h]	-1.754[i]	-1.47[j]	**-3.61**[j]	-1.63
	(0.4629)	(0.086)	(0.080)	(0.151)	**(0.002)**	(0.123)
T = 48	**2.178**[d]	-1.620[f]	1.644[f]	-1.28[k]	**-3.27**[l]	**-3.42**[k]
	(0.029)	(0.105)	(0.100)	(0.216)	**(0.007)**	**(0.004)**

[a] n= 30 [e] n= 5 [i] n= 14
[b] n= 20 [f] n= 6 [j] n= 31
[c] n= 18 [g] n= 16 [k] n= 26
[d] n= 8 [h] n= 10 [l] n= 24

Table 5. Independent t-test results for total *Vibrio* using log growth ratio (log (Ct/C0)).

	July			August (N = 32)		
	≥180μm to <180 μm	≥180μm to Control	<180μm to Control	≥180μm to <180 μm	≥180μm to Control	<180μm to Control
Time (hr)	t (p)	t (p)	t (p)	t (p)	t (p)	t (p)
T = 0	-1.53[a] (0.1363)	-1.13[b] (0.272)	-0.21[c] (0.836)	-0.65 (0.521)	-1.33 (0.200)	-1.40 (0.180)
T = 12 (July) T = 16 (Aug)	**21.49**[d] **(0.0001)**	**48.95**[e] **(<0.0001)**	0.29[f] (0.789)	-0.99 (0.33)	-0.92 (0.369)	0.22 (0.824)
T = 24	**4.26**[g] **(0.001)**	**6.24**[h] **(0.0002)**	1.86[h] (0.100)	0.53 (0.601)	0.46 (0.649)	-0.09[j] (0.932)
T = 36	1.91[b] (0.072)	**4.16**[h] **(0.003)**	**2.62**[i] **(0.023)**	-1.36[j] (0.185)	**-2.41**[j] **(0.029)**	-1.12 (0.273)
T = 48	**11.98**[d] **(<0.0001)**	**6.05**[f] **(0.004)**	**-4.77**[f] **(0.009)**	0.15[k] (0.884)	**-2.33**[l] **(0.038)**	-2.02[k] (0.063)

[a] n= 30
[b] n= 20
[c] n= 18
[d] n= 8
[e] n= 5
[f] n= 6
[g] n= 16
[h] n= 10
[i] n= 14
[j] n= 31
[k] n= 26
[l] n= 24

Table 6. Correlations.

Parameters	July: Log *V. vulnificus* (N = 12)	July: Time (N = 138)	July: Chlorophyll *a* (N = 32)	August: Log *V. vulnificus* (N = 6)	August: Time (N = 277)	August: Chlorophyll *a* (N = 72)
	Spearman Correlation Coefficient, r (P-value)			Pearson Correlation Coefficient, r (P-value)		
Log Total *Vibrio*	**0.777 (0.003)**	**0.524 (<0.0001)**	**0.253 (0.021)**	-0.200 (0.747)	**0.477 (<0.0001)**	0.045 (0.712)
Log Total *Vibrio* growth	--	**0.456 (<0.0001)**	-0.096 (0.639)	--	**0.433 (<0.0001)**	**0.363 (0.002)**
Chlorophyll *a*	--	0.149[a] (0.415)	--	--	-0.185[b] (0.119)	--
≥180μm		0.387[c] (p=0.154)			-0.137[e] (0.397)	
<180μm		**0.532[d] (p=0.500)**			-0.125[e] (0.353)	

[a] n= 32
[b] n= 72
[c] n= 15
[d] n= 14
[e] n= 24

Figures

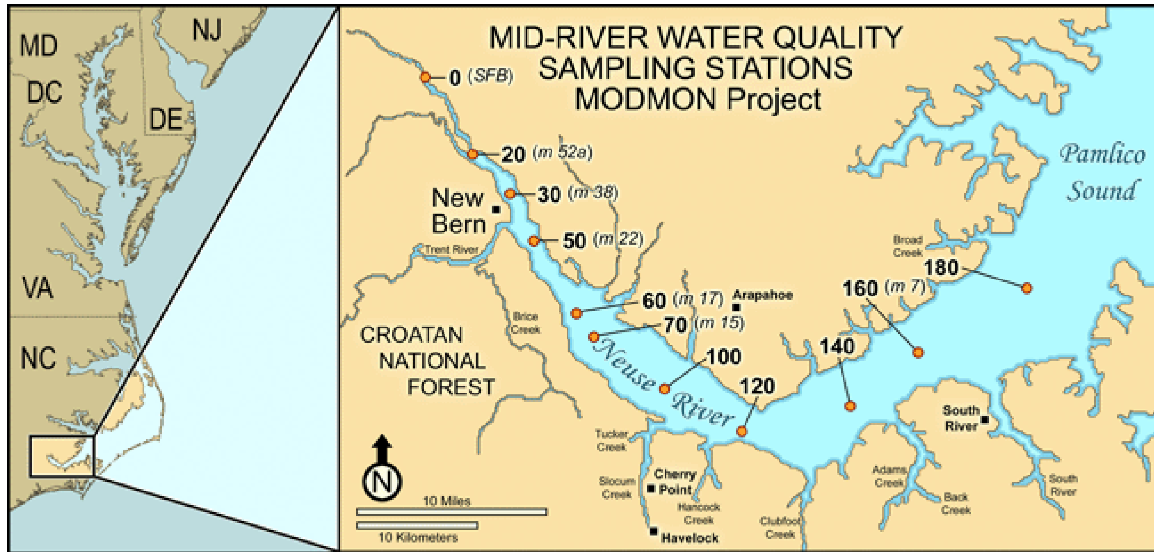
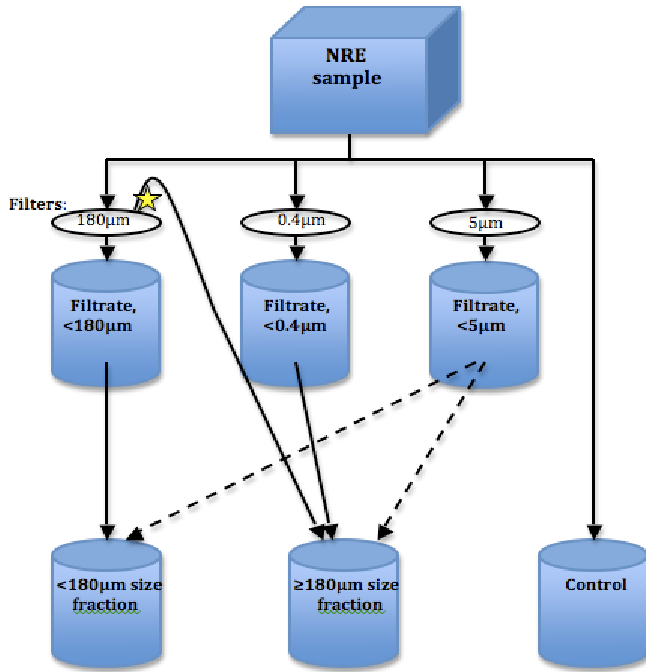


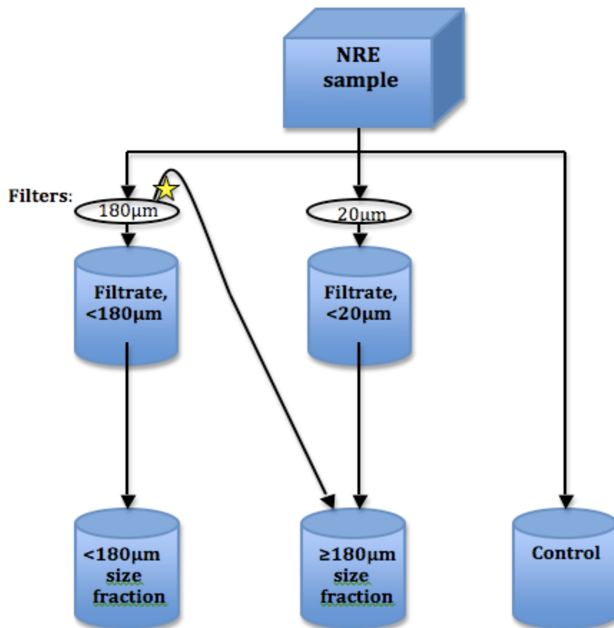
Figure 1. Neuse River Estuary sample sites (from ModMon website, 2009 http://www.unc.edu/ims/neuse/modmon/water_quality.htm)



This step represents material left on the filter being washed off into the 0.4µm filtrate.

Both the <180µm and ≥180µm contain equal volumes of the 5µm filtrate and the 180µm filtrate or 0.4µm filtrate, respectively

a.



This step represents material left on the filter being washed off into the 20µm filtrate.

b.

Figure 2. Experimental procedure for June/July (a) and August (b) samples.

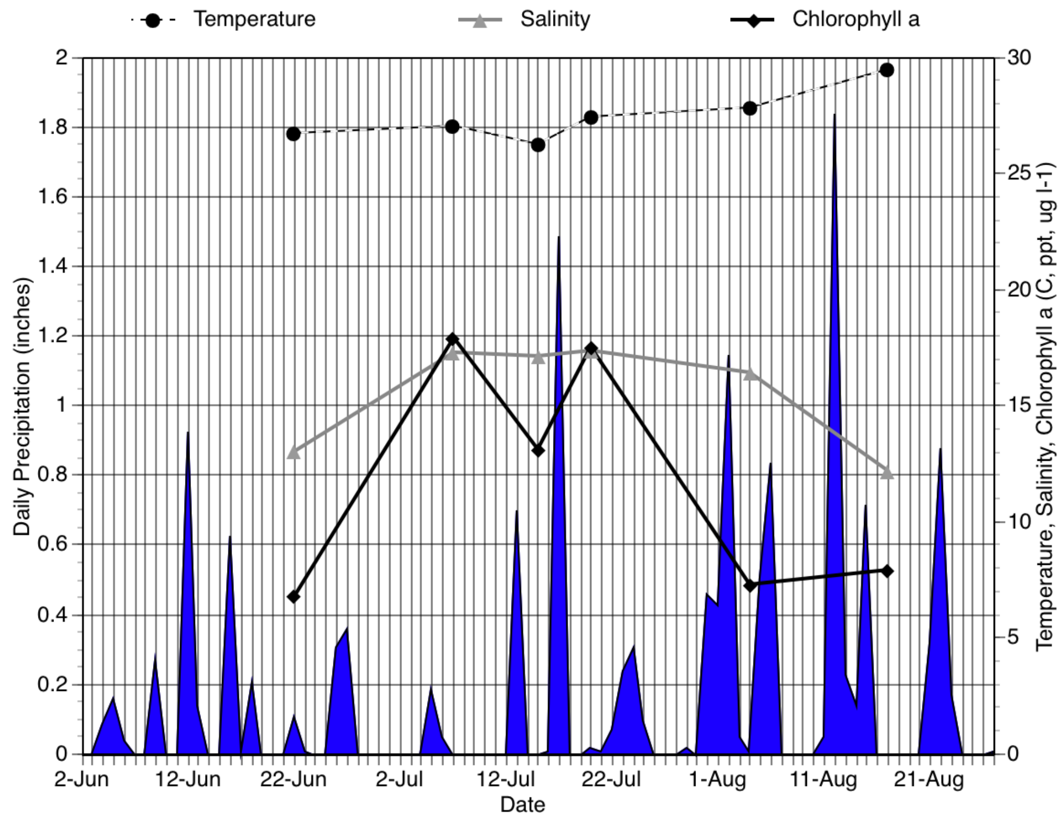
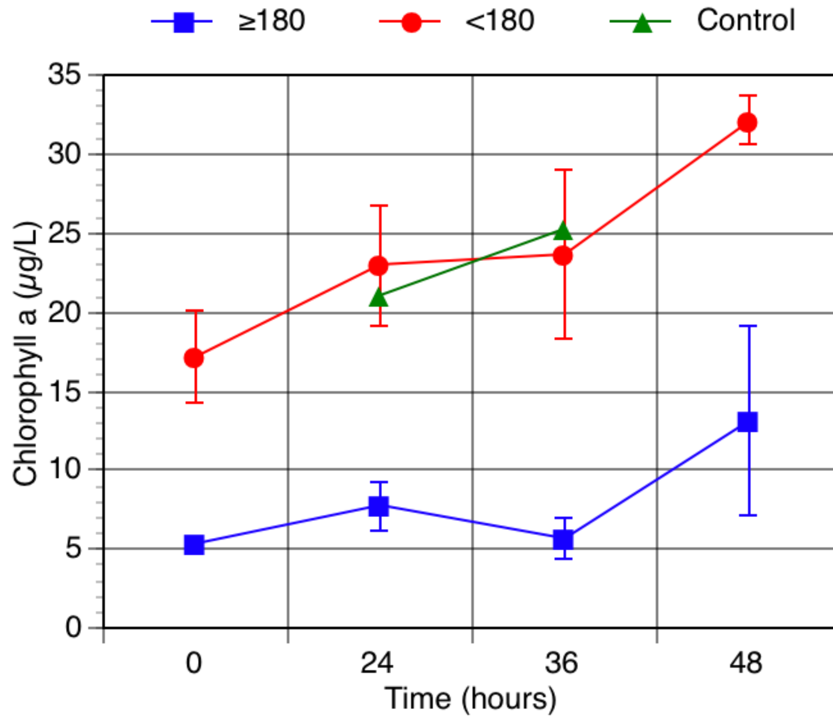
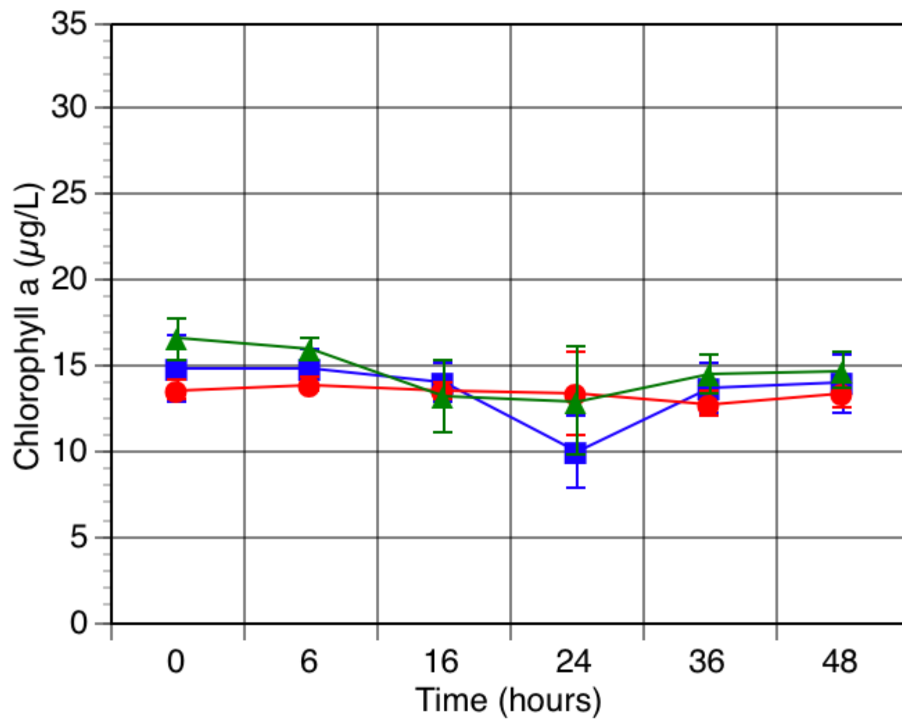


Figure 3. Daily precipitation at New Bern, NC with temperature, salinity and chlorophyll *a* from station 120 of the NRE.



a.



b.

Figure 4. Chlorophyll *a* concentrations over time for June/July (a) and August (b) (error bar = SE).

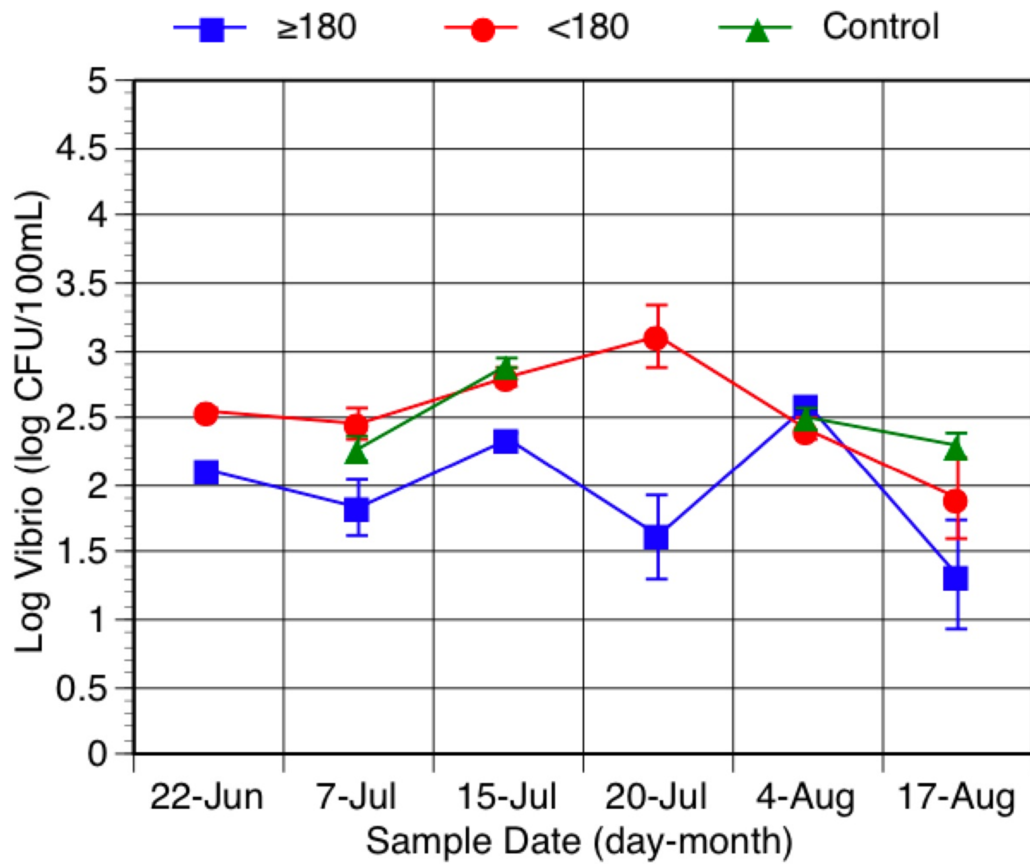
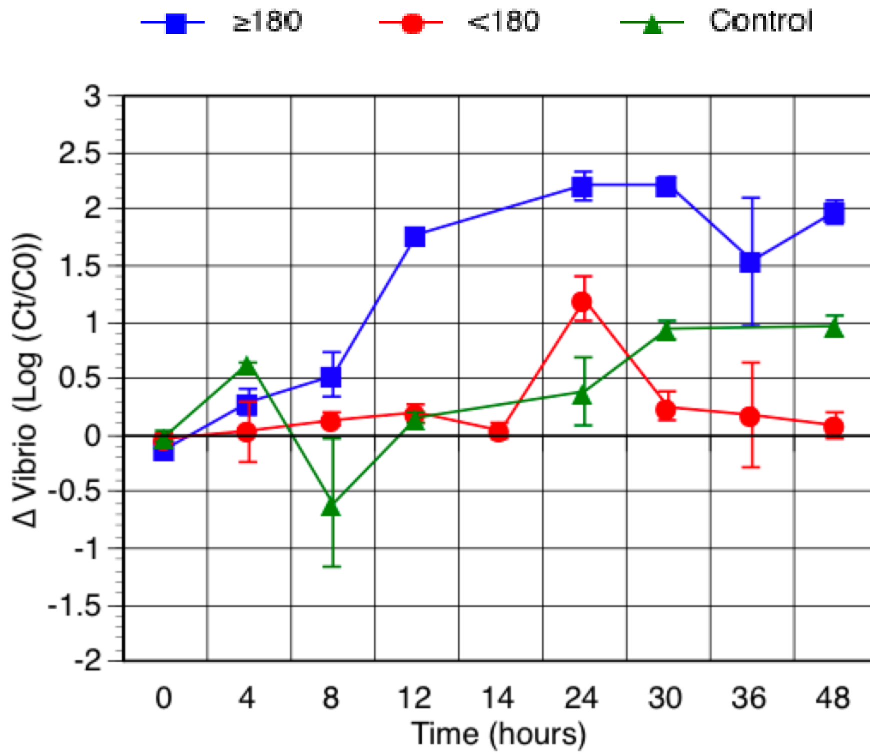
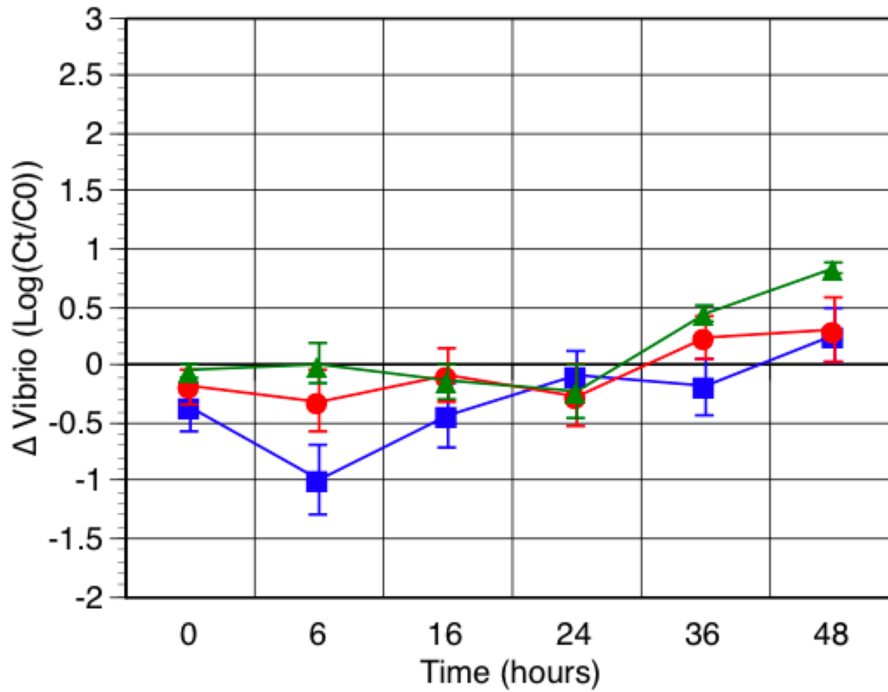


Figure 5. Initial total *Vibrio* concentrations for all sample dates (error bar = SE).

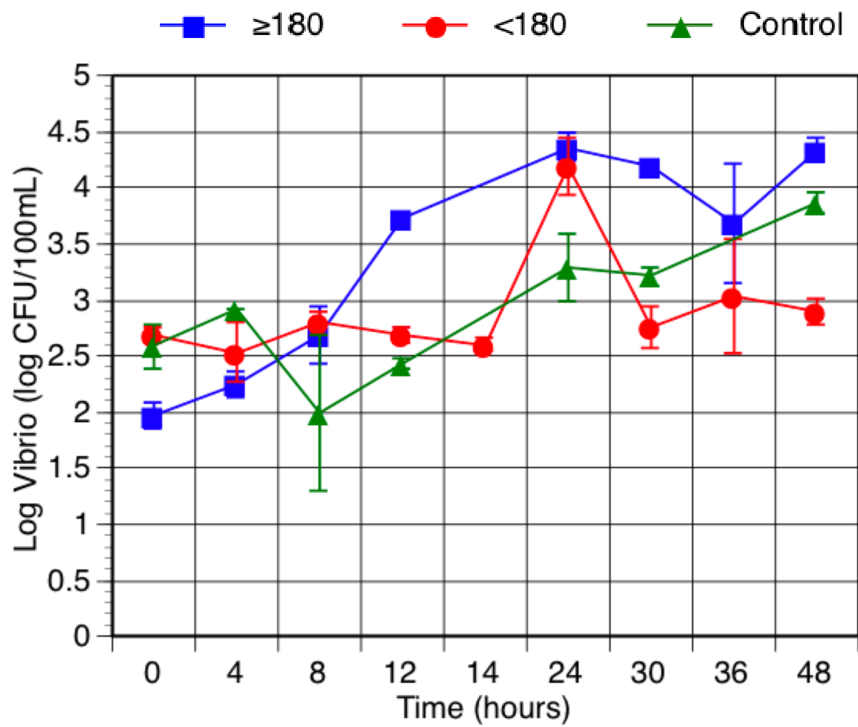


a.

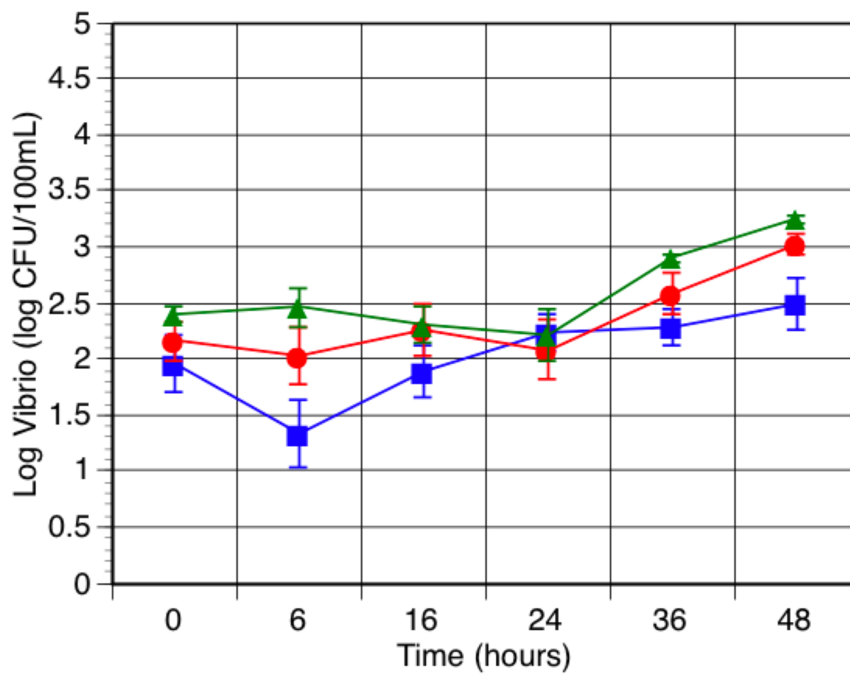


b.

Figure 6. Change in total *Vibrio* concentrations over time for June and July (a) and August (b) samples (error bar = SE).

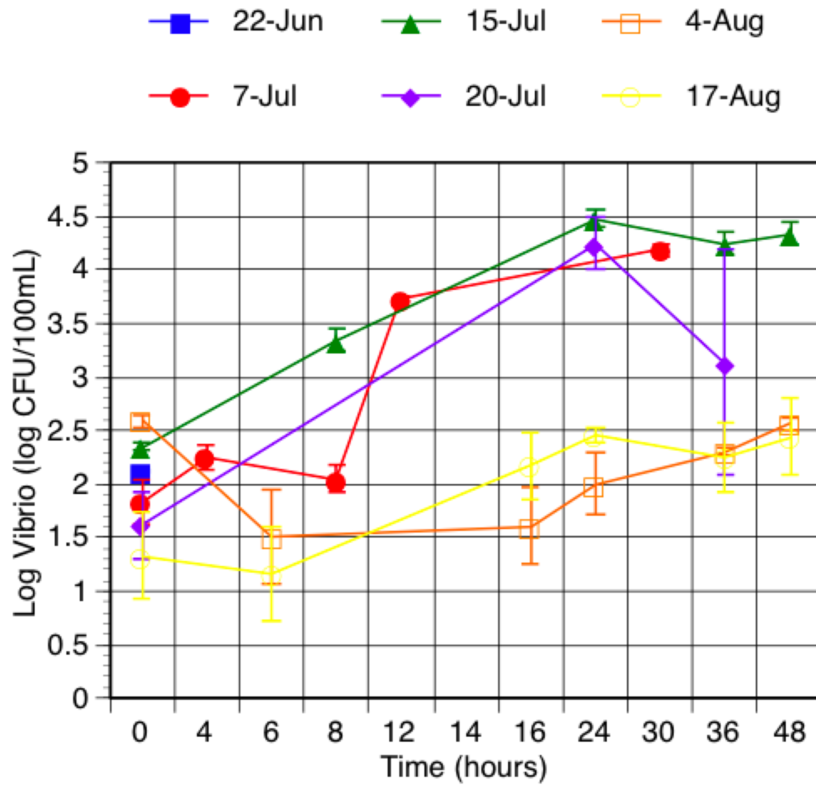


a.

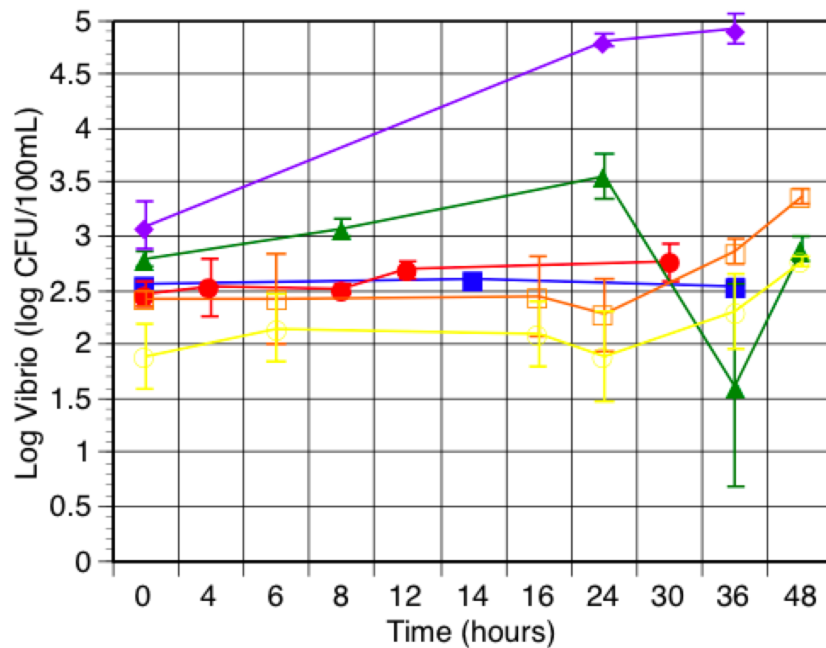


b.

Figure 7. Total *Vibrio* concentrations over time for June/July samples (a) and August samples (b) (error bar = SE).



a.



b.

Figure 8. Total *Vibrio* concentrations over time for ≥180µm (a) and <180µm (b) (error bar = SE).

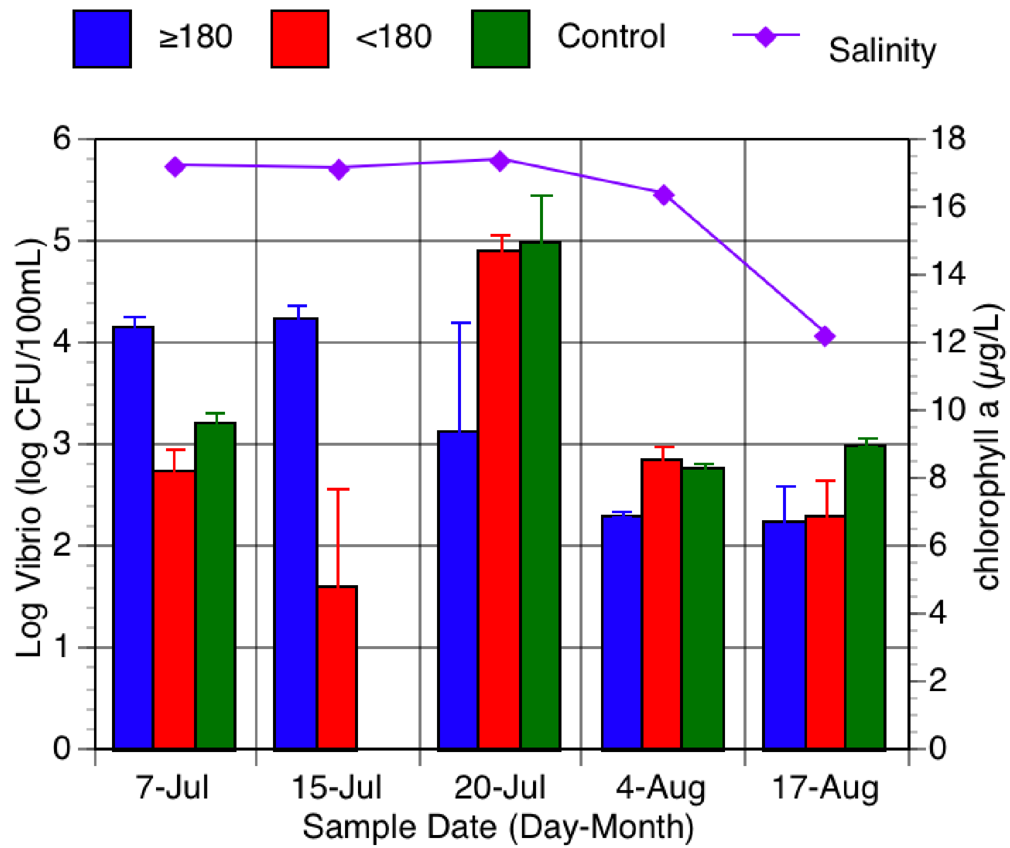
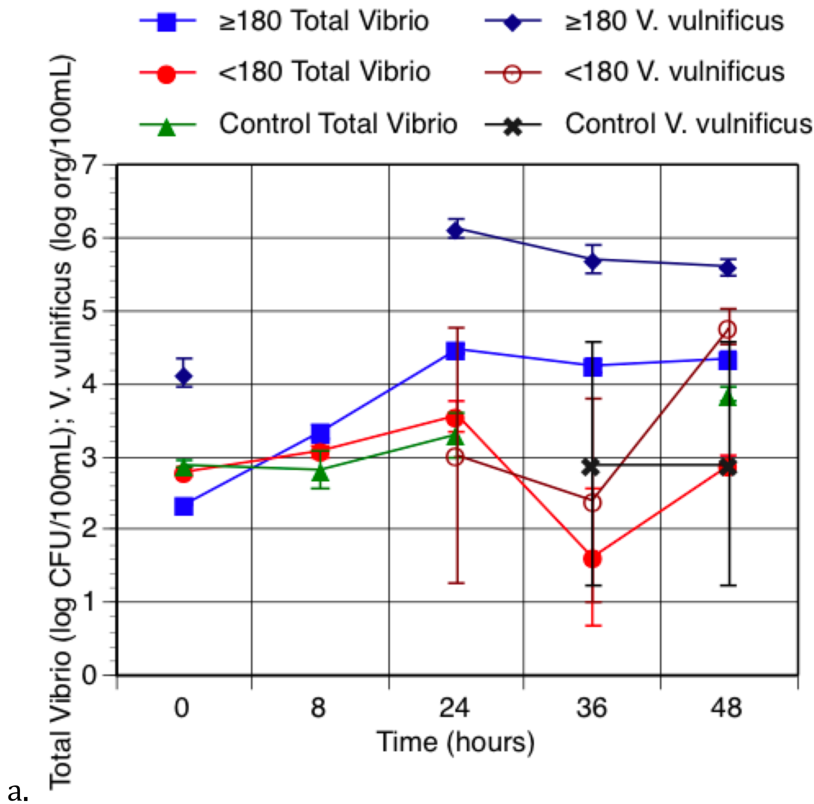
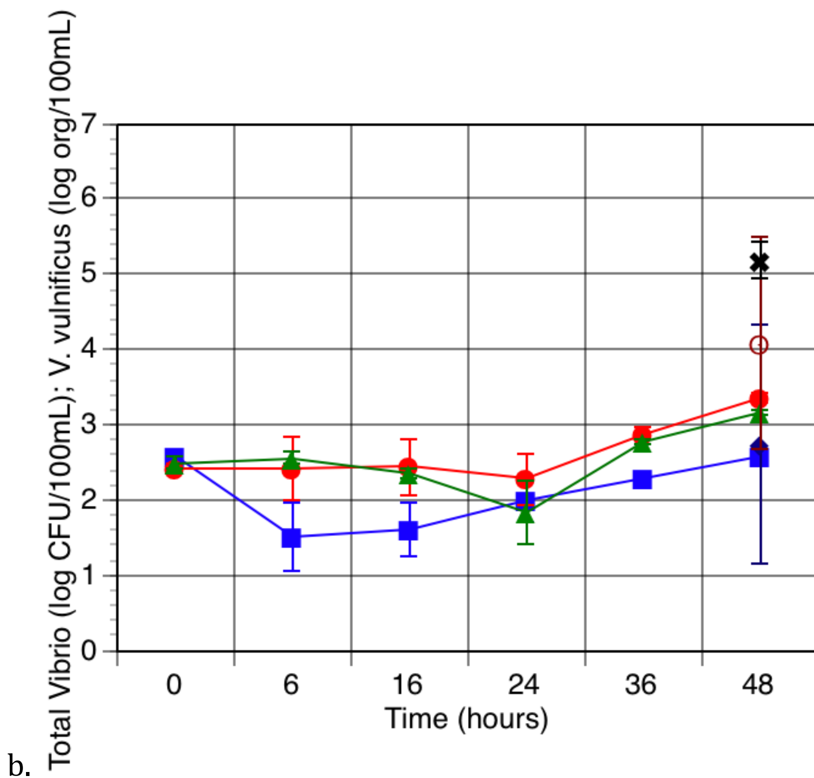


Figure 9. Initial salinity and total *Vibrio* concentration after 36 hours (error bar =SE).



a.



b.

Figure 10. *V. vulnificus* and total *Vibrio* concentrations over time for July 15th (a) and August 4th (b) (error bars= SE).

Appendix A. Total *Vibrio* Concentrations

Samples in June and July were separated into multiple size fractions. For the June 22nd date, samples were divided into ≥180μm, ≥100μm, ≥20μm, <180μm, <100μm and <20μm size fractions. Creation of the ≥100μm, <100μm, ≥20μm and <20μm followed the sample procedure as the ≥180μm and <180μm described in the methods section and Figure 2, except for the change in filter pore size. The same method was used for July samples, except size fractions included ≥180μm, <180μm, <100μm, <20μm and raw water controls.

Samples in August were also separated into size fractions, but with a slight variation on the method used in June and July, and less size fractions were used. The <180μm, <100μm and <20μm did not show significant differences in *Vibrio* or chlorophyll *a* concentration, and a decision was made to focus on fewer size fractions with more replicates. In August, the samples were separated into ≥180μm, <180μm and raw water control with no 5μm inoculant. Also, 'greater than' samples were created by rinsing the material on top of the filter into 20μm filtered water, serving as the base water with nutrients. This change was made from 0.4μm to 20μm due to feasibility as larger volumes were used in August and it would have taken too long to filter through the 0.4μm filter.

Table A-1. Total *Vibrio* concentrations for June 22nd.

		Total *Vibrio* (CFU/100mL)	
Time (hours)	**Size Fraction**	Average n=4	St. Dev.
0	≥180μm	131.3	12.50
	≥100μm	140.0	20.41
	≥20μm	163.8	17.50
	<180μm	363.8	33.26
	<100μm	361.3	14.93
	<20μm	385.0	40.62
14	≥180μm	TNTC	--
	≥100μm	422.5	201.53
	≥20μm	TNTC	--
	<180μm	412.5	129.39
	<100μm	450.0	171.10
	<20μm	242.5	23.27
36	≥180μm	TNTC	--
	≥100μm	715.0	14.14
	≥20μm	TNTC	--
	<180μm	361.3	115.35
	<100μm	330.0	7.07
	<20μm	306.3	119.12

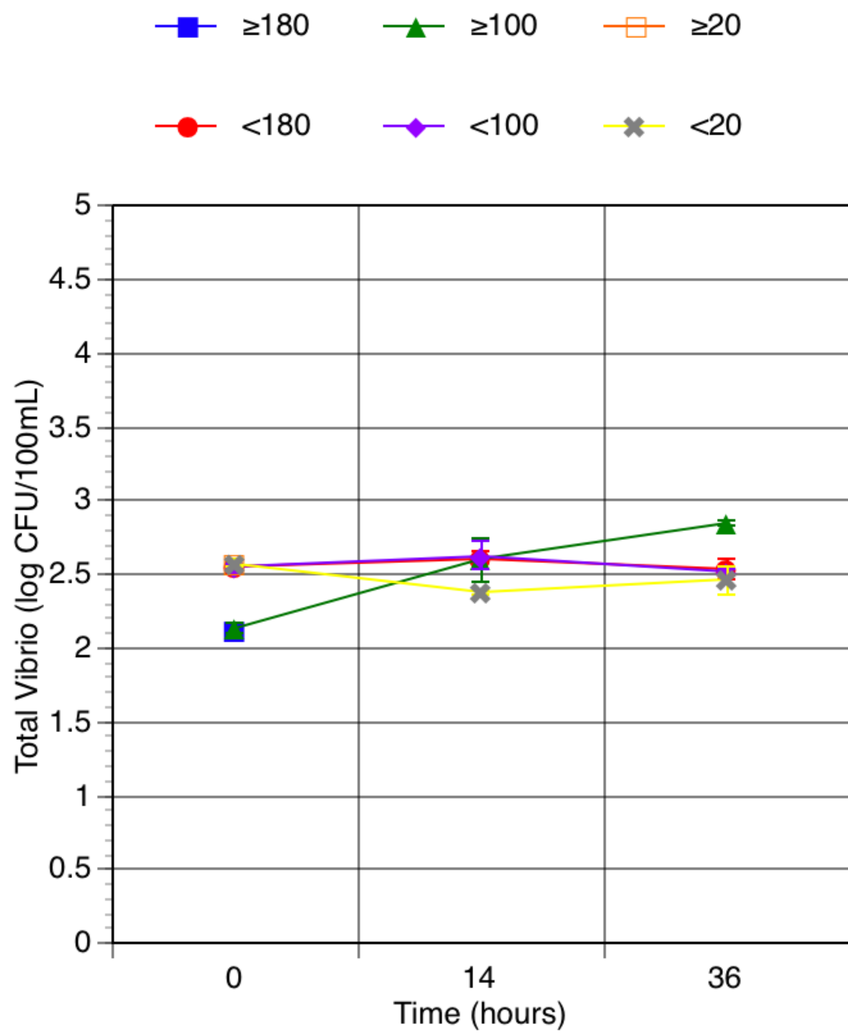


Figure A-1. Total *Vibrio* concentrations over time for June 22nd (error bar = SE).

Table A-2. Total *Vibrio* concentrations for July 7th.

Time (hours)	Size Fraction	Total *Vibrio* (CFU/100mL) Average n=4	St. Dev.
0	≥180µm	91.3	75.6
	<180µm	316.3	131.4
	<100µm	127.5	56.6
	<20µm	200.0	146.7
	Control	142.5	64.0
4	≥180µm	200.0	108.0
	<180µm	481.3	284.8
	<100µm	397.5	99.5
	<20µm	483.8	65.1
	Control	737.5	259.4
8	≥180µm	125.0	64.5
	<180µm	331.3	61.6
	<100µm	485.0	47.1
	<20µm	440.0	56.7
	Control	287.5	272.0
12	≥180µm	5375.0	368.6
	<180µm	511.3	173.2
	<100µm	426.3	152.3
	<20µm	727.5	257.0
	Control	625.0	405.2
30	≥180µm	15700.0	4413.6
	<180µm	750.0	637.7
	<100µm	2512.5	580.8
	<20µm	637.5	616.9
	Control	2425.0	907.8

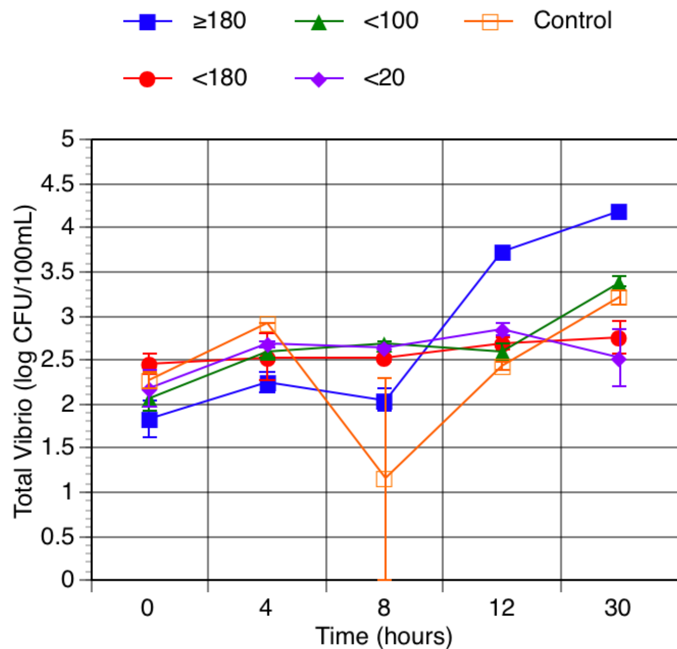


Figure A-2. Total *Vibrio* concentrations over time for July 7th (error bar = SE).

Table A-3. Total *Vibrio* concentrations for July 15th.

Time (hours)	Size Fraction	Total *Vibrio* (CFU/100mL) Average n=4	St. Dev.
0	≥180µm	226.3	25.9
	<180µm	650.0	184.8
	<100µm	556.3	117.4
	<20µm	665.0	31.4
	Control	596.3	242.5
8	≥180µm	2395.0	1103.6
	<180µm	1250.0	488.7
	<100µm	1387.5	401.7
	<20µm	912.5	334.5
	Control	1000.0	529.6
24	≥180µm	31250.0	10523.8
	<180µm	5025.0	4098.1
	<100µm	3775.0	963.1
	<20µm	3000.0	1445.1
	Control	18125.0	20282.9
36	≥180µm	19500.0	9037.0
	<180µm	2000.0	1414.2
	<100µm	27000.0	25459.1
	<20µm	250.0	500.0
	Control	5250.0	6116.9
48	≥180µm	23500.0	9609.0
	<180µm	875.0	478.7
	<100µm	17125.0	18957.7
	<20µm	1250.0	1658.3
	Control	34000.0	30843.7

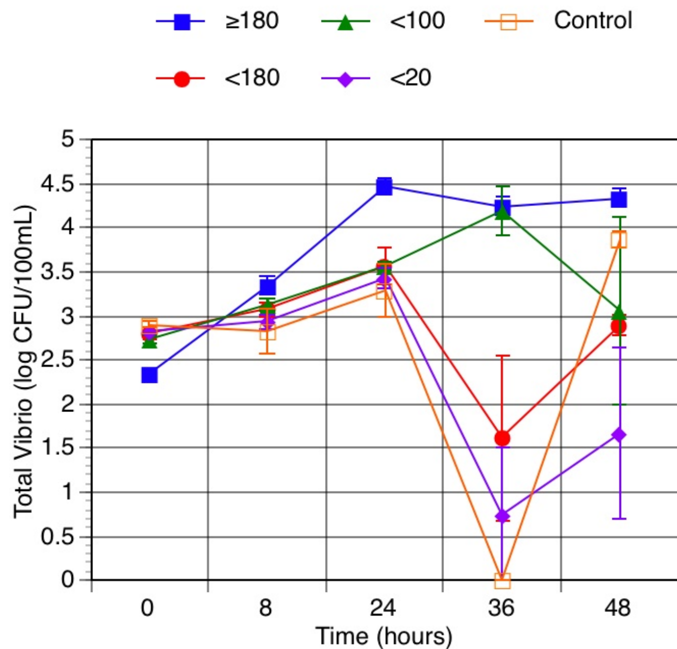


Figure A-3. Total *Vibrio* concentrations over time for July 15th (error bar = SE).

Table A-4. Total *Vibrio* concentrations for July 20th.

Time (hours)	Size Fraction	Total *Vibrio* (CFU/100mL) Average n=4	St. Dev.
0	≥180µm	90.0	127.8
	<180µm	1440.0	961.7
	<100µm	1962.5	138.2
	<20µm	2520.0	320.0
	Control	TNTC	--
12	≥180µm	TNTC	--
	<180µm	TNTC	--
	<100µm	TNTC	--
	<20µm	TNTC	--
	Control	TNTC	--
24	≥180µm	27500.0	24839.5
	<180µm	67000.0	16370.7
	<100µm	36250.0	8995.4
	<20µm	78500.0	2121.3
	Control	204750.0	39651.6
36	≥180µm	14687.5	17511.2
	<180µm	95937.5	50222.2
	<100µm	35312.5	20851.0
	<20µm	254666.7	299067.4
	Control	176500.0	88925.1
48	≥180µm	90.0	127.8
	<180µm	1440.0	961.7
	<100µm	1962.5	138.2
	<20µm	2520.0	320.0
	Control	0	0

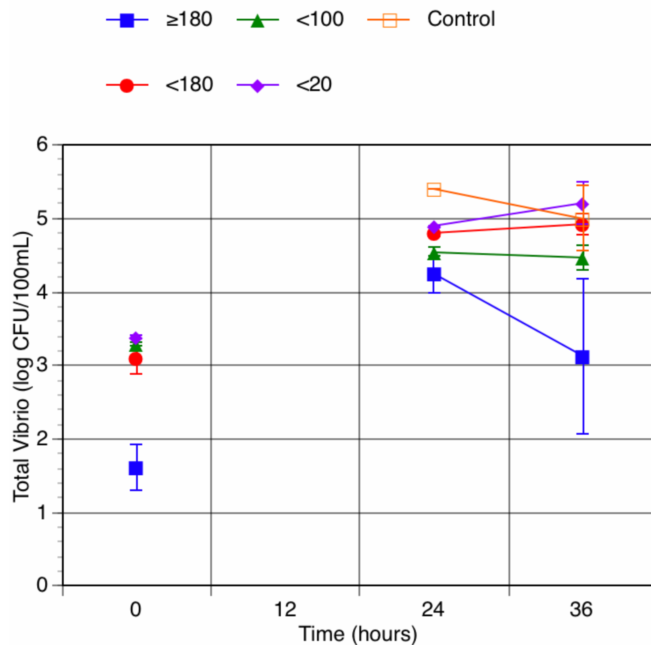


Figure A-4. Total *Vibrio* concentrations over time for July 20th (error bar = SE).

Table A-5. Total *Vibrio* concentrations for August 4th.

Time (hours)	Size Fraction	Total *Vibrio* (CFU/100mL) Average n=8	St. Dev.
0	≥180µm	417.5	167.1
	<180µm	287.5	113.1
	C	350.0	143.7
6	≥180µm	280.0	58.9
	<180µm	386.7	142.9
	C	417.5	228.6
16	≥180µm	120.0	36.5
	<180µm	707.5	522.8
	C	252.5	144.2
24	≥180µm	213.3	104.1
	<180µm	543.3	377.0
	C	360.0	329.1
36	≥180µm	206.7	53.2
	<180µm	912.5	647.3
	C	620.0	160.0
48	≥180µm	377.5	90.0
	<180µm	2510.0	1044.0
	C	1515.0	237.3

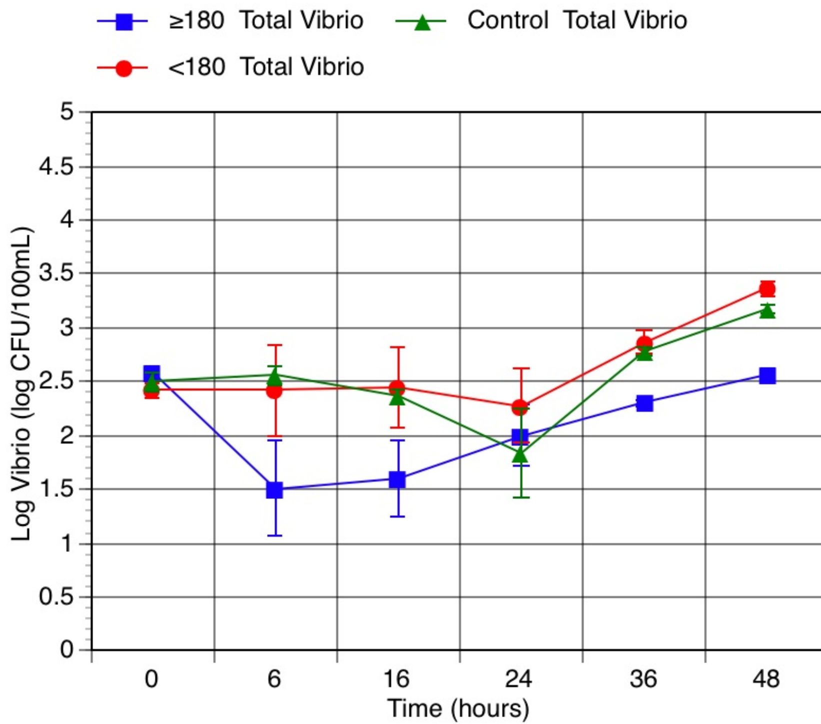


Figure A-5. Total *Vibrio* concentrations over time for August 4th (error bar = SE).

Table A-6. Total *Vibrio* concentrations for August 17th.

Time (hours)	Size Fraction	Total *Vibrio* (CFU/100mL) Average n=8	St. Dev.
0	≥180µm	422.5	170.6
	<180µm	396.7	235.3
	C	357.5	228.3
6	≥180µm	272.5	134.0
	<180µm	441.7	202.2
	C	697.5	678.2
16	≥180µm	391.7	252.2
	<180µm	245.0	143.6
	C	705.0	586.3
24	≥180µm	400.0	199.1
	<180µm	760.0	683.2
	C	647.5	716.9
36	≥180µm	737.5	646.6
	<180µm	295.0	397.8
	C	227.5	396.1
48	≥180µm	597.5	737.5
	<180µm	491.3	179.3
	C	562.5	741.8

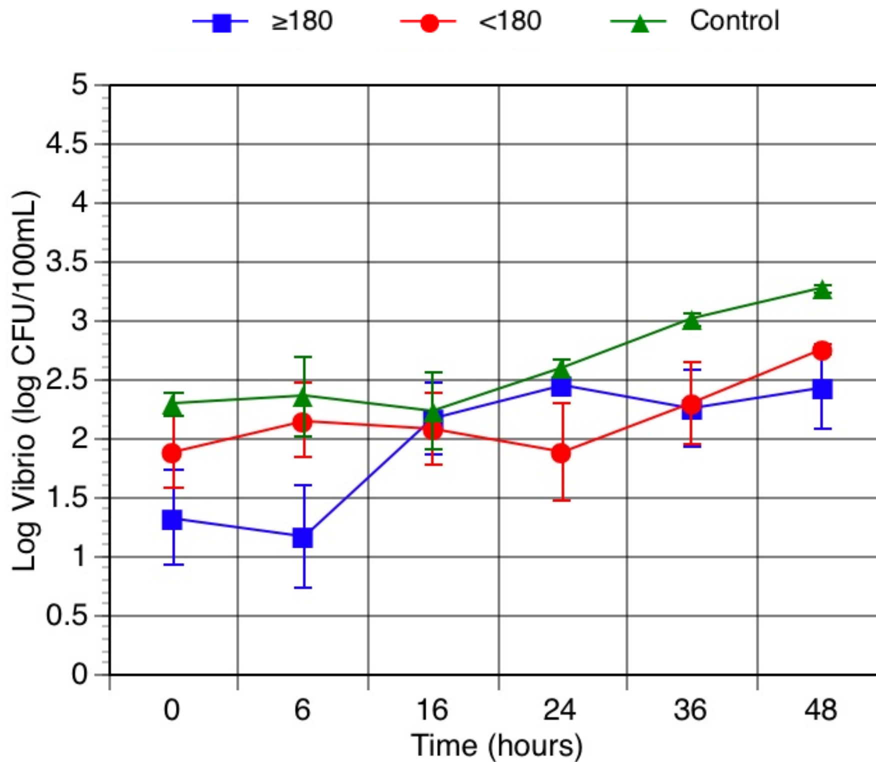


Figure A-6. Total *Vibrio* concentrations over time for August 17th (error bar = SE).

Appendix B. QPCR and *V. vulnificus* Concentrations

V. vulnificus concentrations were quantified using QPCR. *V. vulnificus* concentrations are quantified by creating a standard curve with known amounts of *V. vulnificus* and using the standard equation to calculate the amount of target cells/sample from cycle time values (CT values). When *V. vulnificus* could not be quantified, non detect (ND) was entered. See the following equation:

Target cells / 100mL= (10^[(standard equation slope*sample CT value)+standard equation y-intercept])*dilution factor

Table B-1. QPCR results for July 15th.

Time (hour)	Size Fraction	Standard Equation	R-squared	Efficiency	CT value rep 1	Ct value rep 2	Average CT	Target cells per 100mL
0	≥180a	y = -0.294x + 13.711	0.991	0.97	35.67	37.41	36.54	9294.80
	≥180b	y = -0.294x + 13.711	0.991	0.97	35.58	34.37	34.975	26813.28
	<100b	y = -0.315x + 14.05	0.993	1.07	ND	41.67	41.67	83.94
8	<100a	y = -0.315x + 14.05	0.993	1.07	38.96	ND	38.96	599.24
24	≥180a	y = -0.294x + 13.711	0.991	0.97	29.56	29.52	29.54	2124564.87
	≥180b	y = -0.294x + 13.711	0.991	0.97	30.87	31.15	31.01	785398.37
	<180b	y = -0.294x + 13.711	0.991	0.97	30.53	30.45	30.49	1145750.34
	<100a	y = -0.315x + 14.05	0.993	1.07	ND	37.34	37.34	3880.88
	<100b	y = -0.315x + 14.05	0.993	1.07	41.85	ND	41.85	147.33
36	≥180a	y = -0.294x + 13.711	0.991	0.97	32.78	32.6	32.69	251866.26
	≥180b	y = -0.294x + 13.711	0.991	0.97	30.35	30.63	30.49	1116786.09
	<180a	y = -0.294x + 13.711	0.991	0.97	34.29	34.78	34.535	72233.72
	<100b	y = -0.315x + 14.05	0.993	1.07	ND	35.59	35.59	13809.56
	Controlb	y = -0.294x + 13.711	0.991	0.97	31.46	31.23	31.345	626036.99
48	≥180a	y = -0.294x + 13.711	0.991	0.97	32.46	32.69	32.575	626036.99
	≥180b	y = -0.294x + 13.711	0.991	0.97	31.63	31.13	31.38	626036.99
	<180a	y = -0.294x + 13.711	0.991	0.97	33.98	33.38	33.68	626036.99
	<180b	y = -0.294x + 13.711	0.991	0.97	36.88	34.41	35.645	626036.99
	<100a	y = -0.315x + 14.05	0.993	1.07	36.74	38.73	37.735	2914.10
	Controlb	y = -0.294x + 13.711	0.991	0.97	31.3	31.48	31.39	607253.46

ND = None Detect

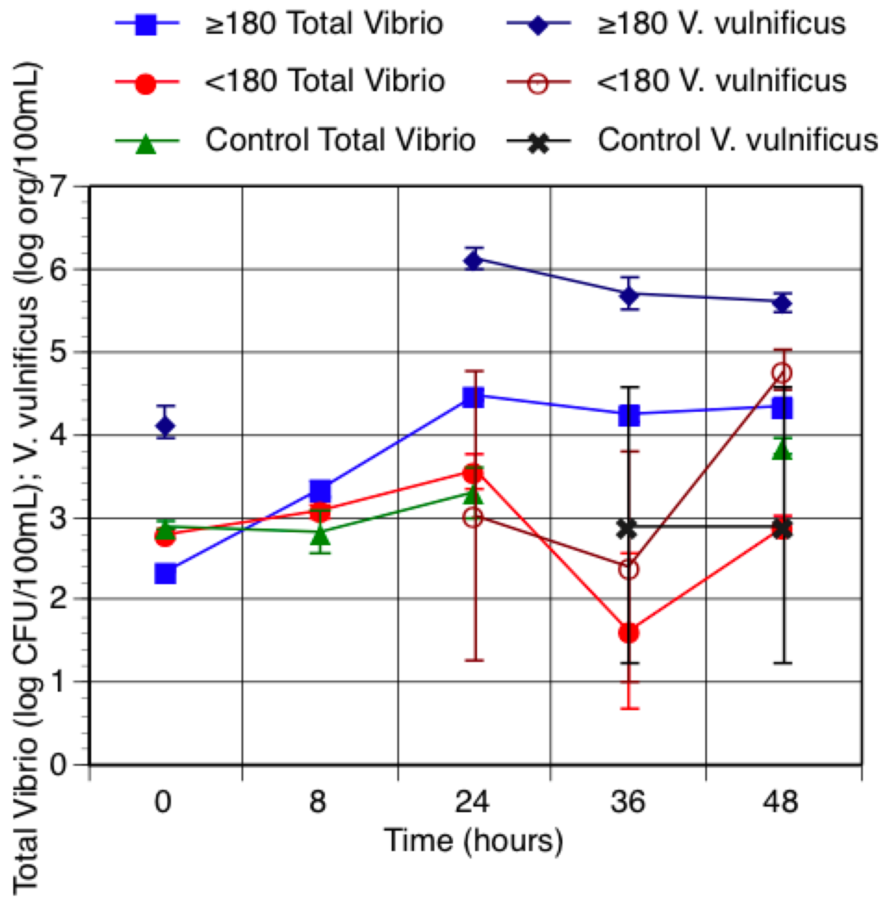


Figure B-1. Total *Vibrio* and *V. vulnificus* concentrations over time for July 15th (error bar = SE).

Table B-2. QPCR results for August 4th.

Time (hour)	Size Fraction	Standard Equation	R-squared	Efficiency	CT value rep 1	CT value rep 2	Average CT	Target cells per 100mL
36	<180a	y = -0.306x + 14.088	0.99	1.02	37.54	ND	37.54	7976.09
	Controla	y = -0.306x + 14.088	0.99	1.02	ND	38.81	38.81	3259.64
48	≥180b	y = -0.306x + 14.088	0.99	1.02	32.07	32.72	32.395	299336.72
	<180a	y = -0.306x + 14.088	0.99	1.02	ND	35.93	35.93	24799.90
	<180b	y = -0.306x + 14.088	0.99	1.02	30.97	30.6	30.785	930722.03
	Controla	y = -0.306x + 14.088	0.99	1.02	31.78	32.14	31.96	406696.09
	Controlb	y = -0.306x + 14.088	0.99	1.02	35.02	34.42	34.72	58171.46

ND = None Detect

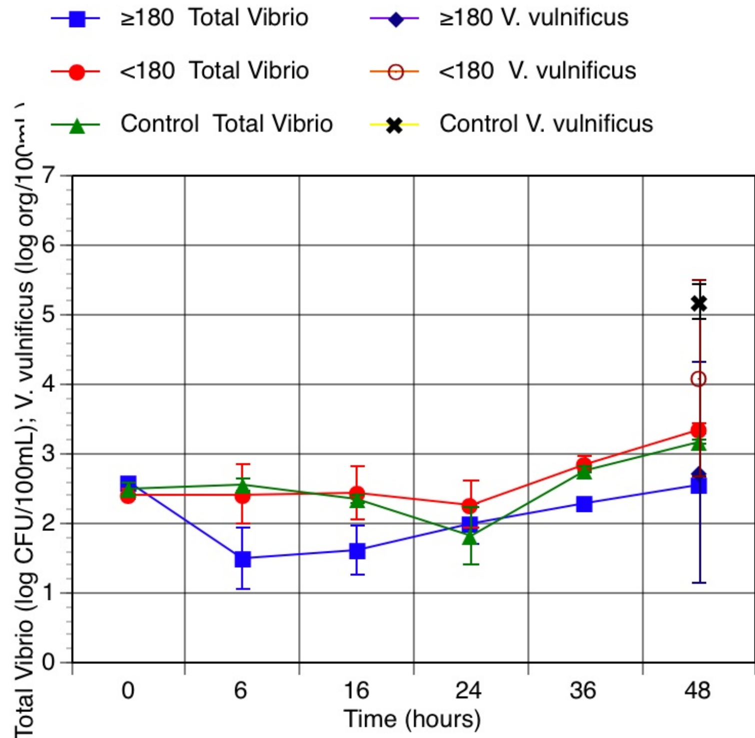


Figure B-2. Total *Vibrio* and *V. vulnificus* concentrations over time for August 4th (error bar = SE).

Appendix C. Chlorophyll *a* Concentrations and Phytoplankton

Chlorophyll *a* concentrations were measure using a fluorometer. Output from the fluorometer was then converted into μg of chlorophyll *a* / 1L of sample using the following equation:

Chlorophyll *a* (μg/L) = output * [volume extracted (L)/volume filtered (L)]

See Tables C-1, C-2, C-3 and C-4 for chlorophyll *a* data for July 15th, July 20th, August 4th and August 17th respectively.

Table C-1. Chlorophyll *a* concentrations for July 15th.

		Volume (L)		Fluorometer Value	Chlorophyll *a*
Time	Sample	Filtered	Extracted	(μg/L)	(μg/L)
0	≥180a	0.050	0.01	23.25	4.65
	≥180b	0.050	0.01	27.79	5.56
	<180a	0.050	0.01	70.5	14.10
	<180b	0.050	0.01	65.93	13.19
	Ca	0.050	0.01	--	--
	Cb	0.050	0.01	38.59	7.72
24	≥180a	0.050	0.01	27.61	5.52
	≥180b	0.050	0.01	26.45	5.29
	<180a	0.050	0.01	79.19	15.84
	<180b	0.050	0.01	85.34	17.07
	Ca	0.050	0.01	105.4	21.08
	Cb	0.050	0.01	22.3	4.46
36	≥180a	0.050	0.01	20.19	4.04
	≥180b	0.050	0.01	17.3	3.46
	<180a	0.050	0.01	71.89	14.38
	<180b	0.050	0.01	72.2	14.44
	Ca	0.050	0.01	126.4	25.28
	Cb	0.050	0.01	31.8	6.36
48	Ca	0.050	0.01	16.71	3.34
	≥180a	0.050	0.01	125.3	25.06

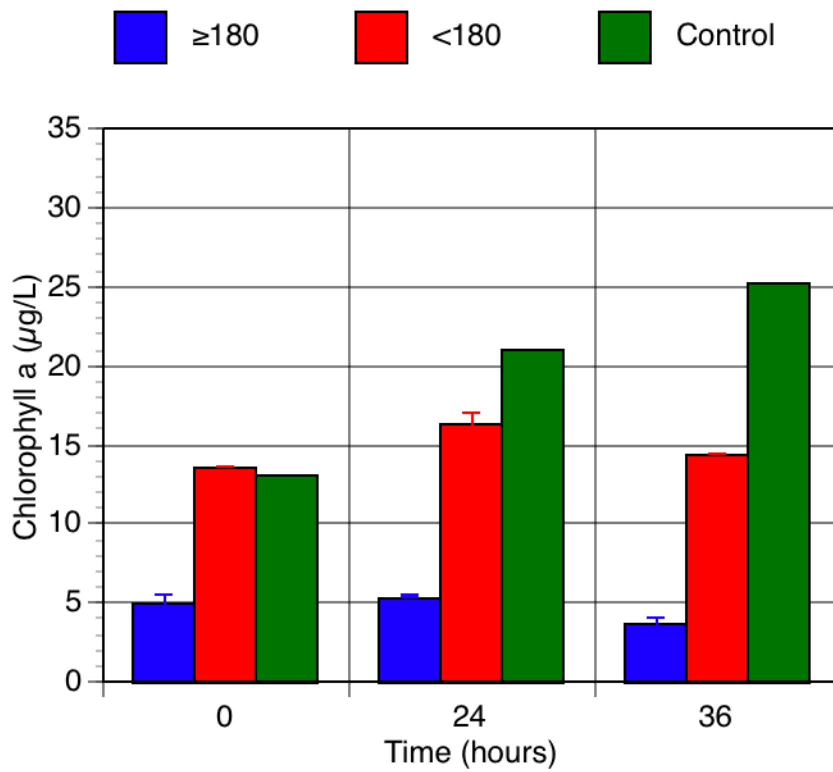


Figure C-1. Chlorophyll *a* concentrations over time for July 15th (error bar = SE).

Table C-2. Chlorophyll *a* concentrations for July 20th.

Time	Sample	Volume (L) Filtered	Volume (L) Extracted	Fluorometer Value (μg/L)	Chlorophyll *a* (μg/L)
0	≥180a	0.050	0.01	30.89	6.18
	≥180b	0.050	0.01	26.27	5.25
	<180a	0.050	0.01	129.6	25.92
	<180b	0.050	0.01	77.29	15.46
24	≥180a	0.050	0.01	41.34	8.27
	≥180b	0.050	0.01	59.09	11.82
	<180a	0.050	0.01	147.1	29.42
	<180b	0.050	0.01	148.8	29.76
36	≥180a	0.050	0.01	31.15	6.23
	≥180b	0.050	0.01	46.27	9.25
	<180a	0.050	0.01	167.4	33.48
	<180b	0.050	0.01	162.3	32.46
48	≥180a	0.050	0.01	30.2	6.04
	≥180b	0.050	0.01	41.19	8.24
	<180a	0.050	0.01	168.6	33.72
	<180b	0.050	0.01	153	30.60

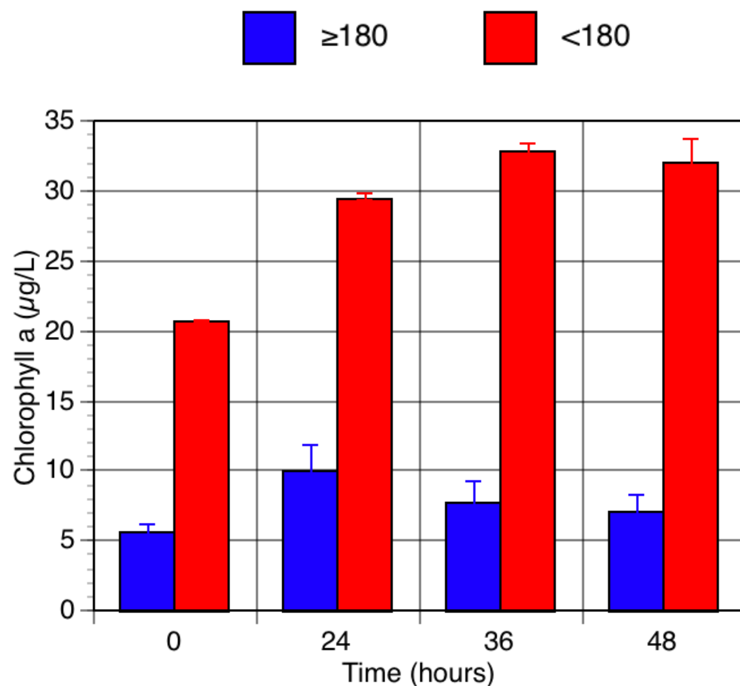


Figure C-2. Chlorophyll *a* concentration over time for July 20th (error bar = SE).

Table C-3. Chlorophyll *a* concentrations for August 4th.

		Volume (L)		Fluorometer Value	Chlorophyll *a*
Time	Sample	Filtered	Extracted	(µg/L)	(µg/L)
0	≥180a	0.050	0.01	57.53	11.51
	≥180b	0.050	0.01	57.81	11.56
	<180a	0.050	0.01	63.24	12.65
	<180b	0.050	0.01	62.49	12.50
	Ca	0.050	0.01	72.52	14.50
	Cb	0.050	0.01	72.8	14.56
6	≥180a	0.050	0.01	65.47	13.09
	≥180b	0.050	0.01	64.31	12.86
	<180a	0.050	0.01	67.96	13.59
	<180b	0.050	0.01	65.43	13.09
	Ca	0.050	0.01	74.24	14.85
	Cb	0.050	0.01	75.31	15.06
16	≥180a	0.050	0.01	63.25	12.65
	≥180b	0.050	0.01	57.67	11.53
	<180a	0.050	0.01	67.05	13.41
	<180b	0.050	0.01	61.55	12.31
	Ca	0.050	0.01	35.65	7.13
	Cb	0.050	0.01	70.69	14.14
24	≥180a	0.050	0.01	35.76	7.15
	≥180b	0.050	0.01	28.8	5.76
	<180a	0.050	0.01	64.01	12.80
	<180b	0.050	0.01	33.39	6.68
	Ca	0.050	0.01	20.85	4.17
	Cb	0.050	0.01	64.75	12.95
36	≥180a	0.050	0.01	54.46	10.89
	≥180b	0.050	0.01	56.38	11.28
	<180a	0.050	0.01	58.88	11.78
	<180b	0.050	0.01	56.04	11.21
	Ca	0.050	0.01	62.02	12.40
	Cb	0.050	0.01	64.3	12.86
48	≥180a	0.050	0.01	57.82	11.56
	≥180b	0.050	0.01	53.09	10.62
	<180a	0.050	0.01	59.89	11.98
	<180b	0.050	0.01	60.35	12.07
	Ca	0.050	0.01	61.93	12.39
	Cb	0.050	0.01	66.81	13.36

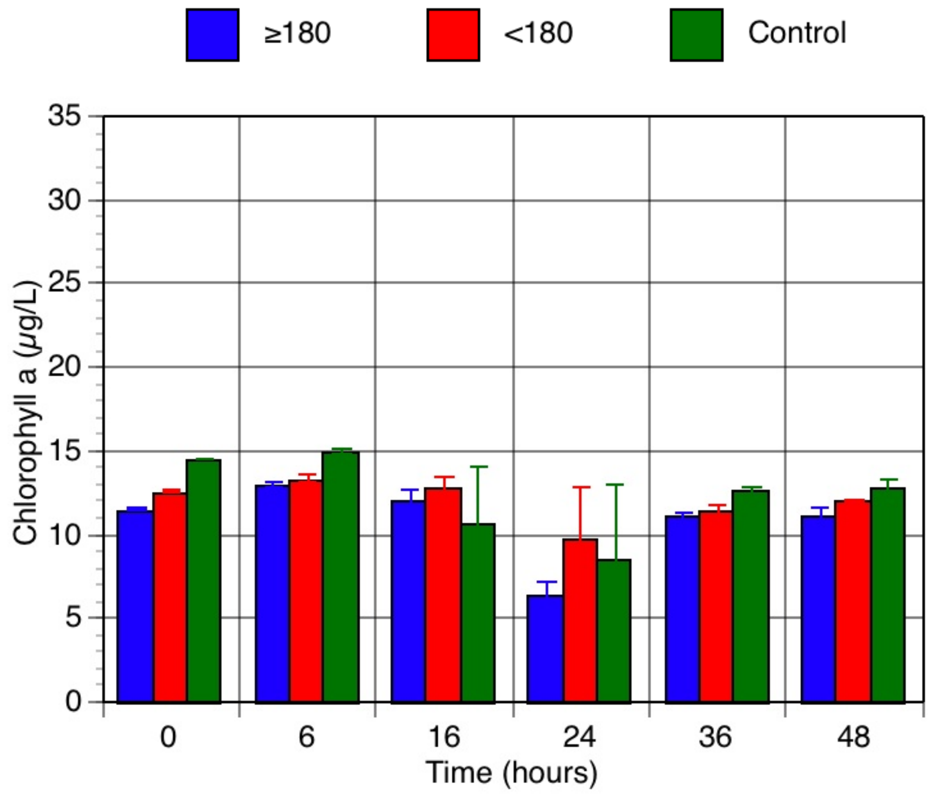


Figure C-3. Chlorophyll *a* concentrations over time for August 4th (error bar = SE).

Table C-4. Chlorophyll *a* concentrations for August 17th.

Time	Sample	Volume (L) Filtered	Volume (L) Extracted	Fluorometer Value (µg/L)	Chlorophyll *a* (µg/L)
0	≥180a	0.050	0.01	91.57	18.31
	≥180b	0.050	0.01	89.75	17.95
	<180a	0.050	0.01	75.37	15.07
	<180b	0.050	0.01	71.38	14.28
	Ca	0.050	0.01	91.06	18.21
	Cb	0.050	0.01	95.61	19.12
6	≥180a	0.050	0.01	80	16.00
	≥180b	0.050	0.01	86.87	17.37
	<180a	0.050	0.01	75.34	15.07
	<180b	0.050	0.01	69.5	13.90
	Ca	0.050	0.01	84.28	16.86
	Cb	0.050	0.01	86.41	17.28
16	≥180a	0.050	0.01	80.91	16.18
	≥180b	0.050	0.01	78.13	15.63
	<180a	0.050	0.01	72.41	14.48
	<180b	0.050	0.01	71.34	14.27
	Ca	0.050	0.01	76.8	15.36
	Cb	0.050	0.01	81.52	16.30
24	≥180a	0.050	0.01	66.25	13.25
	≥180b	0.050	0.01	70.25	14.05
	<180a	0.050	0.01	85.03	17.01
	<180b	0.050	0.01	85.89	17.18
	Ca	0.050	0.01	89.94	17.99
	Cb	0.050	0.01	83.98	16.80
36	≥180a	0.050	0.01	81.12	16.22
	≥180b	0.050	0.01	81.94	16.39
	<180a	0.050	0.01	71.04	14.21
	<180b	0.050	0.01	69.44	13.89
	Ca	0.050	0.01	82.59	16.52
	Cb	0.050	0.01	82.79	16.56
48	≥180a	0.050	0.01	84.52	16.90
	≥180b	0.050	0.01	84.77	16.95
	<180a	0.050	0.01	71.47	14.29
	<180b	0.050	0.01	74.54	14.91
	Ca	0.050	0.01	84.84	16.97
	Cb	0.050	0.01	81.5	16.30

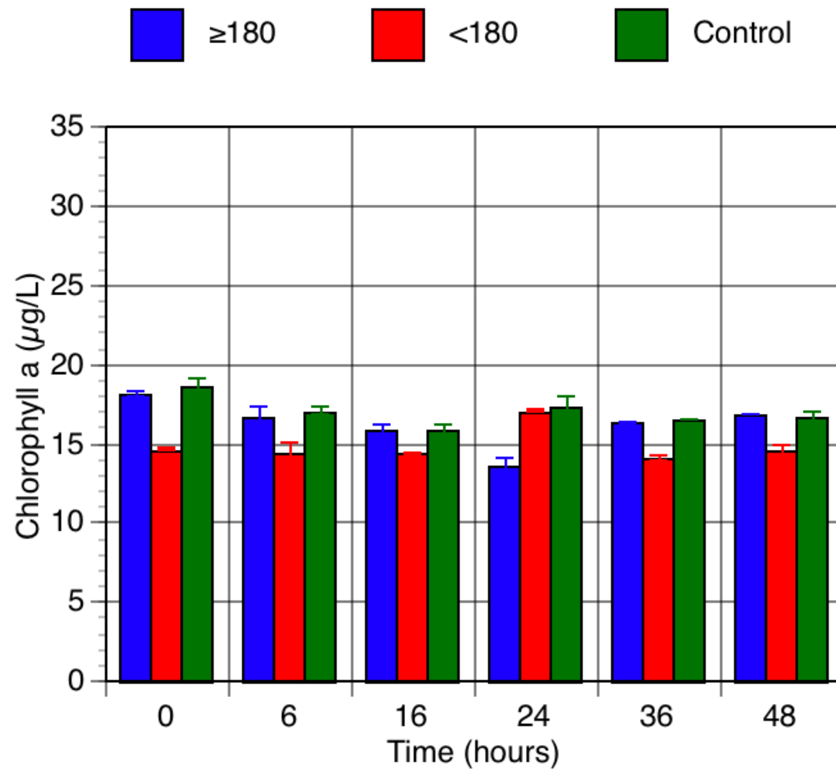


Figure C-4. Chlorophyll *a* concentrations over time August 17th (error bar = SE).

In addition to chlorophyll *a* concentrations, phytoplankton populations were also estimated by microscopic enumeration. 50mL of sample was added into brown bottles with an overall 1% lugols solution to preserve and stain the phytoplankton. 18mL subsamples were then added into settling chambers for 24 hours. Phytoplankton groups were then counted for 10 microscopic fields. Groups enumerated include diatoms, dinoflagellates, cyanobacteria and chlorophytes/cryptophytes/other.

Table C-5. Microscopic Phytoplankton Counts

	≥180μm					**<180μm**					**Control**				
	Total	Diatom	Dinoflagellate	Cyanobacteria	Chlorophyltes/ Cryphytes	Total	Diatom	Dinoflagellate	Cyanobacteria	Chlorophyltes/ Cryphytes	Total	Diatom	Dinoflagellate	Cyanobacteria	Chlorophyltes/ Cryphytes
Date	Count n=10	Percent				Count n=10	Percent				Count n=10	Percent			
7.7*	4.06e5	7.2	2	84.8	6	8.27e5	17.8	1.1	76.5	4.6					
7.15*	5.58e5					1.34e6									
7.20*	3.65e5					6.49e5									
8.4	7.54e5	2.2	3.5	78.2	16.1	6.85e5	6.4	3.4	72.8	17.4	6.57e5	7.8	4.1	74.3	14.0
8.17	8.27e5	50.3	7.1	9.1	33.5	6.76e5	60.7	7.9	4.6	26.8	7.10e5	53.5	9.6	4.6	32.3

* <100μm instead of <180μm for July samples.

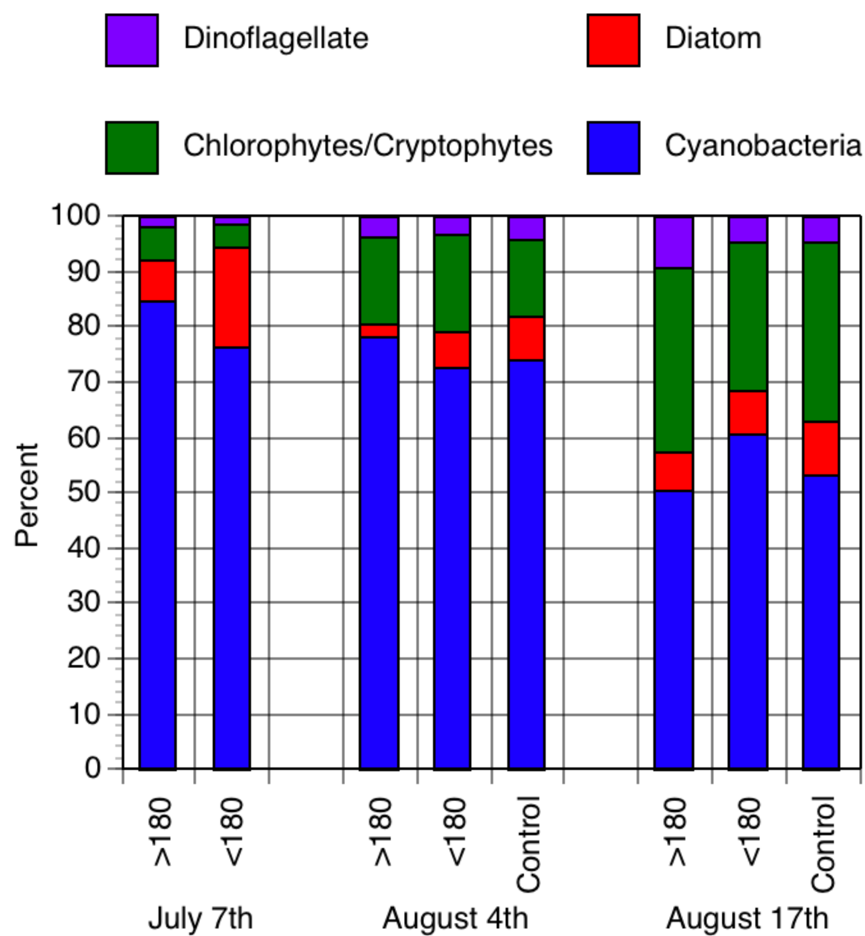


Figure C-5. Percent Phytoplankton Composition

Appendix D – Statistical Analysis

Statistical analyses were conducted in Excel and SAS. Total *Vibrio* and *V. vulnificus* concentrations were log transformed for data analysis. Total *Vibrio* concentrations were also converted into log growth measures using the following formula:

log growth = log (concentration at time X / concentrations at time 0).

Data was then examined for normality. All data was found to be normally distributed except for the log transformed total *Vibrio* concentrations for June/July. Parametric analyses, including independent two-tailed, two sample t-tests and Pearson correlation coefficient, were used for normally distributed data. Samples not normally distributed were analyzed using nonparametric methods, including wilcoxon-mann-whitney two-sample test and Spearman correlation coefficient. The t-test and wilcoxon-mann-whitney test compare the means of two populations. The Pearson and Spearman correlation coefficient compares the variation of the linear relationship between two populations.

Table D-1. Statistical analyses.

Question	Statistical Method	Result
Are total *Vibrio* concentrations initially different between the ≥180μm, <180μm and control?	June/July: wilcoxon-mann-whitney two-sample Z statistic August: independent t-test statistic	June/July: ≥180 & <180: Yes (p=0.0001) ≥180 & Control: Yes (p=0.037) <180 & Control: No August: ≥180 & <180: No ≥180 & Control: No <180 & Control: No
Do total *Vibrio* concentrations grow differently over time between the ≥180μm, <180μm and control?	June/July: independent t-test statistic August: independent t-test statistic	June/July: ≥180 & <180: Yes (p<0.0001) ≥180 & Control: Yes (p=0.004) <180 & Control: Yes (p=0.009) August: ≥180 & <180: No ≥180 & Control: Yes (p=0.038) <180 & Control: Potentially (p=0.063)
Do *V. vulnificus* concentrations correlate with total *Vibrio* concentrations?	June/July: Spearman correlation coefficient August: Pearson correlation coefficient	June/July: Yes (0.003) August: No
Are initial chlorophyll *a* concentrations different between the ≥180μm, <180μm and control?	June/July: independent t-test statistic August: independent	June/July: ≥180 & <180: Yes (p=0.028) ≥180 & Control: N/A <180 & Control: N/A

	t-test statistic	August: ≥180 & <180: No ≥180 & Control: No <180 & Control: No
Do total *Vibrio* concentrations correlate with chlorophyll *a* concentrations?	June/July: Spearman correlation coefficient August: Pearson correlation coefficient	June/July: Yes (p=0.021) August: No
Is total *Vibrio* growth correlated with chlorophyll *a* concentrations?	June/July: Spearman correlation coefficient August: Pearson correlation coefficient	June/July: No August: Yes (0.002)
Is total *Vibrio* growth correlated with time?	June/July: Spearman correlation coefficient August: Pearson correlation coefficient	June: Yes (p<0.0001) August: Yes (p<0.0001)

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